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Chemical markers for the evaluation of sensory and antioxidant properties of wines

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Abstract

Sotolon (3-Hydroxy-4,5-dimethyl-2(5)-furanone) is a chiral lactone responsible for the oxidized flavour in fortified wines and wine produced by oxidative ageing. The perception threshold of the racemic mixture in wine is 8 – 10 μg/L and the flavour is reported as curry, aged honey, aged sake and fenugreek. Though it is considered a typical flavour note in Madera, Porto and Sherry, it is an off-flavour in dry white wine where its oxidative note is detrimental for the fresh taste and odour. The sotolon formation pathways, during winemaking, are affected by chemical and physical factors such as: oxygen concentration, storage temperature and time, reducing sugar concentration and antioxidant compounds concentration (e.g. sulphur dioxide or (GSH) glutathione). Due to the number of chemical and physical factors affecting the Sotolon formation in wine this compound has been suggested as chemical marker of white wine shelf-life.
A fast, sensitive and easy to apply analytical method (UHPLC-UV) and it was applied to the evaluation of SO_2-free Franciacorta DOCG wines in order to assess the effect of different disgorgement conditions (antioxidant additives, ageing time and temperature) on the sotolon formation. The sotolon concentration was measured in sparkling wine stored at 15°C and 25°C for 6 months added to three different antioxidant preparations (2 g/hL and 4 g/hL) potentially substituting the sulfur dioxide. Furthermore, we investigated the chemical and physical factors could affect the sotolon formation in synthetic wine. Model solution conitaing increasing concentration of pentoses, GSH, amino group, catechin, oxygen, ethanal, tartaric acid and iron are stored at two temperatures (70°C and 5°C) for five days in order to clarify the compositive factors affecting the sotolon synthesis in white wine. Finally, we compared the performances of analytical methods (HPLC-UV and UHPLC-MS) for sotolon quantification, which were previously developed. Separately, we developed a fast, sensitive and easy to apply analytical method (UHPLC-UV) for the biogenic amines (BAs) assessment in red wine treated with different malolactic fermentation condition (Spontaneous MLF; Inoculum and Co-inoculum techniques). Moreover was checked the trend of intra and extra-cellular glutathione and their effect on the aromatic matrix of South African Sauvignon blanc (Stellenbosch) must and wine during the alcholic fermentation and aging. The Must was treated with GSH and a GSH-enriched inactive dry yeast preparation (GSH-IDYs).
The proposed analytical methods (UHPLC-UV; HPLC-UV and UHPLC-MS) provide a sample preparation faster and easier-to-apply than those previously reported for the routine analyses of sotolon. The methods (HPLC-UV; UHPLC-MS/MS; UHPLC-UV) were proved suitable for the determination of sotolon concentrations in white wine and in model solution under its sensory perception threshold. Two analytical methods compared (HPLC-UV; UHPLC-MS/MS) were successfully used for the screening of 70 commercial South African wines' sotolon levels. The samples of Franciacorta sparkling wines treated with SO_2 show the best protection against wine oxidation whereas the other commercial antioxidants tested caused detrimental effects due to the sotolon production. The phenolic composition of commercial antioxidants has influenced the production of sotolon. The sotolon formation tests carried out showed which sotolon can be formed by several formation pathways indeed it was generated under both reducing and oxidative conditions. Sotolon formation is enhanced by simultaneous presence of Fe^{++} and O_2, of amino groups and of phenols. Glutathione inhibited the formation of sotolon only when it was simultaneously added with amino groups and phenols in an oxidizing environment or when it was present in oxidative conditions. Under anoxic conditions the sotolon formation test has been demonstrated that there is a high dependence between sotolon and reducing sugar contents, whereas tartaric acid and acetaldehyde didn't affect the formation of sotolon. The formation of

sotolon in anoxic environment has not yet been clarified and further tests will be conduct to understand the role of tartaric acid, ethanol, ethanal and ribose on sotolon production in synthetic wine. The analytical method for the BAs quantification showed good linearity and repeatability and was able to quantify the ABs in red wine. The preliminary results concerning the different malolactic fermentation conditions, suggest that co-inoculum technique does not seem to prevent BAs formation in wine. The trial data on GSH (intra and extra-cellular) have yet to be processed and will be assessed in the future.

Marcatori chimici per la valutazione delle proprietà sensoriali e antiossidanti dei vini.

Il sotolone (3- idrossi -4, 5 - dimetil - 2 (5) - furanone) è un lattone chirale responsabile del sapore ossidato nei vini liquorosi e nei vini prodotti da invecchiamento ossidativo. La sua soglia di percezione della miscela racemica nel vino è 8-10 µg/L e la sua nota aromatica tipica è curry, miele invecchiato, saké invecchiato e fieno greco. Anche se il sotolone è considerato il tipico aroma dei vini Madeira, Porto e Sherry, nei vini bianchi secchi è considerato un off- flavour, dove la sua nota ossidativa è dannosa per il gusto fresco e l'aroma del vino. La sua formazione durante l'affinamento e la vinificazione, è influenzata da molti fattori chimici e fisici quali: concentrazione di ossigeno, temperatura di conservazione e tempo, concentrazione di zuccheri riducenti e la concentrazione di composti antiossidanti (anidride solforosa o (GSH) glutatione). A causa dell'elevato numero di fattori chimici e fisici che influenzano la sua formazione nel vino questo composto è stato suggerito come marcatore chimico della shelf-life del vino bianco. Un veloce, sensibile e facile da applicare metodo analitico (UHPLC-UV) è stato applicato per la valutazione di vino spumante SO_2-free Franciacorta DOCG con lo scopo di valutare l'effetto di diverse condizioni di sboccatura (additivi antiossidanti, tempo e temperatura di invecchiamento) sulla formazione sotolone. La concentrazione di sotolone è stata misurata in vino spumante conservato a 15° C e 25° C per 6 mesi, e trattato con tre diverse preparazioni di antiossidanti (2 g/hL e 4 g/hL) che potenzialmente potrebbero sostituire l'anidride solforosa. Inoltre, abbiamo studiato i fattori chimici e fisici che potrebbero influenzare la formazione di sotolone in vino sintetico. Le soluzioni modello contenevano concentrazioni crescenti di pentosi, GSH, gruppi amminici, catechina, ossigeno, acetaldeide, acido tartarico e ferro. I campioni sono conservati a due temperature (70° C e 5° C) per cinque giorni, al fine di chiarire gli elementi compositivi del vino sintetico che potrebbero influenzare la sua formazione nel vino bianco. Infine, abbiamo confrontato le prestazioni di due metodi analitici (HPLC-UV e UHPLC-MS/MS) per sotolone quantificazione, precedentemente sviluppati. Separatamente, abbiamo sviluppato un metodo analitico veloce, sensibile e facile da applicare (UHPLC-UV) per la valutazione delle amine biogene (BAs) nel vino rosso trattato con diverse condizioni di fermentazione malolattica (FML spontanea; tecniche di inoculo e co-inoculo). Inoltre è stato verificato l'andamento del glutatione intra ed extra-cellulare e il suo effetto sulla matrice aromatica di mosti e vini di Sauvignon blanc (Sud Africa; Stellenbosch) trattati con GSH e una preparazione di lievito secco inattivo arricchito di GSH (GSH-IDYs) durante la fermentazione alcolica e l'invecchiamento.

I metodi proposti analitici (UHPLC-UV, HPLC- UV e UHPLC–MS/MS) evidenziano una preparazione del campione veloce e più facile da applicare rispetto a quelli precedentemente riportati per le analisi di routine del sotolone. I metodi analitici sviluppati (HPLC-UV; UHPLC-MS/MS; UHPLC-UV) si sono dimostrati adatti per la determinazione del sotolone, in vino bianco e in soluzione modello, sotto la sua soglia di percezione sensoriale. I due metodi analitici confrontati (HPLC-UV; UHPLC-MS/MS) sono stati utilizzati con successo per lo screening dei livelli di sotolone in 70 vini commerciali Sudafricani. I campioni di vini spumanti Franciacorta trattati con SO_2 mostrano la migliore protezione contro le ossidazioni, mentre gli altri antiossidanti commerciali causano effetti negativi dovuti alla formazione di sotolone nel vino. La composizione fenolica degli antiossidanti commerciali ha influenzato la produzione di sotolone. Le prove di formazione di sotolone hanno dimostrato che può essere formato da più vie di formazione infatti è stato generato sia in condizioni riducenti che ossidanti. La formazione sotolone era favorita dalla presenza simultanea di ferro, ossigeno, gruppi amminici e di fenoli. Il glutatione ha inibito la

formazione di sotolone solo quando è stato aggiunto simultaneamente con gruppi amminici e fenoli in ambiente ossidante o quando erano presenti le condizioni ossidanti. In condizioni anossiche laformazione di sotolone è stata fortemente influenzata dal contenuto di zuccheri riducenti, mentre l'acido tartarico e acetaldeide non incidono sulla formazione di sotolone. La
formazione di sotolone in ambiente anossico non è ancora stata chiarita e ulteriori test saranno condotti per comprendere il ruolo dell'acido tartarico, etanolo, acetaldeide e ribosio sulla produzione sotolone nel vino sintetico. Il metodo analitico per la quantificazione delle BAs ha dimostrato una buona linearità e ripetibilità ed era in grado di quantificare le ABs in vino rosso. I risultati preliminari relativi alle diverse condizioni di fermentazione malolattica, suggeriscono che la tecnica di co-inoculo non sembra prevenire la formazione BAs in vino. I dati relativi al GSH (intra e extra cellulare) devono essere ancora processati e verranno valutati in futuro.

Index

0 Preface

Wine is a fermented beverage produced through crushing, pressing and fermented of Vitis vinifera grapes. During the winemaking, several compounds can be involved in different and complex chemical trasformations, which can affect the aromatic matrix of wine and its shell-life.
The oxidative alterations are firstly characterized by the presence of ethanal which is responsible of the oxidized green apple odour. The chemical production of acetaldehyde in wine is strictly related to the presence of oxygen and catalysts such as copper and iron. A further formation pathway rises from the dissociation of hydrogensulfide from combined ethanol following to the oxidation of free sulfur dioxide. The main compounds responsible of the untypical-aging are: thiazols, thiofurans and sotolon. Both thiazols and thiofurans confer maderized aroma. They are produced by the Maillard reaction, specifically by the Strecker degradation between cysteine and methylgliossal (a α-di-carbonyl compound deriving from Maillard reaction). 2-acetylthioazol (Olfactory Threshold (S. T.) 3 μg/l), thiophen-2-thiol (S.T. 1 μg/l) and thiomethylfuran (S.T. 1 μg/l) are described as major responsible compounds. When the 2-aminoacetophenone content in wine exceeds 1 μg/l the wet paperboard odour can be clearly perceived . Its formation from the Streker degradation starting from tryptophan has been described.
Sotolon was reported to cause maderized, old honey, Greek hay and maediterranean scrub aroma (S.T. 8 μg/l) in wine. Though it is considered a typical flavour note in Madera, Porto, Sherry and long aged sweet wines, it is an off-flavour in dry white wine where its oxidative note is detrimental for the fresh taste and odour. Sotolon develops in wine making and wine ageing from a number of chemical pathways: thermal degradation of intermediate compounds of the Maillard reactions) involving piruvic acid and glycine, glycolic acid and alanine. 4-hydroxy isoleucine is also described as precursor of Sotolone. Reducing sugars as glucose, ribose and rhamnose are reported to be involved. as well as ascorbic acid, dehydroascorbic acid, ethanal and ethanol. In spite of the wide number of precursors described the final chemical steps are likely due to ethanal and α-chetobutirric acid. Sotolon formation increases as wine exposure to oxygen, storage temperature and time, reducing sugar concentration increase as well as when antioxidant compounds (e.g. sulphur dioxide or glutathione) are not carefully used in wine making. Due to the number of chemical and physical factors affecting the Sotolone formation in wine this compound has been suggested as chemical marker of white wine shelf-life. Nevertheless, the analytical methods proposed in literature for the Sotolone assessment are often tedious and solvent consuming. Due to the low levels occurring in dry white wines extraction and purification steps by organic solvents (liquid-liquid extraction) or solid-phase extraction (SPE solid-liquid extraction) are always needed. All of above mentioned methods require long extraction times (up to 48 h) and/or high wine and solvent volumes (up to 500 ml) which implies high analytical costs or long processing times. Purified samples are finally processed by GC though an HPLC separation method has been proposed. Therefore, a fast, sensitive and easy to apply analytical method has been developed and validated in this PhD work and it was applied to the evaluation of SO2-free Franciacorta DOCG wines in order to assess the effect of different wine making conditions, antioxidant compounds, ageing time temperature on the sotolone formation. Moreover, it was evaluated the influence of chemical and physical factors (reducing sugars, glutathione (GSH), amino group, catechin, oxygen, ethanal, tartaric acid, iron and temperature), which could affect the sotolon formation.
I hope this research could represent a useful tool for a rapid evaluation of the off-flavours which can affect the wine aromas and it may also clarify the mechanisms of formation of sotolon in white dry wine.
Mario Gabrielli

Chemical markers for the evaluation of sensory and antioxidant properties of wines

1 Introduction

1.1 Wine and oxygen

Ageing is a process in the wine leading to profound changes in chemical composition and aroma. The term "aeging" is used with various connotations. The aging of wine is often associated with a negative development of the sensory characteristics. Oxidation/reduction (REDOX) phenomena plays an important roles in many steps of the winemaking process, causing alterations in wine composition. During the aging the oxidation alterations are generally described as "oxidized" in dry wines, "rancio" in sweet fortified red wine and "maderized" in sweet fortified white wines.

It is generally accepted that the exposure to the air deteriorates the aroma, taste and color of white dry wine (Simposon, 1978). It is known that the aromatic deterioration occurs prior to the changes of chromatic properties (Singleton et all., 1976; Escudero et all., 2000). The presence of oxygen is related with the appearance of the maderized aroma and the browning pigments which deteriorate the sensorial properties of dry white wine (Lavigne et al., 2008; Escudero et al., 2002). Briefly, the aging determines a degradation of aroma compounds mainly associated with fruit and floreal aromas and a formation of compounds leading a new aroma profile.

On the contrary the maderized odors characterize the aroma of the fortified wines such as: Madeira, Vin Jauven and botrytised wines (Masuda et al., 1984; Silvia Ferreria et al., 2003; Camara et al., 2004). Further examples where the aging process plays an important role in winemaking technologies are production of Porto and Sherry wines (Pham et al., 1995; Martin et al., 1992).

In the later case, there are even two different aging procedures: oxidative aging for the Oloroso style wines and biological aging with the influence of flor yeast, risulting in fino Sherry.

The oxidative alterations are firstly characterized by the presence of ethanal which is responsible of the oxidized green apple odour. Major compounds responsible of the untypical-aging are: thiazols, thiofurans, 2-aminoacetophenone and sotolon.

Both thiazols and thiofurans confer maderized aroma. They are produced by the Maillard reaction, specifically by the Strecker degradation (Ledl & Schleicher, 1990) between cysteine and methylgliossal (α-di-carbonyl compound deriving from Maillard reaction). 2-acetylthioazol (Olfactory Threshold (S. T. 3 μ g/l), thiophen-2-thiol (S.T. 1 μ g/l) and thiomethylfuran (S.T. 1 μg/l) are described as major responsible compounds (Honggao Xu et al., 2008). When the 2-aminoacetophenone content in wine exceeds 1 μg/l the wet paperboard odour can be clearly perceived. Its formation from the Streker degradation starting from tryptophan has been described.

Sotolon was reported to cause maderized, old honey, Greek hay and maediterranean scrub aroma (S.T. 8 μ g/l) in wine. It is produced by the reaction of alpha-ketobutyric acid with ethanal/ethanol (deriving from the ethanol oxidation) (Pons et al., 2010). The former arises from the oxidation of ascorbic acid. However, significative amounts of sotolon were detected also in ascorbic acid-free wine samples therefore, other formation pathways should be evaluated such as: Maillard reaction (Guerra et al., 2011); reaction of 4-hydroxy –L- isoleucine with methyglyoxal/phenilyglyoxal (Blak Et al., 1996).

The untypical aging of wine is characterized by the presence of one or more of the above described compounds. Reagents, concentration, oxygen amount, pH and temperature can affect the formation and the level of these compounds.

1.2 Chemical oxidation in wine

1.2.1 REDOX potential

Reduction potential (also known as redox potential) is a measure of the tendency of a chemical species to acquire electrons and thereby be reduced. Reduction potential is measured in volts (V), or millivolts (mV). Each species has its own intrinsic reduction potential; the more positive the potential, the greater the species' affinity for electrons and tendency to be reduced. (Trevor Suslow, 2004). In aqueous solutions, the reduction potential is a measure of the tendency of the solution to either gain or lose electrons when it is subject to change by introduction of a new species. A solution with a higher (more positive) REDOX potential than the new species, the system will have a tendency to gain electrons from the new species whereas a solution with a lower (more negative) reduction potential will have a tendency to provide electrons to the new species.

1.3 REDOX potential in wine

Though it is thought that the measurement of wine reduction potentials is of very limited value the consideration of the REDOX potential of individual wine compounds can provide useful insights into the mechanism of oxidative processes.

The reduction potential of wines is used to indicate their level of oxidation or reduction. Reduction potentials are generated by redox couples; the magnitude of the potential for any couple is determined by the relative proportion of the oxidized and reduced component. It might be imagined that a wine with a high reduction potential can be in an oxidized state in which reductants such as sulfur dioxide, ascorbic acid and polyphenols are reacting. Subsequently when the reduction potential is low the wine can be in a reduced state and is most likely to contain reduced sulfur compounds as thiols. Nevertheless, redox couples associated with polyphenols, which are most likely to determine reduction potentials, are not at equilibrium in wine due to the instability of quinones. Ribéreau-Gayon et al., (2000) showed that reduction potentials are strongly dependent on the oxygen, infact a system of oxygen added shows an increase of the potential from 200 to 300 mV at saturation. Joslyn, (1949); Ribéreau-Gayon et al., (2000) examined the effect of the wine-making and the winery operations on the potential which showed to be very sensitive to oxygen concentration.

A well-known example is the oxidative reaction of ascorbic acid to produce dehydroascorbic acid and hydrogen peroxide with the simultaneous reduction from quinone to catechol such as (+)-catechin. The oxidation product, dehydroascorbic acid, is unstable, decomposing to many reactive substance including L-xylosone (**Figure 1.1**).

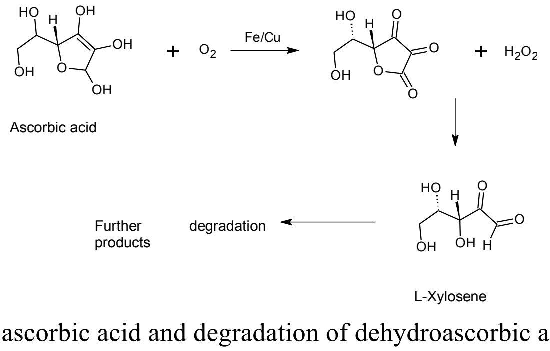

Figure 1.1 Oxidation of ascorbic acid and degradation of dehydroascorbic acd (Danilewicz, 2012).

The aldehydic group of L-xylosone can react with (+)-catechin to form adducts, which generate yellow xanthylium cation pigments under wine-like conditions. The sulfite can remove hydrogen peroxide and carbonyl derivatives, that are formed by the oxidative changes induced by ascorbic acid, thus preventing the appearance of browning and deterioration aromatic (Barril et al., 2009; Bradshaw et al., 2011).

Another a well-known example is polyphenols oxidation, which is reported by (Danilewicz, 2011). The oxidation of polyphenols, such as (+)-catechin, caffeic acid, and gallic acid and its esters, with redox couples of similar reduction potential to the O_2/H_2O_2 couple, is determined, by the catalytic action of iron and cupper and by substances that can react with their quinones. It is widely known that the oxidation of polyphenols is regulated by the catalytic action of iron, which is further enhanced by cupper. The absence of these transition metals from synthetic wine prevents polyphenol oxidation and their removal from wine can slow or stop oxygen consumption. It is proposed that the polyphenols oxidative process is started by the reaction of Fe^{2+} with oxygen, which is able to produce hydroperoxyl radicals and subsequently hydrogen peroxide. O-phenols are able to coordinate ferric ions, electron transfer within the complex returning Fe^{3+} to the ferrous state to continue the redox cycle, whereas the phenolsare oxidized to the semiquinone radical and quinone (Danilewicz, 2011).

1.4 Oxidation of wine's constituents by Fenton's reaction and the role of SO_2

With relatively few exceptions, the oxidation of food causes a detrimental effect on food quality. Wine can be an exception to this rule, however, particularly in the case of maderized wines (such as: Porto wine Madeira and Sherry) wherein the oxidation is critical to the flavor and aroma characteristics of these products. On the contrary in white dry wine the oxidation of aromatic compounds involves the a loss of the original aromatic properties of wine whereas in the red wine a certain degree of oxidation could lead to reduce astringency and enhance color (Danielewicz, 2003). Recent findings have demonstrated that nonenzymatic wine oxidation is strongly influenced by the presence of transition metals (Danielewicz, 2003).

The oxidation of wine's constituents (e.g., ethanol, tartaric acid) could be coupled to the reduction of dioxygen to water. These reactions are closely linked to the interaction between free radical and oxygen which being reduced to hydrogen peroxide by the transfer of electrons from the constituents of the wine to oxygen (Elias et al., 2009).

The direct oxidation of the organic compounds is kinetically disadvantaged because these organic compounds (singlet state) and dioxygen (triplet state) is spin forbidden. The transition metals are thought to catalyze these processes due to their ability to redox cycle (i.e., readily donate and accept electrons) (Miller et al., 1990). Phenolic substances have a primary role as oxidation compoinds in wine. Singleton & Esau, (1969) showed that the reaction between oxygen and phenols in wine, not only, produced the quinone compounds from ortho phenols but also produced, a strong oxidant, as hydrogen peroxide, which in turn oxidized the constituents of wine, such as ethanol to acetaldehyde. As the primary substrates for oxygen in wine, polyphenols are the major substrates of hydroperoxyl radicals, which are formed from the reaction between dioxygen and ferrous and/or copper (I) ions. This reaction produces hydrogen peroxide (H_2O_2), which is able to give other oxidation reactions and its fate is dependent on a number of factors. H_2O_2 is expected to react with a ferrous or cuprous ion to produce a hydroxyl radical, that is a potent species capable to oxidize (non-specific oxidative power) the majority of organic constituents of wine in proportion to their abundance (Fukuzawa et al., 1988).

However, in the presence of excess SO_2, H_2O_2, the sulfite appears to react irreversibly, under acidic conditions, to yield sulfate (HSO_4-/H_2SO_4) and water, thereby leaving the organic fraction of wine protected by oxidative reactions. Other authors (Ribereau-Gayon, 2000) reported that SO_2 explains its antioxidant function reacting directly with oxygen, but this reaction is described as slow and prohibitively in oenological conditions (Danilewicz, 2003). The sulphur dioxide, in the simultaneous presence of oxygen and transition metal (e.g. iron or copper), involves a radical-chain reaction. The reaction is initiated by the oxidation of sulfite by Fe (III) to produce sulfite radicals ($SO_3^{\bullet-}$). Thel radical produced reacts rapidly with oxygen to produce the peroxomonosulfate radical ($SO_5^{\bullet-}$), which is a stronger oxidant capable of oxidizing polyphenols (Das et al., 1999). It is reported by Danilewicz, (2007); Danilewicz & Wallbridge, (2010) that polyphenols matrix, by intercepting the peroxomonosulfate radical, prevents radical-chain propagation and so prevents sulfite autoxidation in wine (Figure 1.2). In the absence of polyphenols the sulfate radical is produced, which is also capable of oxidizing ethanol (Danilewicz, 2007).

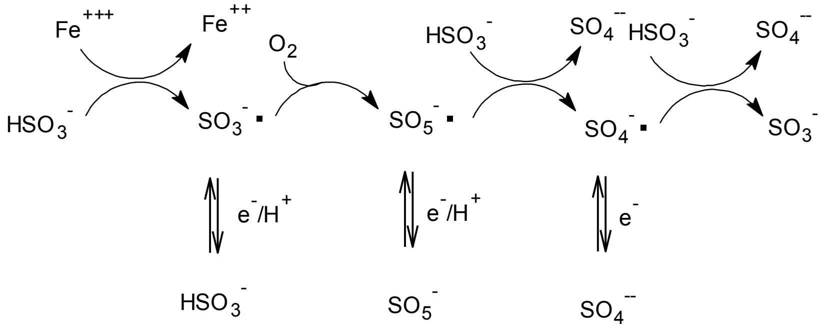

Figure 1.2 Radical-chain involved in sulfite autooxidation (Danilewicz, 2012).

Interestingly sulfite and polyphenols, described as antioxidant compounds, activate oxygen to oxidize ethanol. Accordingly, even if polyphenols and sulfites alone are capable of promoting the oxidation, when combined, are antioxidants compounds in wine. Polyphenols are considered antioxidant because they will able to intercept damaging free radicals to produce less reactive semiquinones. Moreover the quinones produced are potentially harmful as they can combine with amines and thiols in proteins at physiological pH and so disrupt vital biological processes (Bolton et al., 2000). The damaging condensation reactions can be prevented with the addition of sulfite avoiding the appearance of browning and loss of aroma constituents.

The rate of the Fenton reaction is influenced by the presence of metallic binders, concentration of oxygen, pH and concentration of reactants.

The wine contains a number of organic molecules (eg, polyphenols, organic acids) capable of complexing metal ions, which may affect their reactivity with H_2O_2 (Voelker & Sulzberger, 1996). The pH of the solution or wine can also affect the rate of the SO_2/H_2O_2 reaction (McArdle & Hoffmann, 1983) and consequently the amount of H_2O_2 available to the Fenton reaction. The dissolved oxygen may also affect the Fenton reaction, as previously reported (Voelker & Sulzberger, 1996). In addition the relative concentrations of reactants (SO_2 and H_2O_2) are not static. The formation of H_2O_2 in wine is linked with the rate at which hydroperoxyl radicals react with polyphenols. The kinetics of this reaction is influenced by a number of factors such as: pH, oxygen concnetration, composition of the polyphenolic matrix and temperature.

1.5 Flavours deterioration

The aromatic deterioration due to aging and storage is the result of both degradation and formation of the aromatic matrix. In young wine, the oxidative degradation of aromatic compounds leads the loss of the fruity and floreal aroma. The formation of compounds, up to concentration above their perception threshold, involves to the appearance of new perceptible flavours. During the oxidative aging, more than 50 aroma compounds above the threshold are formed. In the Table 1.1 are reported the compound groups involved in aging.

Group	**Compounds**
Terpenes	Nerol; linalool; α -terpineol
Norisoprenoid compounds C_{13}	TDN (1,1,6-trimethyl-1,2-dihydronaphathalene); β -damascenone vitispirane
Higer alcohols	Hexanol; n-butanol; benzyl alcohol;
Fatty acids	2-phenyl ethanol; amyl alcohols Octanoic acid; hexanoic acid
Esters	Isoamylacetate; diethylsuccinate
Aldehydes	Acetaldehyde; branched aldehyde
Furanoic compounds	Furfural; furfuryl alcohol; HMF (5-hydroxy-methyl furfural
Nitrogen compound	2-aminoacetophenone
Lactone	Sotolon

Table 1.1 Odour-active compounds affected by aging.

The single flavour impact of the aromatic compounds can be suppressed or enhanced by the interaction between different aroma volatiles. Furthermore, low amounts of a substance could be positive, whereas higher amount can have a negative effect for the aroma quality, and even sub-threshold concentration can influence sensory perception due to the synergic effects (Culleré et al., 2007).

1.5.1 Aromatic compounds degraded during aging

1.5.1.1 Terpenes

The monoterpene alcohols characterize the wine aroma of many varieties, such as: Muscat and Riesling (Rapp & Mandery, 1986). During the storage, due to the oxidation phenomena, the monoterpens alcohols, such as geraniol, linalool, decrease to dihydric alcohols and terpene dioxides which have a higher perception threshold. (Simpson, 1979; Simpson & Miller, 1983). In many cases, the changes of their concentration result from biochemical rearrangement and hydrolysis. In the aged wine an increased concentration of nerol oxide, linalool oxide, hotrienol and alpha-terpineol is observed (Zoecklein et al., 1999; Williams et al., 1980; Chisholm et al., 1995); moreover, 1,8-terpine was produced by the degradation of alpha-terpineol. Alpha-terpineol was proposed as aging marker because its concentration increased linearly during the storage time (Rapp & Mandery, 1986).

The major amount of the monoterpens are detected as bound forms and as non-volatile moterpene glycosides. Some of these compounds in aged wine are esterified with glycosides, but also with other compounds such as hydroxylated linalool (Simpson & Miller, 1983; Gunata et al., 1986; Zoecklein et al., 1999). The storage not only decreased the free monoterpenes, but also the bond forms of linalool, nerol, geraniol and alpha-terpineol (Zoecklein et al., 1999).

1.5.1.2 Norisoprenoid compounds C_{13}.

During wine aging, some of the compounds are produced by the carotenoid degradation which are important contributors to the aroma of aged wines. An important exemple of this oxidative degradation is 1,1,6-trimethyl-1,2-dihydronaphathalene (TDN), which is responsable for the kerosene-like note in Riesling wines (Marais et al., 1992). Apart from TDN, vitispiranes can also contribuite to the bottle-aged character (Zoecklein et al., 1999). Franciolo et al., (2003) showed that the concentration of TDN and vitispiranes increased with the storage time in sparkling wines. The precursors of TDN are formed by an acid-catalyzed rearrangement of glycosylated norisoprenoids and possibly non glycosylated compounds (Winterhalter et al., 1990; Zoecklein et al., 1990).
Another important norisoprenoid compound is β-damascenone which shows a large decrease upon aging (Rapp et al., 1985). β-damascenone is positively correlated to pleasant aromatic descriptor (cooked apple aroma) (Aznar et al., 2003) but is also described as off-flavour with a strong note of prunes in red wine (Pons et al., 2008).

1.5.1.3 Higer alcohols and fatty acids

Changes in the concentration of fatty acid and higher alcohols depend on the degradation or formation of corresponding esters. An increase of higher alcohols, during the aging, can be explained by acid-catalyzed ester hydrolysis (Rapp et al 1985) as well as decrease or an increase of fatty acids is linked to the chemical esterification or hydrolysis (Ramey & Ough, 1980; Rapp et al., 1985; Zoecklein et al 1999).
Rapp et al., (1985) reported that, during the wine storage in bottle, the higer alcohols such as: hexanol, n-butanol and free benzyl alcohol increased whereas 2-phenyl ethanol and amyl alcohols showed a decrease. On the contrary other author reported that the higher alcohols remain pratically constant in wine (Marais & Pool, 1980). The concentration of octanoic acid and hexanoic acid show no changes during the storage of wine (Marais & Pool, 1980).

1.5.1.4 Esters

The acetate esters and ethyl esters are the main volatile esters and they are mainly formed during the alcoholic fermentation in wine. Diaz-Maroto et al., (2005) reported that the acetate esters are of particular importance for aromatic matrix of wine, but also the ethyl esters of straight-chain fatty acid with an even number of carbon atoms, such as ethyl decanoate, ethyl actanoate and ethyl hexanoate are described as positive flavours of young wine and they give floreal and fruity flavour note. Their concentration can increase or decrease, during the aging, due to chemical esterification or hydrolysis towards chemical equilibria (Simpson et al., 1979). The levels of ethyl esters of straight-chain fatty acid and the acetate esters also, decrease during storage (Diaz-Maroto et al., 2005). This trend is confermed by Zoecklein et al (1999); Simpson et al., (1979); Perez-Coello et al (2003) that reported a decrease of both ethyl and acetate esters such as: isoamylacetate; hexyl acetate, 2-phenylethyl acetate. ethylcaprate, ethyl butyrate, ethyl caproate, ethyl hexanoate, ethyl capryate and hexyl hexanoate. The acetate esters are hydrolyzed more rapidly than the ethyl ester, so fruity and floreal aromas will not lost very quickly. On the other hand, Marais & Pool, (1980) showed that the concentrations of ethyl acetate, diethyl malate, ethyl lactate, diethyl succinate and ethyl monosuccinate, increased during storage.

1.6 Aromatic compounds formed during aging

1.6.1 Aldehydes

During the aging of wine, volatiles aldehydes represent a group of compounds which are subjected to concentration changes. The aldehydes are formed by the oxidation alcohols. The oxidative deterioration of wine aroma is often related to the synthesis of aldehydes (Culleré et al., 2007). The chemical production of acetaldehyde in wine is strictly related to the presence of oxygen and catalysts such as copper and iron (Elias et al., 2010). A further formation pathway rises from the dissociation of hydrogensulfide from combined ethanol following to the oxidation of free sulfur dioxide. Inceases have been attributed to abnormal conditions of wine exposure to oxygen or have been found in the case of Sherry aged with the solera system (Escudero et al., 2002).
The Gas chromatography-olfactory studies of Silva Ferreira et al., (2003b); Escudero et al., (2000) showed that the wine aged at high levels of dissolved oxygen and high temperature generated a rapid oxidative spoilage, which was linked to the presence of strong-smelling aldehydes such as: methional and phenylacetaldehyde.
Aznar et al., (2003) reported that the pleasant aroma descriptor were negatively correlated to phenylacetaldehyde. Sensory investigation (Silvia Ferreira at al., 2003b) showed that the formation of phenylacetaldehyde was correlated to honey-like aroma of oxidative wine.
(E)-2-nonenal aldehyde is reported to be particularly important, since it can be responsable for "plank" or "sawdust" off-flavour (Ferreira et al., 2004; Chatonnet & Dubourdieu, 1998). Moreover branched alphatic aldehydes such as, methylpropanal, 2-methybutanal and 3-methylbutanal were detected in Porto and Sherry wines (Culleré et al., 2007). Further aldehydes are formed during aging such as, hexanal and hexenal (Chisholm et al., 1995). These later oxidative degradation compounds are generally not present above their perception threshold but, together with other aldehydes, can be considered as markers for the oxidation.

1.6.2 Furanoic compounds

2-ethoxyfuran, and ethyl 2-furoate, 2-furfural and 5-hydroxymethyl furfural showed to increase in concentration with storage time (Simpson & Miller, 1983; Fernandez de Simon et al., 2006). Furthermore, it is showed that the volatile 2-furanmethanthiol is produced from the precursor furfural and is detected in barrel-fermented white wine, but the 2-furanmethanthio and furfural levels can also increase during the bottle storage of the sparkling wine. However, (Fernandez de Simon, et al., 2006) reported that furfural can also be produced in wine during bottle aging by hexoses and pentoses sugars.
Sugars are also reported as the precursors of furfural, a reaction catalyzed by heat and acid environment, but oxygen seems not be involved (Simpson, 1979; Simoson & Miller, 1983).

1.6.3 2-aminoacetophenone

The gas chromatography-mass spectrometer studies (Rapp at al., 1993) reported that 2-aminoacetophenone (2-AAP) is identified as a character impact compound for the untypical aging off-flavors. In *Vitis labruscana* grapes, 2-AAP is responsable for the foxy note (Nelson et al., 1977; Acree et al., 1990). In Vitis vinefera grapes is also present in a concentration > 0,2 ug/L (Hoenicke et al., 2002).
The sensory evaluation by Christoph et al., 1995 showed that the percepiction threshold in water is 0,2 ug/L whereas in white wine the threshold is generally 0,5-1,0 ug/L. 2-AAP is mainly generated during the storage of wine. After the alcholic fermentation are detected only low

concentration of 2-AAP in wine; it is formed after the sulphiting. Moreover, in unsulphited wine 2-AAP was not found (Christoph et al., 1998; Hoenicke et al., 2002). The 2-AAP formation pathways in wine are still not known. The 2-AAP seems to be formed to the presence of precursor indolacetic acid due to sulphiting. The oxidation of sulphite induces the generation of superoxide radicals which results in the cleavage of the indole ring and the formation of 2-AAP from indolacetic acid (Figure 1.3) (Christoph et al., 1998; Hoenicke et al., 2002). Further compounds can be cooxidized to 2-AAP due to the sulphiting such as: kynurenine, tryptophane and indole latic acid, but the rate formation of 2-AAP is lower than that from indolacetic acid (Christoph et al., 1998/1999; Hoenicke et al., 2002).

2-APP is also generated by yeast metabolism from tryptophane but normally only amounts below the percepition threshold (Ciolfi et al., 1995; Rapp et al., 1995; Sponholz et al., 1997). Sponholz et al., (1997); Huhn et al (1999) showed that 2-AAP formation is weakly affected by the yeast strain. A low storage temperature (< 10°C) can strongly reduce but not prevent 2-APP formation (Hoenicke et al., 2002). The addition of antioxidant additives is the only suitable enological method to reduce the risk of untypical aging off-flavours. The use of ascorbic acid before or immediately after the sulphiting, is an effective method to decrease the formation of 2-AAP (Gessner et al., 1998/1999).

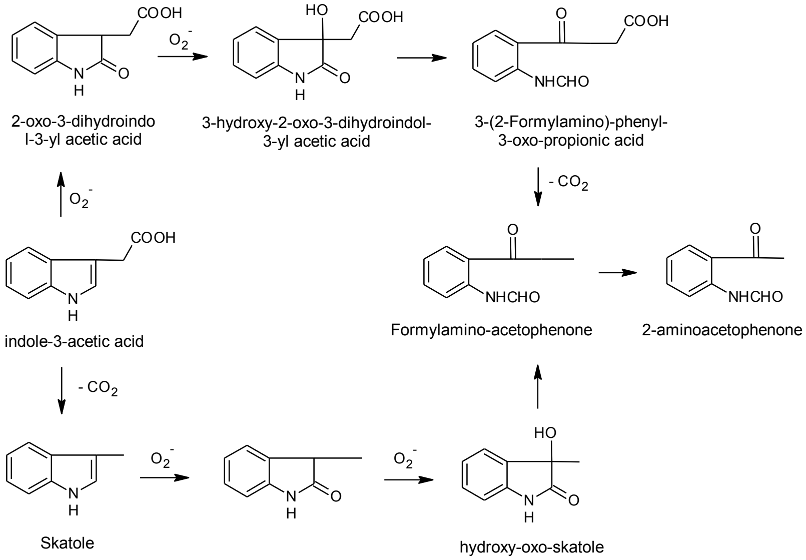

Figure 1.3 Proposed formation of 2-aminoacephenone (2-APP) by co-oxidation of indolacetic acid (according to **Hoenicke et al., 2002**).

1.7 Sotolon

Sotolon (3-hydroxy-4,5-dimethyl-2(5H)-furanone) is a powerful flavour compound with an intense spicy /curry odor (Girardon et al.,1986). Sotolon has been identified and quantified in different wines such as: Botrytised (or noble rot) wines (Masuda et al., 1984), Port (Silvia Ferreira et al., 2003), Vin Jauen (Pham et al., 1995), Sherry (Martin et al., 1992), Madeira (Camara et al., 2004) Vins Doux Naturels (Schneider et al., 1998) and in barrel-aged white wines (Lavigne et al., 2008). Its perception threshold in wine is 10 µg/L (Guichard et al., 1993). The levels of sotolon concentration detected in different wines are reported in Table 2. Although it is considered a typical flavour note in Madeira, Port, Sherry and long aged sweet wines, sotolon is considered to be one of the compounds responsible for the atypical aging and oxidative off-flavour in dry white wines, when its concentration is higher than the perception threshold.

Wine	**Type of wine**	**Sotolon concnetration (µg/L)**
Botrytised (or noble rot) wines	sweet wine	5-20
Port wines	fortified wine	5-958
Vin Javen	Flor-wine	120-268
Sherry wines	fortified wine	0-500
Madeira wines	fortified wine	0-2000
barrel-aged white wines	White dry wine	0-140
Vins Doux Naturels	fortified wine	0-26

Table 1.2 Concentration of sotolon in different type of wines (Masuda et al., 1984; Silvia Ferreira et al., 2003; Pham et al., 1995; Martin et al., 1992; Camara et al., 2004; Lavigne et al., 2008).

1.7.1 Chemical and physical characteristics of sotolon

Sotolon is a a chiral lactone with e very powerfull aroma of burnt/spicy. Its boiling point is at 184°C and its density is 1.049 g/cm^3. It contributes significantly to the burnt note of roasted coffee (Blank et al., 1992), aged saké (Takahashi et al., 1976) and sugar cane (Tokitomo et al., 1980) to spicy/curry note of fenugreek (Girardon et al., 1986), lovage (Blank & Schieberle, 1993) and condiments, as well as to the nutty flavor of botrytised wines (Masuda et al., 1984), Porto wines (Silvia Ferreira et al., 2003) and Sherry wines (Martin et al., 1992). Its perception threshold is extremely low, 0.02 ng/L in air (Blank et al., 1996) and 10 µg/L in wine (Pons et al., 2008). The following threshold values were found in water: detection/nasal 0.3 µg/L (Blank et al., 1996) detection/retronasal 0.01 µg/L (Tokitomo et al., 1980) and recognition/nasal 70 µg/L (Rodel & Hempel, 1974). Both enantiomers R and S of sotolon are present in white wine (Figure 1.4) (Pons et al 2008). In sythetic wine the perception threshold of (S)-sotolon (0.8 µ g/L) was 100 times lower than that of the (R) form (89 µ g/L), indicating that (S)-sotolon contributes to the characteristic aroma of untypical-aging of dry white wines. The commercial dry white wines from different vintages showed three types of distribution patterns: the racemic form, an excess of R enantiomer, and an excess of S enantiomer.

The proportions found in these wines may be explained by the slow racemization kinetics (20 months) of optically active sotolon. Sotolon showed to be stable in an ethanolic solution (14% v/v of ethanol) at the pH 3.10 (Martin et al., 1990), but it is unstable at pH 11 as described by Kobayashi (1989). Furthermore the instability was showed to be irreversible when the pH was decreased.

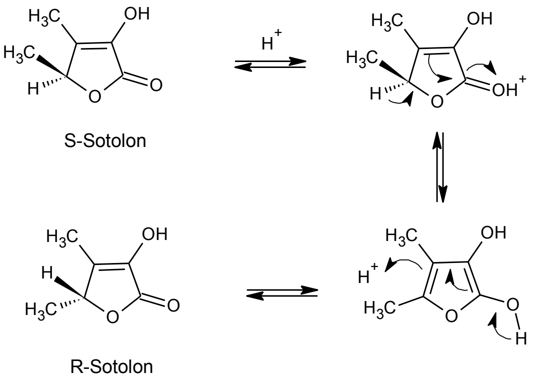

Figure 1.4 Sotolon racemization via enolization (Pons et al., 2008).

1.7.2 Sotolon formation pathways

Sotolon develops in wine making and ageing from a number of chemical pathways among which thermal degradation of intermediate compounds of the Maillard. 4-hydroxy isoleucine is also described as precursor of Sotolon (Figure 1.5). Reducing sugars such as glucose, ribose, rhamnose are reported to be involved as well as ascorbic acid, dehydroascorbic acid, ethanal and ethanol in the formation of sotolon. In spite of the wide number of precursors described the final chemical steps are likely due to ethanal and α -chetobutirric acid (Konig et al., 1999).

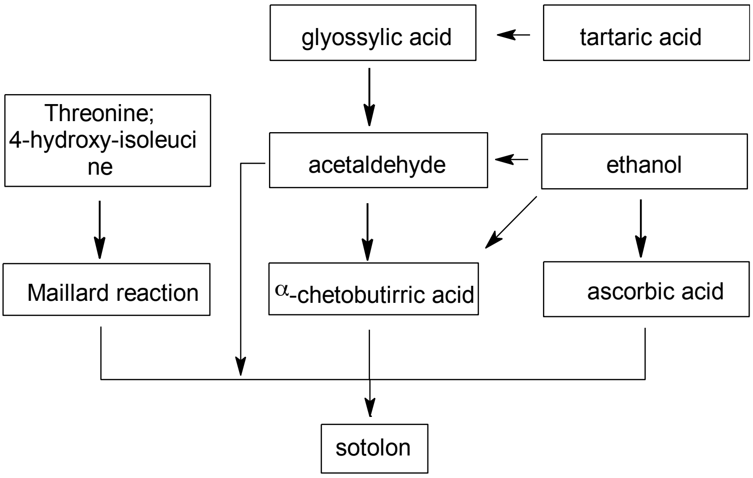

Figure1.5 Summary diagram of the sotolon formation pathways.

Due to the number of chemical and physical factors that influence the sotolon formation and the high number of formation pathways in wine, this compound has been suggested as a chemical marker of white wine shelf-life (Lavigne & Dubourdieu, 2004).

1.7.2.1 Formation of sotolon from 2-chetobutirric acid and ethanal

Pham et al., (1995) studied the sotolon formation simulating the maturation of Vin Jaune under the yeast film, whereas Takahashi et al., (1976) in aged saké. Both authors showed that the sotolon production involves with an aldol condensation reaction between 2-ketobutirric acid and ethanal. Ethanal is produced by the oxidation of ethanol (Elias & Waterhouse, 2010) whereas the formation of 2-ketobutirric resulting from the deamination of threonine or formed by the condensation of two molecules of acetaldehyde (Figure 1.6) (Konig et al., 1999; Cutzach et al., 1999).

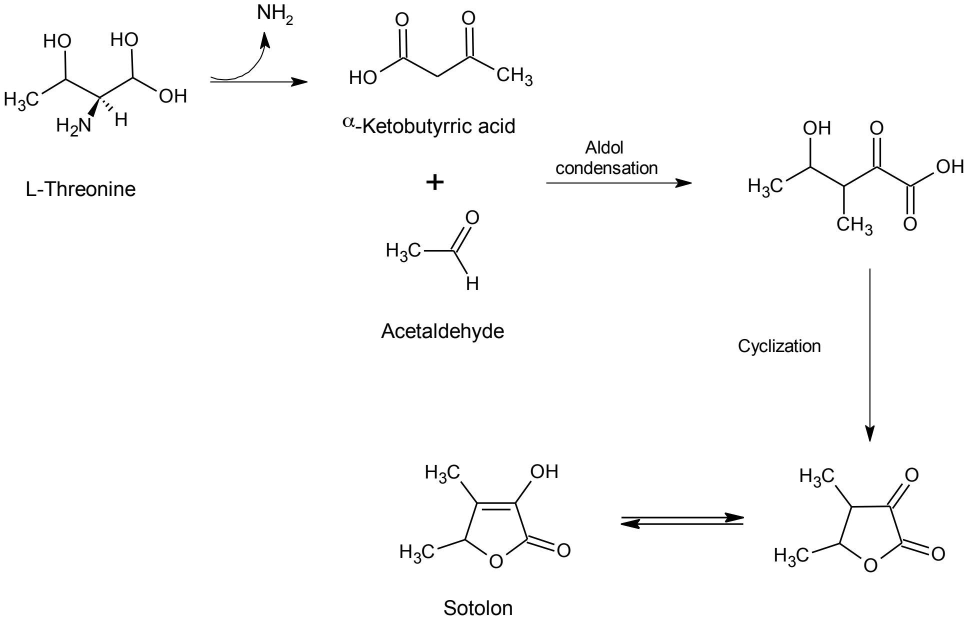

Figure 1.6. Formation of sotolon from α-chetobutyric acid (**Pham et al., 1995).**

1.7.2.2 Sotolon precursor: ethanal procuction by ethanol oxidation

The oxidative reaction from ethanol to acetaldehyde uses a catechol as primary substrate. This system is coupled to the O_2/H_2O_2 system by the Fe^{3+}/Fe^{2+} redox couple (Clark, 1960). Moreover both ferric and ferrous ions are required to complete the oxidative cycle. The ferric ions are reduced by oxidizing the polyphenols, whereas ferrous ions reduce hydrogen peroxide. The oxidation of ethanol to acetaldehyde by oxygen is thermodynamically favored, but is not spontaneous. The formation of H_2O_2 and its reduction by iron (Fe^{2+}) produces highly reactive hydroxyl radicals, which react with ethanol to give ethanal (Figure 1.7).

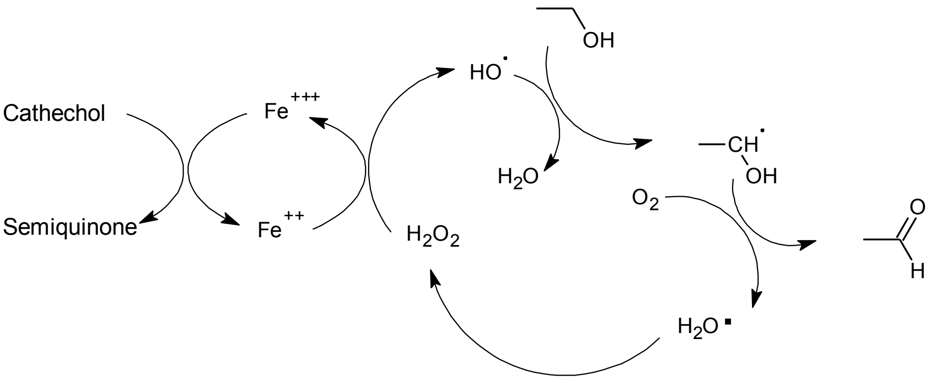

Figure 1.7 Possible additional payhway for the reduction of oxygen in the oxidation of ethanol in oxygenated wine (Danilewicz, 2003).

1.7.2.3 Thermal generation of sotolon from amino acid model systems with glycolic and pyruvic acid.

The study of (Guerra Et al., 2011) investigated the role of 2-keto acid (pyruvic and pyruvic glyoxylic acids) in the Maillard reaction, alanine and glycine were chosen as model amino acids. The results of this work showed that such mixture could generate 3-amino-4,5-dimethyl-2(5H) -furanone (A) which is a precursor of sotolon. The synthesis of compound (A) can predict two step formation pathway. The first step involves the formation of 4,5-dimethylfuran-2,3-dione (B) and the second step involves its conversion in compound (A) through the reaction with amino acids. The 4,5-dimethylfuran-2,3-dione (B) can be produced by two differents pathways such as: reaction between pyruvic acid and formaldehyde source and reaction between self-aldol product of ethanal and glyoxylic acid (Figure 1.8).

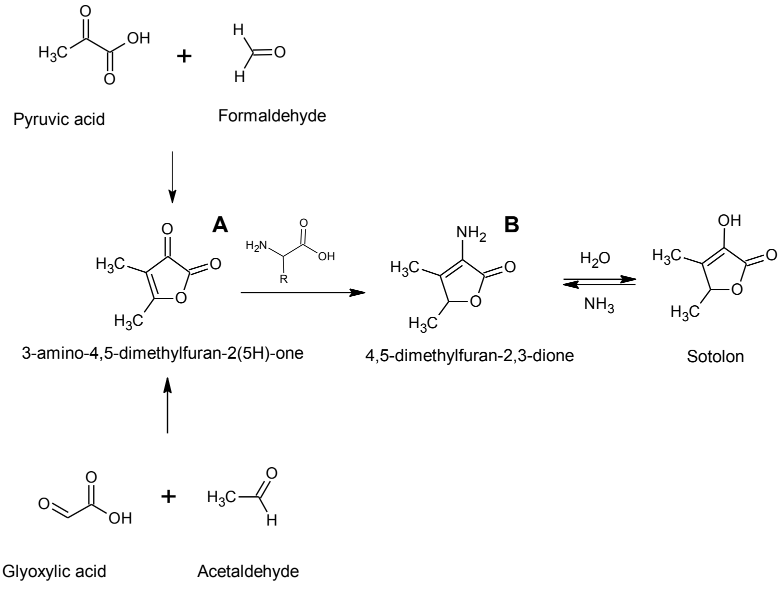

Figure 1.8 General pathway of formation of sotolon (Guerra Et al., 2011).

1.7.2.4 Sotolon precursor: glyoxylic acid production by tartaric acid oxidation

The α-hydroxyacids of wine are also substrates of the Fenton degradation. L(-)-malic acid and lactic acid are degraded oxidatively to 2-oxobutanedioic acid and pyruvic acid , respectively, under Fenton conditions (Merz & Waters, 1949; Oszmianskym et al., 1996).
Fenton, (1894) shewed that iron-catalyzed oxidation of tartaric acid needs a limited amounts of hydrogen peroxide to form dihydroxymaleic acid (A) (Fenton, 1899; Fenton, 1905), although afterwards other authors reported that the reaction intermediate was identified as the trans-isomer dihydroxyfumaric (B) (Hartree, 1953; Gupta Et al., 1953). The oxidative degradation of (B) involves to a further oxidation to diketosuccinic acid with the simultaneous reduction of Fe^{3+} to Fe^{2+} (Fenton, 1905). This acid is unstable in aqueous solution and its decarboxylation leads to hydroxymalonic acid (Chow & Vennesland, 1958). Dihydroxyfumaric acid is also unstable and its decomposition at room temperarure leads to the formation of glycolaldehyde. The formation pathway of glyoxylic acid is unclear (Oszmianski et al., 1996).

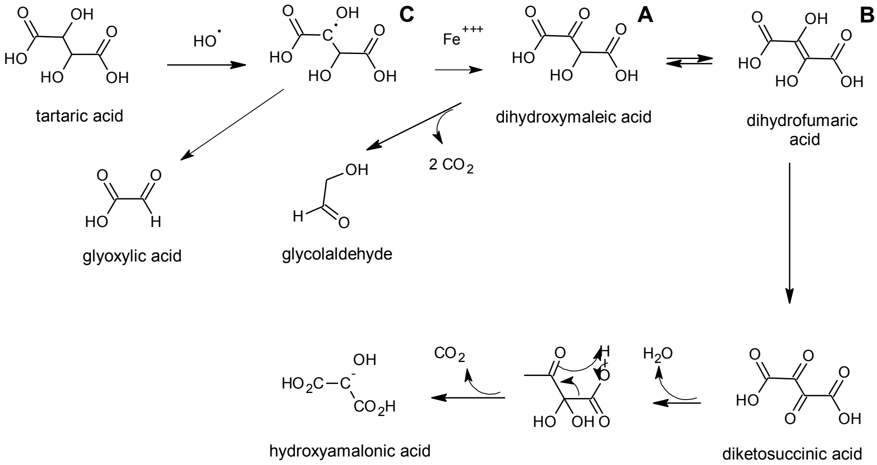

Figure 1.9 Fenton oxidation of tartaric acid (Danilewicz, 2003).

A possibility is 2C-3C bond cleavage of the initial radical (C). Such a reaction is showed with ethylene glycol, where formaldehyde is a minor oxidation product (Merz & Waters, 1949). Another potential source for glyoxylic acid formation could be glycolaldehyde. Fulcrand et al., (1997); Santos-Buelga et al., (1999) reported that in synthetic wine tartaric acid was oxidized to glyoxylic acid to a significant amount despite the presence of a large molar excess of ethanol (Fulcrand et al., 1998). The tartaric acid degradation could involve a reaction with the hydroxyl radical to give glyoxylic acid (Figure 1.9).

1.7.2.5 Production of sotolon from ethanol and ascorbic acid

Konig at al., (1999) showed that the sotolon formation, in model solution (ethanol, ascoric acid and water) stored for 2 week at 70 °C, is strictly linked to the presence of ethanol and ascorbic acid. The generation of sotolon involves two differents pathways: in one pathway, two molecules of ethanol react with ascorbic acid whereas in the second pathway only one molecule of ethanol are incorporated in sotolon molecule.

1.7.2.6 Sotolon production by Maillard reaction

The Maillard reaction is one of the most common reactions that occur in food products during their processing and storage. The reaction occurs between reducing sugars and compounds containing a free amino group including amino acids, peptides and proteins, producing a wide range of colouring (typically brown) and aromatic compounds. Therefore, it can have a strong impact on the appearance, flavour, and nutritional value of many food products. Hoffman & Schieberle, 1997; Hoffman & Schieberle, 1995 reported that the Maillard reactions in diferent combinations of mixtures of L-cysteine (3.3 mmol) and three sugars such as D-glucose or L-rhamnose or D-ribose (10 mmol) have generated a significant amount of sotolon (Table 1.3). L-rhamnose showed more ability to form sotolon than D-glucose and D-ribose.

	FD Factor		
Odorant	D-glucose	L-rhamnose	D-ribose
3-hydroxy-4,5-dimethyl-2(5H)-furanone (sotolon)	16	128	64

Table 1.3 Formation of sotolon by differents reducing sugars The flavor dilution (FD) factors of the odor-active compounds were determined by AEDA (Hoffman T. and Schieberle P. 1997 and Hoffman T. and Schieberle P. 1995)

Moreover, sotolon is also suggested to be generated by Maillard reaction as a result of condensation of molecules such as butane-2,3-dione (diacetyl) and hydroxyacetaldehyde (Silvia Ferreira et al., 2003a).

1.7.2.7 Formation of sotolon from 3-Hydroxy-L-isoleucine

Blank et al., 1996; Blank et al., 1992 explained the sotolon formation by thermal induced oxidative deamination of 4-Hydroxy-L-isoleucine (HIL). The presence of sotolon was assessed by reacting HIL with different mono- and alpha-dicarbonyl compounds in a phophate-buffered model system at 100°C for 60 minutes (Table 1.4).

Carbonyl compounds	Sotolon (µg/mg HIL)	Yield (mol %)
2,3-butandione	0,34 +/- 0,03	<0,1
2,3-pentadione	0,30+/- 0,03	<0,1
methylglyoxal	64,2+/- 0,3	7,4
phenyglyoxal	22,2+/- 0,3	2,5
propionaldehyde	1,00+/- 0,06	0,1
phenylacetaldehyde	0,24+/- 0,03	<0,1

Table 1.4 Formation of sotolon from HIL (Blank I. et al 1996).

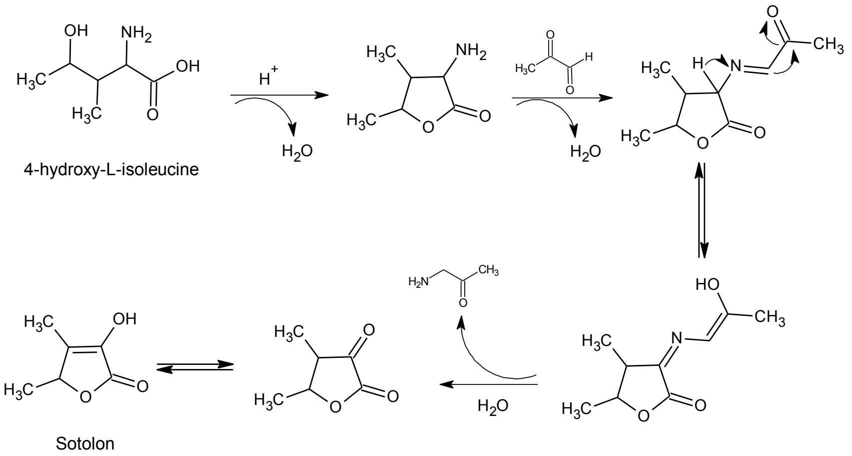

Figure 1.10 Formation of sotolon from 4-Hydroxy-L-isoleucine (HIL) using methylglyoxal as the carbonyl reactant (Blank et al 1996).

Table 1.4 shows that both 2,3-butandione and 2,3-pentadione produced lower ammount of sotolon whereas the highest yield of sotolon was from methylglyoxal and phenylglyoxal. The formation of sotolon involves an acid-catalyzed cyclization of HIL leading to the corresponding lactone which reacts with an alpha-dicarbonyl to form the Schiff base. The later compound is rearranged and hydrolysed, from amino group, to give sotolon Figure 1.10.

1.7.3 Chemical and physical factors affected the sotolon formation in wine

Several papers report the important role of winemaking and storage on the sotolon formation in dry white wines (Lavigne et al., 2008) fortified white wines (Camara et al., 2004) and sweet white wine (Cutzach et al., 2000).

Many authors showed that the formation of sotolon in wine is affected by different chemical and physical factors such as: reducing sugar concentration and storage time (Camara et al., 2004); storage temprature and different containers (Cutzach et al., 2000; Lavigne et al., 2013); concentration of oxygen (Lavigne et al 2008; Cutzach et al ., 1999) and antioxidant compounds concentration (SO2 and glutathione) (Dubourdieu & Lavigne, 2004).

1.7.3.1 Reducing sugar concnetration and storage time

Camara, et al., 2004 evaluated the levels of sotolon and the influence of storage times in 86 aged Madeira wines samples (1-25 years old) with different sugar concentrations such as: 110 g/L for Malvazia , 90 g/L for Boal, 65 g/L for Verdelho varieties and 25 g/L for Sercial.

The level of sotolon was dependent on the storage time in oak barrel with a high correlation coefficient (r = 0,917). The concentration of sotolon increased with aging of wine from 100 μg/L (wines 6 years old) to 1000 μg/L (wines 25 yars old).

The average concentration of sotolon increased from dry wine (Sercial 25 g/L) to sweet wine (Malvazia 110 g/L). Analyzing dry wine samples with the same aging (11 years) it was shownthat the lowest value of sotolon (258,7 μg/L) as opposed to sweet wines that reported a higher content (825,8 μg/L).

Moreover other authors (Schneider et al 1998; Hofmann et al., 1995; Konig at al., 1999) havefurther confirmed that the sugars degradation by Maillard reaction was responsable for producing the furanic compounds. Furthermore the results of this study explained that there is a strong correlation between sotolon and sugar derivatives: furfural, 5-methylfurfural, 5-hydroxymethylfurfural, and 5-ethoxymethylfurfural. These compounds are also well linked with wine aging. These findings indicate that the kinetics of sotolon formation is related with residual sugar contents and storage time.

1.7.3.2 Oxygen

The impact of oxygen and barrel-aging on the formation sotolon were tested by Lavigne et al., (2008); Cutzach et al., (1999). The former author showed that in dry white wines aged in barrels without lees the amount of sotolon was higher that in wines stored with the lees. The lees's capacity to combine oxygen explains their protective effect on the sotolon formation. Moreover the dry white wine demostrated a high correlation (r = 0.939) between dissolved oxygen and sotolon's level.
Cutzach et al., (1999) confermed that the level of sotolon in an oxygenated environment were higher than those measured in absence of air in sweet fortified white wine.

1.7.3.3 Storage temperature and different containers

The relationship between storage temperature, different containers and the level of sotolon was tested in white fortified wines. The winemaking was carried out in three different containers such as: new barrels, 2-years-old barrels and concrete vats (Cutzach et al., 2000). The wines were stored in either air-conditioned or unconditioned environments. The temperature in air-conditioned cellar was kept between 16-18°C, whereas the temperatures in non-air-conditioned winery fluctuated from 8°C (winter season) to 33°C (summer season). The level of sotolon was checked at 6 and 30 months. The results showed that amount of volatile compounds (isomers of methyl-γ-octalactone; sotolon; ethoxymethylfurfural) were affected by the storage temperature and type of container.
In both range of temperature the highest concentration of sotolon was detected in the wines aged in new barrels (Lavigne et al., 2013). The highest level of sotolon (105 ug/L) after 30 months was assessed in the wines aged in new barrels and in non-air-conditioned environment.
The best protection by formation of sotolon was assessed in wine aged in concrete vats stored air-conditioned cellar (16-18°C). The results obtained are confermed by a laboratory study of the formation mechanisms of the volatile compounds during the aging of sweet fortified wines (Cutzach et al., 1999).

1.7.3.4 Antioxidant compounds

Dubourdieu & Lavigne, (2004) showed the relationship between sotolon ammount and glutathione (GSH) concentration during the storage of the bottled dry white wine. The wine was added to 10 mg/l of glutathione and stored for 3 years. The sotolon concentration in the samples treated with glutathione presented three times less the sotolon level that the control (without addition). At the bottling the addition of GSH prevent the erosion of the varietal aroma content and the appearance of untypical aging and oxidative off-flavour in dry white wines

1.7.4 Analytical methods

Several analytical methods are described in literature for the sotolon quantification in fortified white and red wines (Martin et al 1992; Silvia Ferreira et al., 2003) and in dry white wines (Lavigne et al., 2008). Nevertheless, the analytical procedures proposed from other authors for the sotolone assessment are often tedious and solvent consuming (Konig et al., 1999; Escudero et al., 2000). Due to the low levels occurring in dry white wines, extraction and purification steps by organic solvents (liquid-liquid extraction) or solid-phase extraction (SPE solid-liquid extraction) are always needed (Escudero et al., 2000). Purified samples are eventually processed by GC (Konig et al., 1999) though an HPLC (Konig et al., 1999) separation method was proposed.

1.7.4.1 Liquid/liquid and solid phase (SPE) extraction in wine

Alcoholic beverages, as wine, can present more than 1300 volatile compounds, some of which are important analytical compound that could influence the quality of wine because of different reasons such as, their aromatic properties (Guth, 1997; Etievant, 1991), their possible use as markers of origin (Ferreira et al., 1996; Alvarez & Cabezudo, 1988), and the fact that some of them can be suggested as chemical markers of white wine shelf-life (Lavigne & Dubourdieu, 2004).
Although the volatile compounds usually have low molecular weight and have hydrophobic properties, the chemical and physical properties of aromatic compounds are represented in the volatile fraction of an alcoholic beverage (Nykänen & Suomalainen, 1982; Maarse & Visscher, 1989). A second and main characteristic of the analytical techniques of volatile compounds is the fact that wine contains eathanol and several other fermentation compounds, which will influence the isolation of volatile compounds. The presence of alcohol affects the extraction yield of the different solvent and adsorbent systems. The most common solution involved the use of liquid–liquid extractions with various selected solvent systems (Ferreira et al., 2000) or the use of different solid-phase extraction (SPE) systems (Zhou et al., 1996; Ferreira et al 2000). Several extraction solvent are reported in literature such as: chloroform (Takahashi et al., 1976); freon 113 (Escudero et al., 2000); dichloromethane: freon113 (1:9) (Martin et al., 1991); pentane: dichloromethane (2:1) (Konig et al., 1999); dichloromethane (Schneider et al., 1998; Drawert & Rapp, 1996); ethil-acetate:pentane (1:3-1:20) (Ferreira et al., 2000); freon-11 (Cobb & Bursey, 1978) and diethiletere:pentane (1:1-1:9) (Ferreira et al., 2000). All of above mentioned extraction procedures require an extraction step with solvents and a following concentration step. Other authors have reported the use of SPE cartridges for the extraction of sotolon. (Ferreira et al., 2000) tested several SPE cartridges (C_8 , C_{18} ; XAD- 2 , XAD -4 , XAD 7 , XAD -16) whereas (Ferreira et al., 2003) used a resin LiChrolut EN. The above cited extraction procedures require long extraction times (up to 48 h) (Escudero et al., 2000), high volumes of sample (up to 500 ml) (Konig et al., 1999; Takahashi et al., 1976) and solvents (up to 250 ml) (Konig et al., 1999) (Schneider et al., 1998) so increasing theanalytical high cost.

1.7.4.2 Instrumental techniques for the sotolon quantification in wine

Many authors described several gas-chromatography methods such as: GC-O, MDGC-MS, HRGC-MS (Konig et al., 1999); HRGC-MS-O-analysis (Escudero et al., 2000); GC-O (Ferreira et al., 2003); 2D-capillary-GC (Martin et al., 1991); GC-MS (Pons et al., 2010; Camara et al., 2004; Lavigne et al., 2008; Ferreira et al., 2003) and 2D-GC (Martin et al., 1992).
Other authors reported (Guichard et al., 1993; Konig et al., 1999) the separation and quantification of sotolon in liquid chromatography (HPLC-UV). (Guichard et al., 1993) obtained

by the chromatographic separation (HPLC-UV) through a reverse phase (RP) column LiChropher 100 Diol (250 * 4 mm 5 uM), mobile phase consisting of CH2Cl2: hexane (60/40) and a detection UV at 232 nm. (Konig et al., 1999) carried out the quantification of sotolon in liquid chromatography (HPLC-UV), using a C_{18} stationary phase (250 uM Europher 5 * 5 mm), mobile phase consisting of water at pH 2.55 and acetonitrile. The analyte were detected at 232 nm. The analysis time was 30 minutes. The two previously reported methods were able to quantify sotolon in Vin Jaune, Vin de Paille and Tokai and in citrus soft drinks, while an analytical method able to detect sotolon in dry white wine was yet not reported.

To have a practical application in sensorial wine investigations, an analysis method should have to be able to measure concentrations below the odour threshold. Guichard et al., (1993) (HPLC-UV) reported a LOD of 10 µg/L in Vin Jaune, Vin de Paille and Tokai, whereas Camara et al., (2004) (GC-MS) reported a LOD of 1.2 µg/L in Madeira wines. Lavigne at al., (2008) (GC-MS) reported a LOD of 1.2 µg/L in dry white wines while Ferreira et al., (2003) (GC-MS) reported a LOD of 0.84 µg/L in white wines.

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1.9 Aim of the study

Sotolon (3-hydroxy-4,5-dimethyl-2(5)-furanone) is the thermolabile chiral lacton, stable at acid pH and in a polar solvent. Though it is considered a typical flavour note in Madera, Porto, Sherry and long aged sweet wines, it is an off-flavour in dry white wine where its oxidative note is detrimental for the fresh taste and odour (Pons et al., 2008).

Recently, the need for many cellars to lower the dose of sulfur dioxide in bottled wine has led to use of antioxidants alternative to SO_2. For this reason, the winemakers often use different types of phenolic antioxidant compounds whose efficacy has not been studied yet. Dubourdieu & Lavigne (2004) reports that the use of SO_2 protects the wine from the formation of sotolon, but the effect of phenolic compounds is not yet clear. The formation of sotolon was checked in SO_2-free sparkling wine because it is characterized by: the presence of residual sugar, the limited content in grape polyphenols, limited content of O_2, low storage temperature and the need to preserve its color and flavor. Furthermore, several authors reported that sugars (Camara et al 2004), exposure to oxygen (Cutzach et al 1999), temperature (Cutzach et al., 2000) and the polyphenolic matrix (Cutzach et al., 1999) could affect the sotolon formation in wine so, the SO_2-free sparkling was considered suitable to study the factors that influence the formation of sotolon.

To better understand the effect of phenolic compounds on the production of sotolon was developed and validated a fast, sensitive and easy to apply analytical method (UHPLC-UV) and it was applied to the evaluation of SO_2-free Franciacorta DOCG wines in order to assess the effect of different disgorgement conditions (antioxidant additives, ageing time and temperature) on the sotolon formation. The sotolon concentration was measured in sparkling wine stored at 15°C and 25°C for 6 months added to three different antioxidant preparations (2 g/hL and 4 g/hL) potentially substituting the sulfur dioxide cointaining ellagic plant extract from Vitis vinifera, gum arabic and enriched with *Saccharomyces cerevisiae* cell wall. Furthermore, we investigated the chemical and physical factors could affect the sotolon formation in synthetic wine. Model solution conitaing increasing concentration of pentoses, GSH, amino group, catechin, oxygen, ethanal, tartaric acid and iron are stored at two temperature (70°C and 5°C) for five days in order to clarify the compositive factors affecting the sotolon synthesis in white wine. Finally, we compared the performances of analytical methods (HPLC-UV and UHPLC-MS) for sotolon quantification, which were previously developed.

1.10 Reference

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Section 1

UHPLC quantification of sotolon in white wine

1.11 Materials and Methods

1.11.1 Chemicals and reagents

Sotolon (purity exceeding 97%), methanol, ethanol, dichloromethane (DCM), heptane, chloroform, sodium chloride (NaCl), anhydrous sodium sulphate were purchased from Sigma-Aldrich (St. Louis, MO, USA). Polyvinylpolypirrolidone (PVPP) was purchased from Dal Cin (Sesto San Giovanni, Milan, Italy). All the chemicals were of analytical grade, at least. HPLC grade water was obtained by a Milli-Q system (Millipore Filter Corp., Bedford, MA, USA). The synthetic wine contained 5 g/L tartaric acid in 12% ethanol/water solution (v/v), adjusted to pH 3.5 with 12 M sodium hydroxide (Sigma-Aldrich).

1.11.2 Commercial wine samples

Sotolon content was assessed in 18 sparkling (Brut-nature *Champenoise* method) and 12 dry white wine samples produced from three different grape cultivars (Chardonnay, Catarratto and Trebbiano) in the vintages 2011-2013.

1.11.3 Sample preparation development

1.11.3.1 Liquid/liquid extraction trials

An aqueous solution containing sotolon 25 µg/L was extracted by using different solvents: heptane, chloroform and DCM. This solution (300 mL) was liquid/liquid extracted once with 100 mL of each solvent stirring for 30 min. The organic phase was separated by a separatory funnel, added with 2 g of anhydrous sodium sulphate and dried under-vacuum. The dry material was dissolved in 2 mL of water which was submitted to UHPLC separation.
Partition coefficient and extraction yield were calculated as follows:

$$K = \frac{C_s}{C_a} = \frac{g_s \times V_a}{(g_0 - g_s) \times V_s} \quad (1)$$

$$g_s^n = \frac{K \times V_s \times g_0}{V_a + K \times V_s} \times \left(1 - \frac{K \times V_s}{V_a + K \times V_s}\right)^{n-1} \quad (2)$$

$$e_y^n = \frac{\sum_1^n g_s}{g_0} \quad (3)$$

Where:
K: partition coefficient;
C$_s$: concentration of analyte in the extraction solvent;
C$_0$: concentration of analyte in the water solution;
$\boldsymbol{g_s}$: mass of solute in the extraction solvent obtained with a single extraction step;
$\boldsymbol{g_0}$: overall mass of solute;
V$_s$: volume of extraction solvent;
V$_a$: volume of water solution;

g_s^n: mass of solute in the extraction solvent following to the n extraction step(s);$\mathbf{n}$: number of extraction steps;
e_y^n: overall extraction yield after n extraction step(s).

The influence of NaCl concentration on the partition coefficient was tested. Thirty milliliters of synthetic wine solution containing 1 mg/L of sotolon were added with NaCl 20 g/L, 50 g/L and 100 g /L. These solutions were extracted once with 30 mL of DCM (30 min shaking with Griffin Flask Shaker) and prepared as described as above.
Moreover, the assessment of the extraction yield after 10, 20, 30 and 40 minutes shaking under the above extraction conditions was carried out on synthetic wine solution (sotolon 1 mg/L) added with NaCl 100 g/L.

1.11.3.2 Sample purification trials

Three SPE resins were tested: C_{18} 360 mg (Waters, Milford, MA, USA), Polymer-based 200 mg (Phenomenex, Torrence, CA, USA) and in-lab packed PVPP 50 mg. The SPE columns were loaded with 2 mL of sotolon (5 mg/L) in aqueous solution and the eluted solutions were collected. Then, methanol 5%, 10%, 20%, 40%, 60%, 80% and 100 % (v/v) were tested as eluting solvent.

1.11.3.3 Sample preparation

Three grams of NaCl were dissolved in 30 mL wine in a 100 mL bottle then 40 mL of DCM were added. The bottle was hermetically closed and shaken for 10 minutes with a wrist action stirrer (Griffin Flask Shaker). The mixture was centrifuged 5 min at 5000 × *g* and the DCM was separated by a separatory funnel and recovered. This solvent extraction procedure was carried out for 3 times; the organic solvent fractions were jointly collected and added with 2 g of anhydrous sodium sulphate. The DCM was evaporated under-vacuum; the dry material was dissolved with 2 mL of methanol 5% which was purified by a PVPP 50 mg SPE cartridge and eluted solution was recovered.

1.11.3.4 Calibration curves

Calibration graphs were obtained by spiking known amounts of sotolon to water (117 µg/L - 438 µg/L) and white wine (2.36 µg/L - 23.4 µg/L).

1.11.4 Validation procedure

1.11.4 .1 Precision parameters

Spiked white wine (young Chardonnay/Trebbiano wine, vintage 2011) samples containing sotolon 2.36 µg/L, 4.67 µg/L, 11.7 µg/L and 23.4 µg/L were prepared to assess the response linearity of the analytical method response. Each sample was submitted to three replicated determinations for three different days (nine determinations in total per sample). Standard aqueous solutions containing sotolon 117 µg/L, 219 µg/L and 438 µg/L were submitted to triplicated UHPLC separations in three different days to assess the response linearity of aqueous solutions.

1.11.4 .2 Recovery

The recovery of the method was assessed by comparing the peak areas obtained from the aqueous solutions (116.8 µg/L, 218.7 µg/L and 437.7 µg/L) directly injected to the spiked wine samples (7.8 µg/L, 14.6 µg/L and 29.2 µg/L equivalent to a 15 dilutions) prepared as above. The recovery was evaluated through triplicated determinations performed in three different days.

1.11.4 .3 Limit of detection and of quantification

The LOD was calculated as indicated as follows:

$$\boldsymbol{LOD = a + 3s_{x/y}} \quad \mathbf{(4)}$$

Where:
a: intercept point of the regression line;
$\boldsymbol{s_{x/y}}$**:** standard deviation of linear regression.

1.11.4 .4 Ultra high performance liquid chromatography

The UHPLC separation was performed by an Acquity HClass UHPLC (Waters, Milford, MA, USA) system equipped photo diode array detector 2996 (Waters). The column used was Kinetex C_{18} 100 x 3 mm, 2.6 µm particle size, 100 Å pore size (Phenomenex, Torrence, CA, USA). The chromatographic separation was carried out with an isocratic elution running 5% methanol for 4 minutes followed by column washing (100% methanol for 1 minute) and column conditioning (4 minutes). Sotolon was detected at 235 nm wavelength. The injection volume was 20 µL and the column was thermostatted at 30° C. All the samples were filtered through a 0.22 mm pore size PVDF membrane (Millipore, Billerica, MA, USA) before injection.

1.11.4 .5 Quantification of sotolon

Sotolon was quantified in synthetic wine solutions and white wine samples by the external standard method. Chromatographic data acquisition and processing were performed by Empower 2 software (Waters).

1.11.4 .6 Statistical analysis

The equations of the calibration curves were assessed by the linear regression analysis. Differences between the calibration curve slopes obtained in aqueous solution and white wine were evaluated by the F-test and considered significant different at $P < 0.05$.

1.12 Results and Discussion

1.12.1 Analytical method development

The physical-chemical properties of sotolon were firstly evaluated in order to fulfill the analytical sensitivity required. The UV-vis spectra of sotolon in aqueous solution (5 mg/L) showed a maximum absorbance at 235 nm (Figure 1.11) and a high molar extinction coefficient (e°=10255 L cm^{-1} mol^{-1}). Preliminary UHPLC runs showed that sotolon concentrations in the range 60-90 µg/L (values corresponding to 8 – 12 folds higher than the perception threshold) were suitable to gain the required detection and quantification levels.
Therefore, wine volume as low as 20-30 mL could allow the sotolon quantification. Such values were lower than the analytical methods reported in the previous literature (Pons et al., 2010; Lavigne et al., 2008). Due to the low amount of sotolon in wine, its detection and quantification requires an extractive step by organic solventwhich needs to be concentrated, as well. Consequently, a liquid/liquid extraction was developed by evaluating the sotolon extraction yield of heptane, chloroform and DCM which were previously employed (Takahashi et al., 1976; Schneider et al., 1998). The DCM showed an extraction yield 32% higher than chloroform, whereas heptane did not extracted detectable amounts of sotolon (Table 1.5).

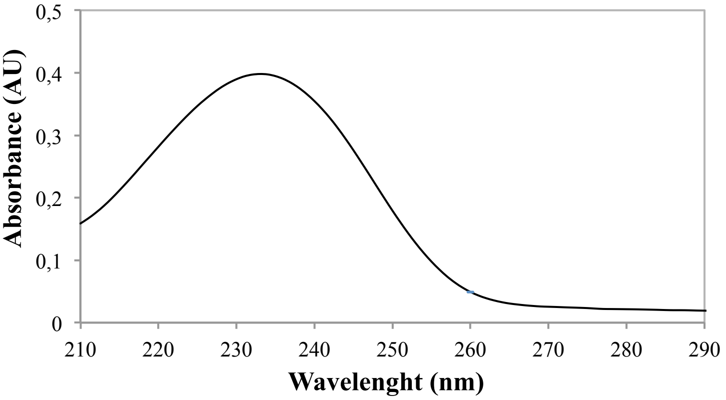

Figure 1.11 Spectrophotometric adsorption spectrum of 39 µM sotolon in aqueous solution.

Solvents	Sotolon extracted (mV*sec.)	Partition coefficient (K)
Heptane	n.d.	0
Chloroform	32	0.81
Dichloromethane	47	2.1

Table 1.5 Sotolon amounts (as peak area) and corresponding partition coefficient obtained with different extraction solvents.

The comparison of the analytical responses (peak areas) obtained from the sotolon in aqueous solution and extracted sample showed DCM had the highest extraction yield (41%) which corresponded to a partition coefficient exceeding 2.1 (Table 1.5). Moreover, DCM volumes ranging from 40 mL to 60 mL were calculated from the partition coefficient (see formulas 1, 2 and 3 reported in Materials and Methods section) allowing the injection of quantifiable sotolon amounts (1.2 – 1.8 μg) taking into account the concentration factor of one order of magnitude for 30 – 40 mL of wine sample, Since sotolon amounts up to 68 - 76 % were extracted by using solvent/wine volume ratios ranging from 1:1 to 2:1, three consecutive extractions were needed to obtain an overall extraction yield approaching 100 %.

Higher extraction yield could be obtained by increasing the ionic strength of the aqueous solution (Xie et al., 1997); therefore, different levels of NaCl (20 g/L, 50 g/L and 100 g/L) were evaluated. As expected, the partition coefficient increased exponentially as the salt concentration increased (Table 1.6). The partition coefficient for the salt-free synthetic wine solution was lower (K=1.8) than the value obtained with the aqueous solution (K=2.1) owing to the solvent properties of ethanol. The highest NaCl content (100 g/L) allowed to double the partition coefficient and decrease the foam formation during the liquid/liquid extraction.

Sodium chloride (g /L)	**Extraction yield (%)**	**Partition coefficient (K)**
0	65.8	1.8
20	86.5	1.9
50	88.5	2.3
100	93.0	3.5

Table 1.6 Extraction parameters of sotolon obtained with different amounts of sodium chloride in synthetic wine solution using dichloromethane as organic solvent.

The effect of the shaking time on the extraction was also investigated. Higher extraction yield was obtained in synthetic wine solution shaken for less of 30 minutes. Up to 30 % sotolon was lost following to 30 minutes shaking (Table 1.7) probably due to its degradation which can occur in DCM (Martin et al., 1990).

Shaking time (min.)	**Peak area (mV*sec.)**
10	258
20	294
30	207
40	222

Table 1.7 Effect of the shaking time on the sotolon amount (as peak area) extracted from a synthetic wine solution with dichloromethane as organic solvent.

In spite of the extraction procedure, some interferences were found in the chromatographic run and the purification step was needed. Three different hydrophobic resins were tested with increasing methanol concentrations as eluting solvent. Sotolon has lower hydrophobic properties than other wine compounds capable to absorb light at 235 nm wavelength, (e.g. polyphenols) owing to the lower molecular weight and the presence of a hydroxyl group in its molecular structure. Therefore, the SPE conditions effective in retaining polyphenols and eluting sotolon were evaluated. Polymer and C_{18} resins showed high retentive properties and sotolon was eluted with methanol 60% and 40%, respectively for Polymer and C_{18} resins. Nevertheless, interfering compounds were still found in the chromatogram. Lower retention property was obtained with PVPP which allowed the sotolon elution by methanol 20%. In order to avoid the sample dilution, the dried sample was dissolved in methanol 5% and 10% before the SPE purification step. Sotolon was not adsorbed on the PVPP resin and it was recovered in the loaded sample. Though the methanol 10% allowed a sotolon recovery 2% higher (Table 1.8), the methanol 5% was chosen because an interference-free chromatogram was obtained (Figure 1.12).

Methanol (%)	**SPE Polymer 200 mg**	**SPE C_{18} (360 mg)**	**SPE PVPP (50 mg)**		
loaded	n.d.	0.4	56.0	56.0	56.0
5	-	-	-	31.2	-
10	-	-	-	-	34.4
20	0.1	37.7	43.2	-	-
40	29.4	54.1	0.6	-	-
60	69.9	0.5	tr	-	-
80	0.4	0.5	tr	-	-
100	0.1	n.d.	tr	-	-
Cumulative	99.9	92.3	99.8	87.2	89.4

Table 1.8 Sotolon recovery (%) obtained with different SPE resins and methanol concentrations as eluting solvent.

Finally, the wine samples were prepared using the optimized analytical conditions: 30 mL of wine added with NaCl 100 g/L, 3 extraction steps with 40 mL of DCM and 10 minutes shaking each, vacuum drying, sample recovery with 2 mL of methanol 5% (v/v), SPE purification on PVPP resin and HUPLC run. The chromatographic separation occurred with low-methanol content (5%) in very short time (2.9 min) due to the low hydrophobicity and molecular weight of sotolon. Moreover, this method was solvents saving for both sample preparation (Konig et al., 1999; Schneider et al., 1998) and chromatographic separation. No interfering peak was found and the sotolon peak was baseline-separated (Figure 1.12).

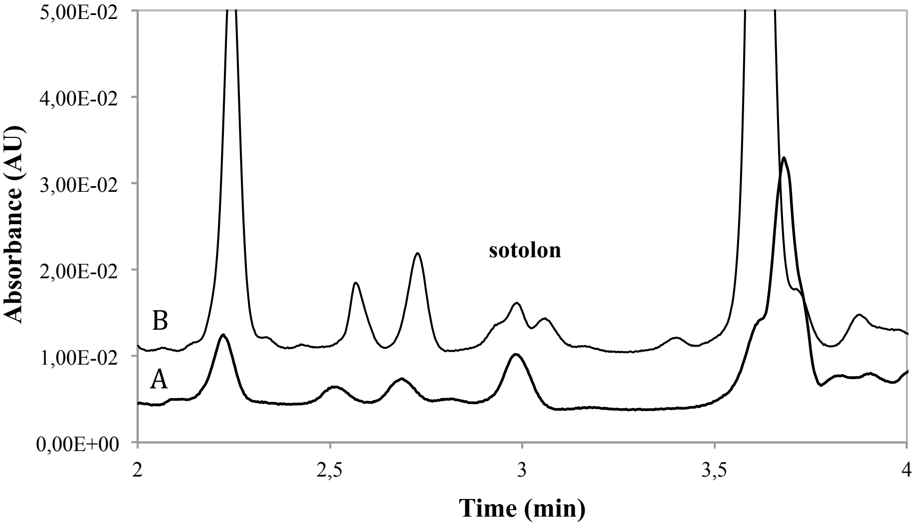

Figure 1.12 UHPLC separation of white wine samples dissolved in methanol 5% (A) and 10% (B) after drying under-vacuum and SPE purification with PVPP.

The precision parameters were evaluated for the sotolon concentrations (up to 25-30 µg/L) usually reported in the literature for the dry white wine (Martin & Etiévant 1991; Cutzach et al., 1999; Lavigne et al., 2008) and in agreement with its perception threshold (< 8 µg/L). Therefore, sotolon levels up to 23 µg/L were evaluated in both aqueous solution and white wine. The analytical method showed linear response (R^2>0.999) in the range tested with both the matrices (Figure 1.13) and the calibration curves obtained were significantly different ($p<0.05$).

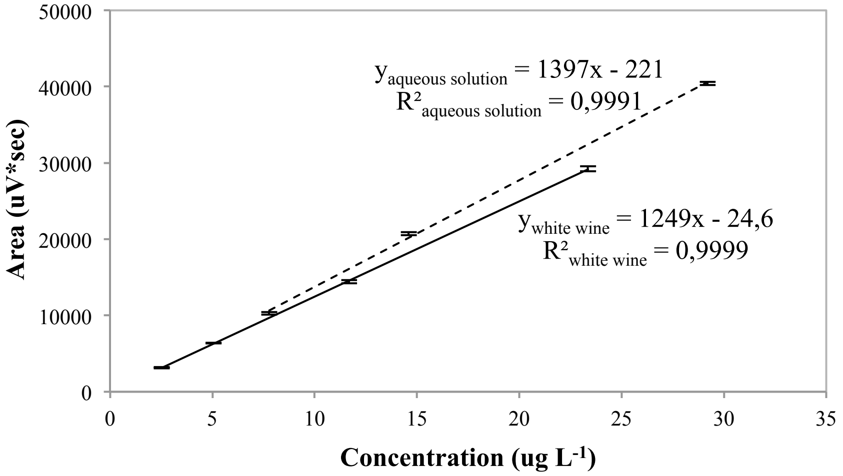

Figure 1.13 Calibration curves for sotolon in aqueous solution (dashed line) and white wine (solid line). Standard deviations are reported by vertical bars (n=9).

The response factor (as calibration curve slopes ratio) was 10% lower for the white wine samples prepared as previously described in comparison to the corresponding aqueous solutions directly injected probably due SPE purification step. This difference needs to be taken into account for the sotolon quantification.

Amounts as low as 2.5 μg/L were accurately quantified in white wine samples though with slightly higher precision values (Table 1.**9**). Nevertheless, relative intermediate repeatability values lower than 10% were calculated for sotolon level as low as 2.3 μg/L and 3.1% for higher concentration values.

Concentration added (μ g/L)	2.34	4.67	11.68	23.36
Concentration calculated (μ g/L)	2.37	4.72	11.55	23.41
Concentration calculated (μ g/L)	2.33	4.43	10.53	21.14
SD repeatability **(** μ g/L)	0.11	0.063	0.18	0.27
SD intermediate repeatability **(** μ g/L)	0.10	0.060	0.19	0.26
Repeatability limit **(** μ g/L)	0.24	0.13	0.48	0.68
RSD (%)	3.36	0.85	1.36	1.08
Average RSD (%)	1.66			

Table 1.9 Precision parameters of sotolon quantification (n=9). Legend: SD: standard deviation (μg/ L); RSD: relative standard deviation (%%).

The LOD of sotolon in white wine was 0.029 μg/L, a value much lower than the perception threshold in white wine. It is lower than the LOD values reported by other researchers also considering the GC techniques (Guichard et al., 1993; Camara et al., 2004; Lavigne at al., 2008; Ferreira et al., 2003). The sotolon recovery was evaluated by spiking white wine and its values were 97.7%, 91.1%, and 89.5% , respectively for sotolon concentration as 7.78 μg/L, 14.60 μg/L and 29.20 μg/L. Such values were higher than those previously reported (Guichard et al., 1993; Martin & Etiévant, 1991; Bailly et al., 2009).

1.12.2 Quantification in commercial white wine

The proposed method was applied to the determination of sotolon in some commercial white wine samples (Brut-nature sparkling and still dry wines). In spite of the very low sugar content and the high CO_2 pressure occurring during bottle corking and storage, the highest sotolon levels (6 μg/L and 13 μg/L) were detected in two samples of sulfur dioxide-free and ascorbate-free sparkling wines (samples 11 and 12, respectevely stored seven months at 25°C (Table 1.10). The role of sulfur dioxide in preventing sotolon formation is well known (Lavigne et al., 2008) even if other SO_2-free sparkling wine samples (e.g. samples 1 and 2 in Table 1.10) did not contained detectable amounts of sotolon. No perceptible concentrations of sotolon were found in two samples of still Cataratto dry wine (samples 19 and 20). Sotolon was not detected or quantified in the others 26 commercial samples of both sparkling and still wines analyzed. Our results were in agreement with the data previously reported (Martin & Etiévant, 1991; Dagan et al., 2006; Riberau-Gayon et al., 2006; Lavigne et al., 2008) and highlight a deeper knowledge on chemical, physical and technological factors affecting sotolon formation in wine is needed.

Code	Grape cultivar	Vintage	Description	Sotolon
1	Chardonnay	2011	Sparkling wine	n. d.
2	Chardonnay	2011	Sparkling wine	n. d.
3	Chardonnay	2011	Sparkling wine	n. d.
4	Chardonnay	2011	Sparkling wine	n. d.
5	Chardonnay	2011	Sparkling wine	n. d.
6	Chardonnay	2011	Sparkling wine	n. q.
7	Chardonnay	2011	Sparkling wine	n. q.
8	Chardonnay	2011	Sparkling wine	n. q.
9	Chardonnay	2011	Sparkling wine	n. q.
10	Chardonnay	2011	Sparkling wine	n. q.
11	Chardonnay	2011	Sparkling wine	6.4
12	Chardonnay	2011	Sparkling wine	13.4
13	Chardonnay	2011	Sparkling wine	n. d.
14	Chardonnay	2011	Sparkling wine	n. q.
15	Chardonnay	2011	Sparkling wine	n. d.
16	Chardonnay	2011	Sparkling wine	n. q.
17	Chardonnay	2011	Sparkling wine	n. d.
18	Chardonnay	2011	Sparkling wine	n. q
19	Catarratto	2011	Dry white wine	3.6
20	Catarratto	2011	Dry white wine	3.0
21	Chardonnay/ Trebbiano	2010	Dry white wine	n. d.
22	Chardonnay/ Trebbiano	2010	Dry white wine	n. d.
23	Chardonnay/ Trebbiano	2011	Dry white wine	n. d.
24	Chardonnay/ Trebbiano	2011	Dry white wine	n. d.
25	Chardonnay/ Trebbiano	2011	Dry white wine	n. d.
26	Chardonnay/ Trebbiano	2012	Dry white wine	n. d.
27	Chardonnay/ Trebbiano	2012	Dry white wine	n. d.
28	Chardonnay/ Trebbiano	2012	Dry white wine	n. d.

29	Chardonnay/ Trebbiano	2012	Dry white wine	n. d.
30	Chardonnay/ Trebbiano	2012	Dry white wine	n. d.

Table 1.10 Sotolon amounts (μg/L) detected in commercial samples of Brut-nature sparkling and dry white wine. Legend: n. d.: not detectable; n. q.: not quantifiable.

1.13 Conclusion

The proposed analytical method provides a sample preparation faster and easier-to-apply than those previously reported for the routine analyses of sotolon. It can be a suitable analytical tool for the investigation of the sotolon formation of in either model solution or white wine. The sensitivity parameters allowed the the investigation of sotolon formation kinetic at an adequate concentration levels. Finally, this method can be considered a suitable analytical tool for monitoring the oxidative aging and storage evolution of commercial white wine and sparkling wine. Our preliminary data in model system showed the suitability of the method for the application to more complex synthetic wine matrices in terms of selectivity in order to assess the role of certain wine compounds (e. g. sugars, acetaldehyde) on sotolon formation (data shown in part 3). Moreover, the analytical method previously developed will be used to evaluate the sotolon amount in Franciacorta sparkling wines that were produced without SO_2. Wine was treated in disgorgement without antioxidant compounds, with SO_2 (50 mg/hl) and with three commercial antioxidant mixture (Ant.ox 1-2-3). All samples were store at two temperatures (25°C-15°C) and the commercial antioxidant compounds were added in two concentrations (2-4 g/hl).
The checking of sotolon amount in sparkling wines produced without SO_2 will allow to better understand the influence of temperature and the composition of different commercial antioxidants on the sotolon production.

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Section 2

Determination of sotolon content in South African white wines by two novel HPLC-UV and UHPLC-MS/MS methods

1.15 Materials and Methods

1.15.1 Chemicals

4,5-Dimethyl-3-hydroxy-2,5-dihydrofuran-2-one (≥97%); dichloromethane (≥99.8%); sodium chloride (≥99.5%); methanol (≥99.9%); acetonitrile LC-MS CHROMASOLV®(≥99.0%) ; iso propanol LC-MS CHROMASOLV® (≥99.0%) and anydrous sodium sulphate (≥99.0%) were purchased from Sigma-Aldrich (St. Louis, MO, USA). UPLC water was obtained from Milli-Q filtration system (Millipore Filter Cor., Bedford, MA, USA). PVPP resin was purchased from Dal Cin Gildo spa (20099-Sesto San Giovanni MILANO). The model wine (M.W.) contained 12% (v/v) ethanol and 5 g/L of tartaric acid, and the pH was adjusted to 3.5 with sodium hydroxide (Sigma-Aldrich St. Louis, MO, USA*).*

1.15.2 Samples

Sotolon analysis was carried out on 70 commercial South African white wines The commercial wines were produced from ten different grape cultivars (Sauvignon Blanc, Chardonnay, Chenin blanc, Viognier, Semillon, Grenache blanc, Pinot Grigio, Colombard, Gewurztraminer and Rhine Riesling) and sixteen different vintages (from 1983 to 2013). The wine samples coded by number (1-65) were sourced directly from local cellars, while the wines coded by letter (a-e) were stored for 2 years at 37°C.

1.15.3 Sample preparation

3 g/L of NaCl were added to 30 mL of white wine. The wine was extracted two times with 20 mL each of CH_2Cl_2 for 10 min with stirring. The organic phases were combined and 2 g anhydrous Na_2SO_4 was added to remove water traces. CH_2Cl_2 was evaporated to dryness under a nitrogen stream and the dry material was re-dissolved in 2 mL of 5% methanol solution. The concentrated extract was further purified with 50 mg of PVPP resin. The solution was filtered (0.22 μm PVDF Millipore) before injection.

1.15.4 Ultra-high-performance UHPLC–MS/MS and HPLC-UV analysis

UHPLC-MS separations were performed with a Waters Acquity HClass UHPLC system equipped with a mass spectrometer, API and microtriple quadropole. The column used was a BEH C18, 2.1 x 100 mm from Waters. Detection (MS) conditions used were MRM mode, positive ionization, parent ion m/z 129, daughter ions 55 and 83. The mobile phases were (A) 1% formic acid in water and (B) methanol:acetonitrile:iso-propanol (49:49:2) and the flow rate 0.4 mL/min. The injection volume was 3 μL and the column temperature was at 30° C. HPLC-UV separations were performed with an Agilent 1260 Series system fitted with a diode array detector. The column used was a Kinetex C18 100 x 3 mm x 2.6 μm from Phenomenex. The sotolon was detected at 235 nm. The mobile phases used were (A) water and (B) methanol and the flow rate 0.45 mL/min. The injection volume was 20μL and the column temperature 30° C. The gradients are reported in **Table 1.11.**

UHLPC-MS/MS gradient				**HLPC-UV gradient**			
Time (min.)	Flow (mL/min)	Eluent A (%)	Eluent B (%)	Time (min.)	Flow (mL/min)	Eluent A (%)	Eluent B (%)
0.0	0.4	91	9	0	0.45	95	5
3.0	0.4	91	9	8	0.45	95	5
3.1	0.4	30	70	9	0.45	0	100
4.0	0.4	0	100	11	0.45	0	100
5.0	0.4	0	100	12	0.45	95	5
5.1	0.4	91	9	20	0.45	95	5
6.5	0.4	91	9				

Table 1.11 Solvent gradients condition. UHPLC-MS/MS (A: water/ formic acid 1% and B: methanol:acetonitrile:iso-propanol (49:49:2)) and HPLC-UV (A: water and B: methanol)

1.15.5 Validation procedure of HPLC-UV and UHPLC-MS/MS methos

The validation of the method was carried out with respect to qualitative (selectivity) and quantitative (linearity, LOD, LOQ, repeatability and accuracy) parameters, as indicated below:

1.15.5.1. Selectivity

Selectivity was tested by spiking model wine and white wine with 10 µg/L sotolon, performing the sample preparation procedure and the separation, and comparing the chromatograms. The selectivity of the method was evaluated by comparing the results obtained for the detection of sotolon in the absence and presence of possible interferences originating from the white wine matrix.

1.15.5.2 Linearity

The linearity interval tested was 5-50 µg/L in wine and in model solution, with extractions done in duplicate. The limit of detection (LOD) and limit of quantitation (LOQ) were calculated as the lowest concentration of analyte in a sample that resulted in a signal-to-noise ratio of 3 and 10, respectively. The baseline noise was calculated by the software.

1.15.5.3 Accuracy (recovery test)

Accuracy was measured for two levels of sotolon, 10.7 µg/L and 21.5 µg/L. Spiked model wine (at the two levels), white wine (blank) and the same spiked white wine (at the two levels) were extracted in duplicate.

1.15.5.4 Precision

Precision was expressed as repeatability (within-day measurements) and intermediate precision (day-to-day measurements). Two levels of sotolon in model wine and in white wine were used (10.7 µg/L and 21.5 µg/L). The extractions were done in triplicate, and the intermediate precision was measured over three days. For the instrumental repeatability, samples containing 21.5 µg/L sotolon extracted from wine and model wine were injected five times. The relative standard deviation (RDS) values were calculated for the peak areas and retention times.

1.16 Results and Discussion

1.16.1 Sample prep

Compared with conventional analytical procedures proposed by other authors, the method described above presents a sample prep step (extraction and purification) which is faster, with higher sensitivity and easier to apply in practice. Other authors have proposed procedures which used high extraction times (up to 48 h), (Escudero et al., 2000), high volumes of wine (up to 100 mL) (Pons et al., 2010 and Lavigne, et al., 2008) and of solvents (up to 250 mL) (Konig et al., 1999; Schneider et al., 1998).

1.16.2 Comparison of the validation results for the UHPLC-MS/MS and HPLC-UV methods

The comparison between the two instrumental methods used was done using the validation parameters for each method.
Selectivity was evaluated by comparing the sotolon peak in the presence and in the absence of interferences from the matrix. The UHPLC-MS/MS and HPLC-UV chromatograms of samples resulting from the sample prep of spiked model wine and dry white wine are reported in Figure 1.14/ 1.15

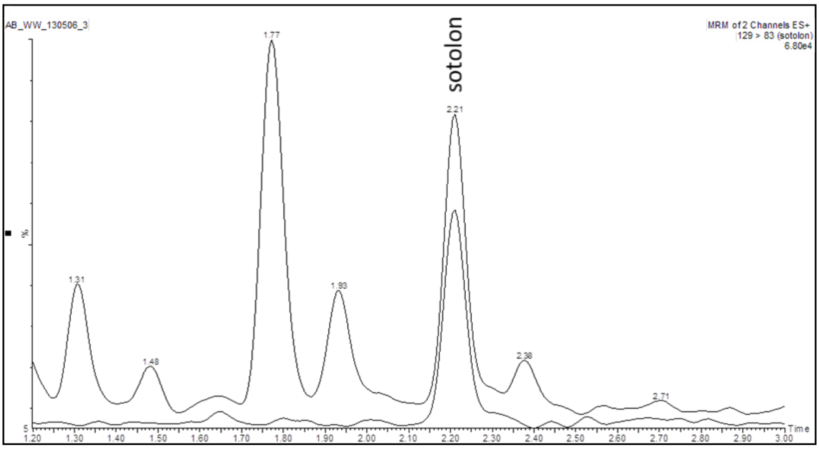

Figure 1.14 Sotolon peak from spiked model wine (bottom trace) and spiked white wine (top trace) in UHPL-MS/MS.

The retention times of sotolon were 2.2 min and 5.7 min in UHPLC-MS/MS and HPLC-UV separation respectively. Even though in the UHPLC-MS/MS chromatogram there can be seen much less interferences and the baseline noise is lower, sotolon could also be measured in with the HPLC-UV method without interferences from the matrix.

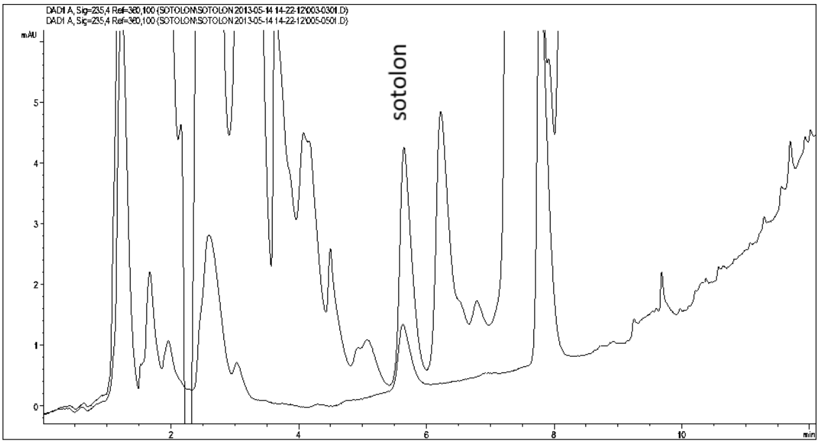

Figure 1.15 Sotolon peak from spiked model wine (bottom trace) and spiked white wine (top trace) in HPLC-UV. detection at 235 nm.

The linearity interval of quantification was evaluated in young dry white wine and in synthetic wine at six concentration levels (5,38; 10,76; 16,14; 21,52; 32,28 and 53,80 µg/L). The samples were prepared and injected in duplicate for the synthetic and dry white wine at each concentration level. The linearity correlation coefficients (R^2) were calculated from the linear regression analysis and are reported in Table 1.12. Both methods showed good linear response; this linear range is in accordance with those previously reported in wine (Camara et al., 2004; Lavigne et al., 2008; Pons et al., 2010).

Method	Matrix	R^2	LOD (µg/L)	LOQ (µg/L)
HPLC-UV	Wine	0.9986	0.259	0.862
	Model wine	0.9957	1.394	4.643
UHPLC-MS/MS	Wine	0.9953	0.001	0.003
	Model wine	0.9598	0.004	0.013

Table 1.12 Linearity parameters of UPLC-MS and HPLC-UV methods.

The odour threshold of sotolon in wine is approximately 10 µg/L. To have a practical application in sensorial wine investigations, an analysis method should be able to measure concentrations below the odour threshold. Guichard et al., (1993) reported a LOD of 10 µg/L in Vin Jaune, Vin de Paille and Tokai, whereas Camara et al., (2004) reported a LOD of 1.2 µg/L in Madeira wines. Lavigne at al., (2008) reported a LOD of 1.2 µg/L in dry white wines while Ferreira et al., (2003) reported a LOD of 0.84 µg/L in white wines. The UHPLC-MS/MS method we developed had a LOD value of 0.001 µg/L and LOQ was 0.003µg/L in wine. The HPLC-UV method had LOD and LOQ values for wine of 0.259 µg/L and 0.862 µg/L, respectively. As can be seen from Table 2, the values for LOD and LOQ in model wine were higher than in white wine; this could be attributed to a higher extraction yield from the wine than from the synthetic medium. The reason for this possible higher yield is not clear at this stage and can be investigate in a follow on study. The precision of a method is a measure of the extent to which individual test results of multiple repeated procedures on a series of standards agree. Therefore it is important to evaluate both the precision of the sample preparation procedure and that of the instrumental method.

The levels chosen for testing the precision were 10 and 20 µg/L; the first close to the odour threshold and the second a medium-high white wine level. The RSD% was calculated for peak areas, and the values are indicated in Table 1.13. For the day-to-day determination there was no observable trend. Generally, the repeatability for wine sample was better than for model wine.

Method	**Matrix**		**Repeatability peak area %RSD**	**Accuracy**
		Level, µg/L	Average ± SD	%
HPLC-UV	Wine	10	7.59±0.70	75.50
		20	7.54±3.15	78.61
	Model wine	10	15.18±5.13	--
		20	12.79±1.73	--
UHPLC-MS/MS	Wine	10	14.32±3.11	77.44
		20	7.45±3.04	87.72
	Model wine	10	8.43±9.72	--
		20	12.16±1.02	--

Table 1.13 Repeatability and accuracy parameters of UPLC-MS and HPLC-UV methods.

The repeatability and intermediate precision values are acceptable for the determination of sotolon, but they could be improved if an internal standard have been included in the procedure.

For the HPLC-UV method the average %RSD for peak areas were 7,56 and 13,98 for wine and model wine, respectively. For the UHPLC-MS/MS method the %RSD for peak areas were 10,89 and 10,29 for wine and model wine, respectively. For retention times, the %RSD was 0.23 for the HPLC-UV method and 5,45 for the UHPLC-MS/MS method.

Accuracy measures the amount of analyte that is quantified relative to the amount present in the sample. In other words, recovery tests will indicate the amount of analyte quantified in the presence and in the absence of matrix interferences. The recovery values (Table 3) were acceptable for both methods (over 75%), but the use of an internal standard would increase the accuracy of the measurements.

By comparison with GC-MS and HPLC methods, the UHPLC-MS/MS method showed improved sensitivity and speed for sotolon quantification in white wines (Guichard et al., 1993; Camara et al., 2004; Lavigne et al., 2008). While the HPLC-UV method was shown to be acceptable for sotolon determination when present even below the odour threshold, the much lower LOD and LOQ values of the UHPLC-MS/MS method makes it appropriate not only for measurements of sotolon at levels around the odour threshold (important from a sensory point of view), but also for metabolic studies, where much lower concentrations could be of interest. However, the majority of the analytical methods described in literature for sotolon determination in wine are based on GC-MS (Silva Ferreira et al., 2003; Camara et al., 2004; Lavigne & Dubourdieu, 2004; Oliveira & Silvia Ferreira, 2008). The setting up of an analytical method allowing sotolon determination by using HPLC-UV could represent an alternative tool for oenological and cellar laboratories for sotolon determination that do not have access to GC-MS.

1.16.3 Sotolon quantification in white wine

The developed methods were satisfactorily applied for the determination of sotolon in 70 South African white wines (sweet and dry wines). Sotolon concentrations as determined with UHPLC-MS/MS and HPLC-UV analysis are shown in Table 1.14

Code	**Cultivars**	**Vintage**	**Description**	**Sotolon (µg/L) UHPLC-MS/MS**	**Sotolon (µg/L) HPLC-UV**
58	Chenin Blanc	2013	Dry white wine	0.62	n.d.
59	Chenin Blanc	2013	Dry white wine	0.68	n.d.
60	Chenin Blanc	2013	Dry white wine	0.49	n.d.
63	Sauvignon Blanc	2011	Dry white wine	n.d.	n.d.
64	Sauvignon Blanc	2011	Dry white wine	n.d.	n.d.
65	Semillon	2011	Dry white wine	1.96	2.56
16	Semillon	2010	Dry white wine	0.67	n.d.
17	Semillon	2010	Dry white wine	5.31	4.68
18	Pinot Grigio	2010	Dry white wine	1.55	n.d.
19	Chenin Blanc	2010	Dry white wine	2.95	3.55
20	Colombard	2010	Dry white wine	0.55	n.d.
49	Chardonnay	2010	Dry white wine	9.11	8.13
50	Sauvignon Blanc	2010	Dry white wine	1.07	n.d.
61	Sauvignon Blanc	2010	Dry white wine	0.79	n.d.
62	Semillon	2010	Dry white wine	0.57	n.d.
52	-	2010	Sweet wine	n.d.	n.d.
10	Chenin Blanc	2010	Dry white wine	2.90	2.97
1	Sauvignon Blanc	2009	Dry white wine	n.d.	n.d.
6	Viognier	2009	Dry white wine	1.36	n.d.
8	Sauvignon Blanc	2009	Dry white wine	n.d.	n.d.
20	Chenin Blanc	2009	Dry white wine	0.55	n.d.
21	Sauvignon Blanc	2009	Dry white wine	4.96	7.17
25	Grenache Blanc	2009	Dry white wine	1.34	n.d.
28	Semillon	2009	Dry white wine	0.88	n.d.
31	Chardonnay/Viognier	2009	Dry white wine	1.08	n.d.
43	Sauvignon Blanc	2009	Dry white wine	n.d.	n.d.
48	Chardonnay	2009	Dry white wine	4.50	5.28
51	Sauvignon Blanc	2009	Dry white wine	n.d.	n.d.
9	Viognier	2009	Dry white wine	1.36	n.d.
3	Chardonnay	2008	Dry white wine	3.39	5.75
4	Chenin Blanc	2008	Dry white wine	4.94	5.86
5	Viognier	2008	Dry white wine	4.72	7.36
12	Chardonnay	2008	Dry white wine	2.03	2.05
23	Chardonnay	2008	Dry white wine	4.73	3.34
24	Sauvignon Blanc	2008	Dry white wine	2.17	n.q.
27	Viognier	2008	Dry white wine	1.31	n.d.
39	Chenin Blanc	2008	Dry white wine	1.78	n.d.
44	Sauvignon Blanc	2008	Dry white wine	n.d.	n.d.
42	Sauvignon Blanc	2008	Dry white wine	n.d.	n.d.
47	Chardonnay	2008	Dry white wine	3.46	3.60
2	Chardonnay	2007	Dry white wine	4.98	7.73
7	Chenin Blanc/Colombard	2007	Dry white wine	1.59	n.d.

14	Chardonnay	2007	Dry white wine	1.56	n.d.
15	Semillon	2007	Dry white wine	0.63	n.d.
29	Semillon	2007	Dry white wine	4.32	6.12
32	Viognier/Chenin Blanc/Chardonnay	2007	Dry white wine	n.d.	n.d
33	Rhine Riesling	2007	Dry white wine	n.d.	n.d.
41	Sauvignon Blanc	2006	Dry white wine	n.d.	n.d
45	Sauvignon Blanc	2006	Dry white wine	n.d.	n.d
11	Chardonnay	2005	Dry white wine	5.41	n.q.
13	Sauvignon Blanc	2005	Dry white wine	4.97	6.16
26	-	2005	Dry white wine	n.d.	n.d.
30	Sauvignon Blanc	2005	Dry white wine	1.34	n.d.
46	Sauvignon Blanc	2005	Dry white wine	n.d.	n.d.
57	Chardonnay	2003	Dry white wine	1.62	n.d.
55	Chenin Blanc	2002	Dry white wine	2.62	n.q.
53	-	2002	Dry white wine	0.80	n.d.
40	Chardonnay	2001	Dry white wine	2.60	n.q.
38	Chenin Blanc	2001	Dry white wine	1.36	n.d.
54	Rhine Riesling	2000	Dry white wine	0.82	n.d.
56	-	1999	Sweet wine	8.72	n.q.
36	Gewurztraminer	1988	Dry white wine	2.19	n.q.
35	Colombard	1984	Dry white wine	0.85	n.d.
37	Colombard	1983	Dry white wine	2.04	n.q.
a	Sauvignon Blanc	2011	Dry white wine	23.97	27.09
b	Sauvignon Blanc	2011	Dry white wine	23.47	15.28
c	Sauvignon Blanc	2011	Dry white wine	21.55	25.87
d	Sauvignon Blanc	2011	Dry white wine	36.08	27.29
e	Sauvignon Blanc	2011	Dry white wine	27.58	24.72

Table 1.14 Description and sotolon quantification of dry and sweet white wines analyzed.

Using the UHPLC-MS/MS method the highest sotolon concentrations were found in wine 49 (9.11 µg/L), which was a dry white wine (vintage 2010), and wine 56 (8.72 µg/L) which was a sweet wine of the 1999 vintage. For both these wines, the sotolon concentration was slightly lower than the perception threshold (10 µg/L). By using this method, sotolon was not detected in 15 other wines and it was lower than the perception threshold for most of the wines analyzed (48).

The HPLC-UV method detected the highest sotolon concentration in wine 49 (8.13 µg/L) as well, even if it was lower (-10.8%) than the concentration measured by UHLPC-MS/MS. Sotolon was not detected in 42 wines, 27 wines more than by UHPLC-MS/MS method, and not quantifiable in 7 wines, including wine 56. These differences are probably due to the major sensitivity of MS detection in comparison to UV detection, especially at these relative low levels.

Different sotolon concentrations were measured using the two chromatographic methods. In 12 wines the HPLC-UV method indicted higher sotolon levels, ranging from 1 to 41% for wines 12 and wine 3 respectively. However, in 3 wines (17, 23 and 49) the UHPLC-MS/MS method indicated 11 to 25% higher sotolon concentrations.

(Martin & Etiévant, 1991; Dagan et al., 2006; Riberau-Gayon et al., 2006; Lavigne et al., 2008). Sotolon content in wine are reported to be related to the winemaking conditions, *e. g.* oxidative/reducing conditions, barrel ageing (Schneider et al., 1998; Cutzach et al., 1999) as well as the sugars content in wine (Camara et al., 2004). However, most of the South African wines' sotolon levels were lower than that of the perception threshold, even in wines containing sugar and/or older than 10 years. These levels thus seems much lower than those previously reported (Martin et al., 1992; Silva Ferreira et al., 2003; Camara et al., 2004; Oliveira & Silva Ferreira, 2008), by determining it by HPLC equipment (Giuchard et al., 1993).

However, wines stored at a higher temperature for 2 years (wines a-e) had sotolon levels 1.5 to 3 times higher than the perception threshold. This finding confirms the significant effect that temperature has on sotolon increases in white wine (Cutzach, et al., 1999).

This study, although performed on a limited number of white wines, indicates that sotolon does not occur at concentrations higher than the perception threshold in South African wines stored under normal cellaring temperatures. Elevated storage temperatures could however increase sotolon levels significantly in South African white wines.

1.17 Conclusions

The analytical method developed uses a sample preparation steps that is faster and easy-to-apply in practice than many other previously reported methods. The validation of the methods (UHPLC-MS/MS and HPLC-UV) for sotolon determination had good accuracy, repeatability and linearity, and an absence of interferences in dry white wine. The UHPLC-MS/MS method showed higher sensitivity, but the repeatability was better with the HPLC-UV method. Even so, both methods were proved suitable for the determination of sotolon concentrations in white wine under the sensory perception threshold. The two methods were successfully used for the screening of 70 commercial South African wines' sotolon levels. In general, sotolon does not seem to occur in South African white wines at levels over the perception threshold. However, sotolon levels can be drastically increased in white wines exposed to prolonged high storage temperatures.

1.18 Reference

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Section 3

Evaluation of SO2-free Franciacorta DOCG wines in order to assess the effect of different disgorgement conditions.

1.19 Material and Methods

1.19.1 Chemicals

All the chemicals were of analytical grade at least. 3-Mercaptopropionic acid (3MPA) and p-benzoquinone (pBQ) were purchased from Fluka (Switzerland). Glutathione, sotolon (purity exceeding 97%), ascorbic acid (AA), dehydroascorbic acid (DHA), 1,2-phenylenediamine dihydrochloride (OPDA), dichloromethane (DCM), sodium chloride (NaCl), anhydrous sodium sulphate, putrescine (Put), ethanol, ethanal, ribose, $Fe(SO)_4*7H_2O$ and trifluoroacetic acid (TFA) were purchased from Sigma-Aldrich (St. Louis, MO, USA). Polyvinylpolypirrolidone (PVPP) was purchased from Dal Cin (Sesto San Giovanni, Milan, Italy). Citric acid was purchased from J. T. Baker (Phillipsburg, NJ, US); HPLC grade methanol was from Panreac (Barcelona, Spain), and HPLC grade water was obtained by a Milli-Q system (Millipore Filter Corp., Bedford, MA, USA). The synthetic wine contained 2.5 g L^{-1} tartaric acid in 12% ethanol/water solution (v/v), adjusted to pH 3.5 with 12 M sodium hydroxide (Sigma-Aldrich). The three antioxidant preparations tested were mixtures of plant gallic and ellagic acids extracted from grape (Vitis vinifera L.) coded as powder 1, plant ellagic acid and biological gum arabic coded as powder 2, and plant gallic and ellagic acids and Saccharomyces cerevisiae cell wall fractions coded as powder 3.

1.19.2 Wine samples

The sparkling wine was industrially-scale produced by a cellar located in Franciacorta area (Lombardy, Italy) in the 2010 vintage from Chardonnay grape applying the rational wine making procedures usually adopted in the winery for the manufacture of sparkling wine according to the *Champenoise* method. Base wine (10 hL) was bottled, the second fermentation was performed and the sparkling wine was maintained 12 months on the yeast lees before the disgorging.

1.19.3 Experimental design of brut nature sparkling wines

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The experimental design is summarized in Table 1.15 Briefly, brut nature sparkling wine was added with the three antioxidant preparations (named AO 1, AO 2 and AO 3) at the concentration levels 20 mg L^{-1} and 40 mg L^{-1} at disgorging. Further samples were added with sulfur dioxide (50 mg L^{-1}) or disgorged only with the wine itself (control samples). The disgorged wine sample were stopped with crown cap and stored in lying position for 7 months at 15°C and 25°C protected from light in the cellar. For each treatment and temperature investigated, the content of glutathione (GSH), sotolon, Ascorbic acid (AA), dehydroascorbic acid (DHA), and the absorbances at 420 nm were evaluated. Moreover, these parameters were also measured in the sparkling wine still maintained on the yeast lees at 12°C during the trial and in the commercial *pas dosé* sparkling wine disgorged. The latter was stored in the cellar at 12°C, as well.

For each commercial antioxidant compound was evaluated the content of glutathione (GSH), sotolon, Ascorbic acid (AA), dehydroascorbic acid (DHA), total polyphenols (T.P.), the cysteine (Cys), cysteine adsorbed (Cys ab.), reactivity to the SO2, antioxidant power and phenolic composition were evaluated.

Sample code	**Product added**	**Dosage (mg L^{-1})**	**Storage temperature (°C)**
1	No addition	--	15
2	No addition	--	25
3	SO_2	50	15
4	SO_2	50	25
5	Powder 1	20	15
6	Powder 1	20	25
7	Powder 1	40	15
8	Powder 1	40	25
9	Powder 2	20	15
10	Powder 2	20	25
11	Powder 2	40	15
12	Powder 2	40	25
13	Powder 3	20	15
14	Powder 3	20	25
15	Powder 3	40	15
16	Powder 3	40	25
17	Wine maintained on yeast lees		12°C
18	*Pas dosé* traded wine		12°C

Table 1.15 Experimental design.

1.19.4 Experimental design of sotolon formation

The sotolon formation was assessed in model wine solutions composed of ethanol (12% vol/vol), tartaric acid (5g/L), ribose (10g/L), putrescine (1 mM), ethanal (50 mg/L) at a pH of 3.20. The samples were added with different levels of (+)-catechin ((+)-cat), glutathione (GSH), iron (Fe^{++}), oxygen (O_2) and putrescine (Put). Moreover model solution was added of increasing concentrations of tartaric acid, ribose, ethanol and ethanol (Figure 1.16). The samples were then stored at 5° C (control sample) and 70° C for five days and glass bottles were hermetically closed. The bottles were filled to half their volume and had a headspace of about 50 cm^3. Moreover the sotolon formation was carried out in synthetic wine added to AO2 powder (100 mg/L), GSH (10 mg/L) in oxidative and anoxic conditions. The samples were then stored at 5° C (control sample) and 30° C for five months and the glass bottles were hermetically closed and filled to half their volume (headspace of about 50 cm^3) Triplicate trials were carried out.

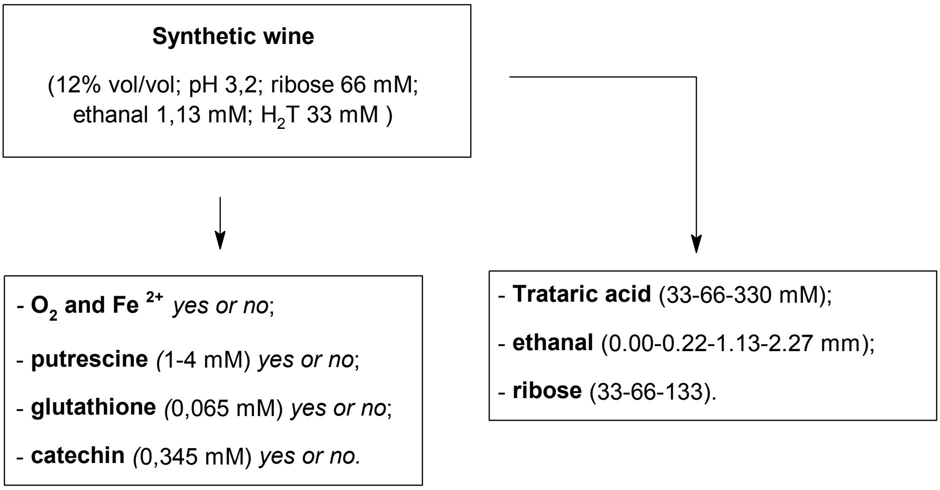

Figure 1.16 Experimental design of sotolon transformations.

1.19.5 Analytical methods

1.19.5.1 Determination of sotolon

Sotolon was measured in both sparkling wines and commercial preparations. For the wine samples preparation, three grams of NaCl were dissolved in 30 mL wine in a 100 mL bottle then 40 mL of DCM were added. The bottle was hermetically closed and shaken for 10 minutes with a wrist action stirrer (Griffin Flask Shaker). The mixture was centrifuged 5 min at 5000 × g and the DCM was separated by a separatory funnel and recovered. This solvent extraction procedure was carried out for 3 times; the organic solvent fractions were jointly collected and added with 2 g of anhydrous sodium sulphate. The DCM was evaporated under-vacuum; the dry material was dissolved with 2 mL of methanol 5% which was purified by a PVPP 50 mg SPE cartridge and eluted solution was recovered.

For the commercial preparations, 200 mg of powder were dissolved in 50 mL of synthetic wine solution; the liquid/liquid extraction of sotolon was carried out as reported as above for the wine samples.

1.19.5.2 Determination of glutathione

Glutathione was evaluated in both sparkling wines and commercial preparations. For the sparkling wine samples, its content was determined as described by . Briefly, the sparkling wine (2 mL) treated with PVPP and centrifuged was derivatized with pBQ followed by the addition of 3MPA. The reaction mix was filtered through 0.22 μm pore size PTFE membrane (Millipore, Billerica, MA, USA) and submitted to the HPLC separation.

Glutathione in commercial preparations was measured as described by Tirelli et al., (2010). Briefly, the powders were suspended in citrate buffer 75 mM at pH 5, derivatized with pBQ and added with 3MPA. The reaction mix was injected in HPLC after the filtration with 0.22 μm pore size PTFE membrane (Millipore).

1.19.5.3 Determination of ascorbic acid and dehydroascorbic acid

Quantification of ascorbic acid and dehydroascorbic acid was carried out as previously described by Zapata & Dufour, (1992) with some modifications.

For the wine samples, 30 mL of wine were added with EDTA (0.03%). For the commercial preparations, 100 mg sample were dissolved in 100 mL of synthetic wine solution added with EDTA (0.03%). The wine and the homogenate were filtered through a 0.45 µm PVDF filter and purified on a C18 Sep-Pak cartridge (Waters, Mil-ford, MA, US).

The HPLC analysis was achieved after derivatization of DHAA into the fluorophore 3-(1,2-dihydroxyethyl) furol [3,4-b]quinoxaline-1-one (DFQ), with OPDA. Standard solutions of both AA and DHAA ranged from 2 mg L^{-1} to 50 mg L^{-1} were prepared in synthetic wine solution. Reversed phase HPLC separation was performed with a Waters Alliance 2695 (Milford, MA, US) equipped with a photodiode array detector Waters 2996 and a C_{18} column (Nova-Pak 150 x 3.9 mm, 4 µm, Waters). The chromatographic separation was carried out with an isocratic elution running acetate buffer 50 mM, pH 4.5 added with 5% methanol for 15 minutes followed by column washing (100% methanol for 2 minute) and column conditioning (4 minutes). The flow rate was 0.9 mL min^{-1}. Column temperature was 25°C and the injection volume was 20 µL. Chromatographic data were acquired from 230 nm and 500 nm and processed at 261 nm and 348 nm respectively for AA and DHAA by Empower 2 software (Waters). The results were expressed as g AA and DHA per liter of wine and per 100 g of powder.

1.19.5.4 Determination of phenolic compounds

1.19.5.4.1 Spectrophotometric analysis

LC-MS analysis

The phenols characterization was carried out on the three commercial preparations added to the sparkling wine. The phenolic compounds were identified and quantified as reported by Fracassetti et al., (2013). The extraction of phenolics was performed as follows: 1 g of the three commercial preparations was added to 25 mL of 50% methanol in water acidified with1% formic acid. Different extractions were carried out in order to achieve the better phenols recovery using variable ratio of water and methanol, with and without formic acid. The use of 50% methanol in water acidified with 1% formic acid allowed the better recovery of phenols. The powders were vortexed for 2 min, sonicated for 15 min (Sonicator Branson 5510, Emerson, Danbury, CT, US) and centrifuged at 3000 rpm for 15 min at 4°C (Centrifuge 5804 R, Eppendorf, Hamburg, Germany). The supernatants were recovered, freeze-dried under vacuum, suspended in 2 mL of the corresponding extraction solvent, then filtered with a PVDF filter 0.22 µm (Millipore) and injected in LC/MS. The identification and quantification of phenols were performed using an Agilent 1100 Series equipment (Agilent, Santa Clara, CA, USA) equipped with G1312A binary pump, G1313A autosampler, G1315B photodiode array detector, and G1322A degasser controlled by the Agilent software v. A08.03. HPLC was coupled with a detector MSD Trap 1100 Series (Agilent) with an electrospray ionisation system (ESI), with the following conditions: the heated capillary was 350°C and 3–3.5 kV voltage, mass scan (MS) and MS/MS were measured from 100 to 1500 m/z. Collision induced fragmentation experiments were performed in the ion trap using helium as the collision gas, and the collision energy was set at 75%. Mass spectrometry data were acquired in the negative ionisation mode. .

A column Pursuit XRs C18 250x40 mm from Varian (Agilent) was used and a flow rate of 0.8 mL min^{-1}. The used solvents were 1% formic acid in water (A) and acetonitrile (B) which was in the following separation gradient: 1% B in A at 0 min, 9% B at 10 min, 35% B at 48 min, and 95% B at 52 min, following by washing and conditioning steps. Data were registered from 250 nm to 700 nm and the phenolic compounds were quantified at 280 mn, 360 mn, and 520 nm, depending on the type of phenolic compound. Integrations were performed by Agilent ChemStation for LC 3D, Rev. B.01.03 SR1. MS trap control was carried out Bruker Daltonic version 5.2. Quantification of gallic acid, ellagic acid, myricetin and their derivatives, and ellagitannins was carried out with the calibration curves obtained for gallic acid (1–300 mg L^{-1}), ellagic acid (1–300 mg L^{-1}), rutin (1–300 mg L^{-1}), and vescalagin (0.1–100 mg L^{-1}), respectively, at the appropriate wavelengths. All the samples and standards were injected in triplicate.
Moreover, samples of the three powders were analysed by UPLC-Q-TOF (Agilent) in order to further confirm the phenolic compounds identified by MS Trap. The Q-TOF equipment had the following conditions: ESI gas temperature 280°C, drying gas 9 L min^{-1}, nebulizer 35 psig, sheath gas temp 400°C, sheath gas flow 12 L min^{-1}. MS TOF fragmentor 100 V, mas range 100–1500, negative mode. The column was Poroshell 120, EC-C18, 2.7 µm, 30x100 mm (Agilent); the eluents were 0.1% formic acid in water (A) and acetonitrile acidified with 0.1% formic acid (B). The separation gradient started with 1% B in A at 0 min, 9% at 3 min, 48% at 20 min, and 95% at 23 min, following by washing and conditioning steps. The volume injected was 2 µL and the flow rate was 0.4 mL min^{-1}. The determinations were carried out in triplicate.

1.19.5.5 Antioxidant capacity assays

The antioxidant capacity of both powders was carried out through two different methods measuring the free radical scavenging capacity (DPPH and ABTS assays).
The free radical scavenging activity determined with DPPH followed the method of **Brand-**Williams et al., (1995**)** with some modifications (Espín, Soler-Rivas, Wichers, & GarcíaViguera, 2000; Llorach, Tomás-Barberán, & Ferreres, 2004). The DPPH solution was diluted with methanol to an absorbance of 1.00 (±0.03) at 515 nm. In a 96-wells micro plate (Nunc, Roskilde, Denmark), 250 µL of DPPH solution were placed in each well and 2 µL sample were added. The sample was dissolved in 70% methanol (20 g L^{-1}) and, after centrifugation, it was serially diluted.
The ABTS method was performed as reported by Mena et al., (2011). The ABTS solution was diluted with water to an absorbance of 1.00 (±0.03) at 414 nm. In a 96-wells micro plate (Nunc, Roskilde), 250 µL of ABTS solution were put in each well and 2 µL sample were added. The sample was dissolved in water (20 g L^{-1}) and, after centrifugation, it was serially diluted. For both assays, the reaction kinetic was monitored for 50 min at 25°C by micro plate reader (Infinite® M200, Tecan, Grödig, Austria). A calibration curve was made by adding increasing concentration of Trolox ranged from 50 to 1000 µM; each concentration was assayed in quadruplicate, as well each sample.

1.19.5.6 Proanthocyanidin analysis (Bate&Smith)

The proanthocyanidin concentration was also determined using the methodology proposed by Bate-Smith, (1981). Each wine sample was diluted at a ratio of 1/50 (v/v) with deionised water. In two separate test tubes, 4 mL of the diluted sample (powder (1g/L) and wine), 2 mL of deionised water and 6 mL of hydrochloric acid were added. One test tube (reaction tube) was placed in a water bath at 100 °C for 30 min, and the other test tube (blank tube) was left to stand in the dark for the same time. After 30 min, 1 mL of ethanol was added to each tube, and the tubes were left in the dark until the heated reaction tube was cooled. The absorbance of each test tube was measured in a spectrophotometer at 550 nm using deionised water as the blank. The absorbance difference was multiplied by the factor 1162,5 and the concentration of proanthocyanidins was expressed in mg/l of cyanidin.

1.19.5.7 Reactivity to the SO_2 of powders

The reactivity to the SO_2 of powders was determined by spectrophotometric analysis. The powders were dissolved in a synthetic wine (12% ethanol, 5 g/L tartaric acid and pH 3,2) and analyzed before and after the addition of sulfur dioxide. The absorbance of each test tube was measured in a spectrophotometer at 280 nm using deionised water as the blank. The absorbance difference was quantified as gallic acid and reactivity to the SO_2 of powders was expressed as mg/g of powders.

1.19.5.8Cysteine and cysteine absorbed

The cysteine (Cys), cysteine absorbed (Cys ab.) and were assessed according to the method reported in (Kritzinger, 2012).

1.19.5.9 Oenological parameters

The following parameters were evaluated according to standard methods in the oenological sector: total polyphenols (T.P.).

1.20 Results and Discussion

1.20.1 Characterization of commercial antioxidant compounds

The experimental antioxidant additives showed three different phenolic compositions (Table 1.16). Sotolon was absent in AO1, AO2 and AO3. The powder AO1 was characterized by the absence of cysteine, cysteine ab., glutathione, ascorbic acid and dehydroascorbic acid. The cysteine ab. was present at 0.09 mg/g and 0.64 mg/g in AO2 and AO3 respectively, whereas 58 mg/g of GSH was quantified in AO3 . The AO2 compound showed a higher reactivity to the SO2 (35 mg/g of gallic acid) when compared to AO1 (25 mg/g of gallic acid) and AO3 (22 mg/g of gallic acid). The AO1 was characterized by the highest amount of total phenol (T.P.) than AO2 which presented the highest concentration of proanthocyanidins (B&S). The AO3 demonstrated the lowest amount of total phenols (T.P.) and the absence of polymeric phenols (B&S). AO1 showed the highest concentration of ellagic tannins (10%) and antioxidant power (88 M trolox/g) whereas the AO2 presented the higher concentration of gallotannins (21%) and the lowest antioxidant power (18 M trolox/g).

Powder	Pol. SO_2	T.P.	B&S	Cys	AA-DAA	Cys Ab.	GSH
		mg(gallic ac-id)/g				mg/g	
AO1	25	347	157	0	0	0	0
AO2	35	245	380	0	0	0,09	0
AO3	22	97	0	0	0	0,64	58

Table 1.16 Characterization of the experimental antioxidant additives (AO1-AO2-AO3).

Powder	Flavonols	Gallic Ac.	Ellagic Ac.	sotolon	DPPH
		(% phenols weight)		µg/g	M trolox/g
AO1	-	7,3	10	0	88
AO2	2	21	.	0	18
AO3	-	15	.	0	60

Table 1.16 Characterization of the experimental antioxidant additives (AO1-AO2-AO3)

The chemical compositions of antioxidant additives demonstrated their different origin. AO1, AO2 and AO3 could be produced by different vegetable matrices such as: vegetable ellagitannins and gallotannins (AO1), vegetable gallotannins and procyanidins (AO2), and vegetable gallotannins and cell walls by S. cerevisiae (AO3).

1.20.2 Brut nature sparkling wines

The analytical procedure allows the separation of an interference-free chromatographic peak and it has a linear response in all the range tested. A repeatability mean value was also obtained. Sotolon concentrations higher than 2 µg/L can be quantified.

The experimental antioxidant additives show a lower protective ability than sulphur dioxide (Table 1.17) since the wines containing them have a darker yellow color (higher absorbance at 420 nm) and contain sotolon. Sotolon was detected in the samples treated with AO1 and AO3 when were stored at 25°C whereas, the wine treated with AO2 showed the higher amount of sotolon at 25 °C and 15 °C. The wines added with 40 mg/l of AO2 contain an amount of sotolon higher than the olfactory perception threshold and a yellow colour more intense than the SO2 containing sample in spite of the high glutathione concentration. In the samples containing large amount of GSH, low level of one of its oxidized form (2-S-glutathionylcaftaric acid: GRP) were detected which suggests an intense oxidation. This data is apparently in contrast to the results arising from wine color, the darker yellow note could be attributed to the presence of oxidized phenols in the additives used. Suprisingly the oxidation of GSH is higher in samples stored at low temperature.

antioxidant compounds	**dose (g/l)**	**storege temperature (°C)**	**Abs at $\lambda_{420\ nm}$ (AU)**	**GSH Conc. (mg/L)**	**GRP Area (µV*sec)**	**sotolon (µ g/l)**
no one	-	15	0,142	1,3	146	-
no one	-	25	0,150	2,7	123	-
SO2	50 ppm	15	0,099	0,9	136	-
SO2	50 ppm	25	0,122	2,3	132	-
AO1	2	15	0,154	1,8	142	-
AO1	2	25	0,158	3,6	123	< 2 µ g/l
AO1	4	15	0,156	2,1	166	-
AO1	4	25	0,160	3,4	152	< 2µg/l
AO2	2	15	0,157	1,3	168	< 2µg/l
AO2	2	25	0,172	2,4	140	< 2µg/l
AO2	4	15	0,170	1,4	146	6
AO2	4	25	0,181	4,3	114	13
AO3	2	15	0,147	1,6	145	-
AO3	2	25	0,147	2,5	128	< 2µg/l
AO3	4	15	0,141	1,6	148	-
AO3	4	25	0,155	3,8	128	< 2µg/l
Undisgorged		12°C	0,226	1,1	167	-
Commercial sparkling wine		-	0,164	2,4	339	< 2µg/l

Table 1.17 Quantification of Abs$_{420\ nm}$ (AU); GSH (mg/l); GRP (µV*sec) and sotolon (µg/l) in Franciacorta sparkling wines product without SO$_2$

The additive AO2 is responsible for the high sotolon amount formed in SO$_2$ free wine. The characterization of the antioxidant compound AO2 showed higher reactivity to the SO$_2$, higher concentration of gallotannins (21%) and the lowest antioxidant power (18 M trolox/g) when compared to the other commercial antioxidant compounds tested. Probably the amount of gallotannins and the poor antioxidant property of AO2 may affect the content of sotolon. Additionally, the antimicrobial properties of the commercial antioxidants tested should be evaluated.

The following work was aimed to evaluate the chemical and physical factors affecting the formation of sotolon in order to achieve further indications concerning the evolution of SO_2-free sparkling wine, to explain the detrimental effect of antioxidant preparations during wine storage. Moreover, research efforts were carried out to explain the detrimental effect of antioxidant preparations during wine storage. Subsequently, will be evaluated the influence of oxygen, iron (Fe^{++}), amino groups, catechin, reducing sugar, tartaric acid, ethanal, temperature and ethanol on the sotolon formation in synthetic wine.

1.20.3 Sotolon formation in synthetic wine

Sotolon is mainly responsible for the aged bouquet, and this study shows the importance of oxygen consumption in the formation of this compound, as also reported in other studies (Ferreira et al., 2005; Lavigne et al., 2008). Towards a better understanding of the chemical and physical factors which affected the formation of sotolon are evaluated the influence of amino group, oxidative conditions and catechin (Table 1.18), secondly we tested the influence of GSH, amino group, oxidative conditions and catechin (Table 1.19). Finally we evaluated the influence of tartaric acid, ethanal and reducing sugars on sotolon formation in anoxic condition (Table 1.20). The formation of sotolon showed to be affected by physical and chemical factors tested. Temperature, catechin, ribose, ethanol, GSH and oxidative conditions have affected the formation of sotolon. Sotolon formation was strongly influenced by the storage temperature in fact in all the samples stored at 5° C was never detected sotolon. The treatments of the synthetic wine are reported in Figure 1.16. during the storage at 70° C of the model wines, a “spicy/curry” off-flavor was observed. The influence of temperature on the sotolon formation is also reported by (Cutzach et al., 1999). Moreover, sotolon has been detected either in the presence of oxidative conditions (Fe^{++} and O_2), than in samples stored at 70° C under anoxic conditions, therefore it is possible to hypothesize two different formation pathways. Other authors (Lavigne et al., 2008; Cutzach et al., 1999) reported that the dissolved oxygen content is highly correlated with the sotolon concentration, both in wine and in synthetic wine, but the mechanisms of sotolon formation tested under anoxic conditions was not clear.

1.20.3.1 Oxidative conditions

The data reported in Table 1.18 show that the sotolon formation is not influenced by individual physical and chemical factors tested, but by their simultaneous presence in synthetic wine. The control sample stored at 70° C under anoxic condition had an odor reminiscent of “old honey/currry” and a sotolon content exceeding its perception threshold (10 μg/L), although it had not been stored under oxidation conditions. This result is confermed by Lavigne et al., (2008); Cutzach et al., (1999) that detected sotolon also in in white dry wines stored in anoxic condition. The amount of sotolon detected in samples added either with putrescine, or catechin or oxygen / iron was not significantly different from the control stored in anoxic conditions, whereas the control simultaneously treated with both putrescine and oxidative conditions (O_2 and Fe^{++}) and stored at 70° C showed a higher amount of sotolon (18 μg/L) than the samples individually treated.

Assay	**Ribose**	**Ethanal**	**Put.**	**O_2/Fe^{++}**	**Cat.**	**Sotolon (µg/L)**	
	(mM)	(mM)	(mM)		(mM)	5° C	70° C
(Control)	-	-	-	-	-	0	**10**^{a}
+ O_2 and Fe^{2+}	66	1.13	1	presence	-	0	**10**^{a}
+ Putrescine	66	1.13	1	absence	-	0	**10**^{a}
+ Putrescine + O_2 and Fe^{2+}	66	1.13	1	presence	-	0	**18**^{c}
+ Putrescine	66	1.13	4	absence	-	0	**13**^{b}
+ Putrescine + O_2 and Fe^{2+}	66	1.13	4	presence	-	0	**19**^{c}
Putrescine + catechin	66	1.13	4	absence	0.35	0	**11**^{a}
Putrescine + O_2 and Fe^{2+}+ catechin	66	1.13	4	presence	0.35	0	**25**^{d}

Table 1.18 Putrescine influence on sotolon formation in synthetic wine (12% v/v ethanol; 5 g/L tartaric acid; pH 3.2) stored five days at 5° C and 70° C.

The increase in the concentration of putrescine in oxidative conditions, from 1 mM to 4 mM, did not generate significant increases in the amount of sotolon (19 µg/L), whereas the addition of cathechin increased the amount of sotolon up to 25 µg/L which was the highest concentration detected.

Assay	**GSH**	**Ribose**	**Ethanal**	**Put.**	**O_2/Fe^{++}**	**Cat.**	**Sotolon (µg/L)**	
	(mM)	(mM)	(mM)	(mM)		(mM)	5° C	70° C
(Control)	0	0	0	0	absence	0	0	**10**^{a}
+ GSH	0.065	66	1.13	1	absence	0	0	**10**^{a}
+ GSH + O_2 and Fe^{2+}	0.065	66	1.13	1	presence	0	0	**6**^{e}
+ GSH + Putrescine	0.065	66	1.13	1	absence	0	0	**12**^{a}
+ GSH + Putrescine + O_2 and Fe^{2+}	0.065	66	1.13	1	presence	0	0	**6**^{e}
+ GSH + Putrescine + catechin	0.065	66	1.13	1	absence	0.35	0	**11**^{a}
+ GSH + Putrescine + O_2 and Fe^{2+}+ catechin	0.065	66	1.13	1	presence	0.35	0	**7**^{e}

Table 1.19 GSH influence on sotolon formation in synthetic wine (12% v/v ethanol; 5 g/L tartaric acid; pH 3.2) stored five days at 5° C and 70° C.

The data reported in Table 1.19 show the influence of GSH on sotolon formation under different redox condition. The control sample showed the same concentration of sotolon (10 µg/L) previously detected (Table 1.18), confirming the hypothesis sotolon could be formed by either oxidative and anoxic conditions. Glutathione causes a decrease of the sotolon formation up to 6 µg/L when it was present with oxygen and iron. Furthermore, GSH inhibited the formation of sotolon (up to 7 µg / L) if it was simultaneously present with putrescine, oxidative conditions and catechin. These results are confermed by Dubordieu & Lavigne, (2004) who detected a higher amount of sotolon in dry white wines not treated with glutathione than wines treated with 10 mg/L of GSH at bottling. On the contrary, in the absence of oxidizing conditions glutathione had no inhibitory effect on the formation of sotolon.

Catechin and glutathione have demonstrated two opposing roles in the formation of sotolon although both express their effect in the presence of iron and oxygen.

Assay	**GSH (mM)**	**Ribose (mM)**	**Ethanal (mM)**	**Put. (mM)**	**O_2/Fe^{++}**	**AO2 (mg/L)**	**Sotolon (µg/L)**	
							5° C	**30° C**
Synthetic wine + GSH	0.065	66	1.13	1	absence	0	0	**0^{g}**
Synthetic wine + GSH + O_2 and Fe^{2+}	0.065	66	1.13	1	presence	0	0	**$2{,}4^{e}$**
Synthetic wine + GSH + AO2	0.065	66	1.13	1	absence	100	0	**$2{,}7^{e}$**
Synthetic wine + GSH + AO2 + O_2 and Fe^{2+}	0.065	66	1.13	1	presence	100	0	**$1{,}4^{f}$**

Table 1.20 GSH influence on sotolon formation in synthetic wine added with AO2 powder (12% v/v ethanol; 5 g/L tartaric acid; pH 3.2) stored five months at 30° C.

The Table 1.20 show the data of the sotolon transformation carried out at 30° C and 5° C. The samples were added of AO2 powder and GSH and stored in anoxic and oxidative conditions for 5 months. Sotolon was not produced in the samples added with GSH, stored in anoxic condition, at 30°C and in the samples stored at 5° C, whereas in others treatments sotolon was produced. The amount of sotolon detected in the samples "synthetic wine + GSH + O_2 and Fe^{2+}" and "synthetic wine + GSH + AO2" stored at 30° C were 2,4 µg/L and 2,7 µg/L respectively. The samples treated with AO2, GSH and oxidative condition at 30° showed a decrease in the concentration of sotolon up to 1.4 µg/L.

1.20.3.2 Anoxic conditions

The data in Table 1.21 shows that the sotolon formation under anoxic condition is strongly influenced by the reducing sugar concentration and ethanol concentration. Data regarding the amount of sotolon produced in anoxic condition showed that the highest sotolon concentration was detected in the samples treated with the highest amount of ribose (330 mM), while the lowest amount of sotolon was found in the model wines, which were treated with 7% and 17% of ethanol.

Assay	**EtOH**	**H_2T**	**Ribose**	**Ethanal**	**Put.**	**O_2/Fe^{++}**	**Sotolon (µg/L)**	
	(mM-%)	(mM)	(mM)	(mM)	(mM)		5° C	70° C
(Control)	1,2	33	0	**0**	0	absence	0	**10**^{a}
+ ethanal	1,2	33	66	**0.26**	0	absence	0	**10**^{a}
+ ethanal	1,2	33	66	**1.13**	0	absence	0	**11**^{a}
+ ethanal	1,2	33	66	**2.26**	0	absence	0	**13**^{b}
+ ribose	1,2	33	**33**	1.13	0	absence	0	**10**^{e}
+ ribose	1,2	33	**66**	1.13	0	absence	0	**11**^{a}
+ ribose	1,2	33	**330**	1.13	0	absence	0	**28**^{d}
H_2T	1,2	**33**	66	1.13	0	absence	0	**10**^{a}
H_2T	1,2	**66**	66	1.13	0	absence	0	**11**^{a}
H_2T	1,2	**133**	66	1.13	0	absence	0	**10**^{a}
CH_3CH_2OH	**1,2**	33	66	0	0	absence	0	**3,5**^{e}
CH_3CH_2OH	**2,06**	33	66	0	0	absence	0	**9**^{a}
CH_3CH_2OH	**2,90**	33	66	0	0	absence	0	**3**^{e}

Table 1.21 Tartaric acid, ribose, ethanol and ethanal influence on sotolon formation in synthetic wine (12% v/v ethanol; 5 g/L tartaric acid; pH 3.2) stored in anoxic conditions for five days at 5° C and 70° C.

Acetaldehyde influenced the sotolon amount only when it was added to the highest concentration (2,26 Mm), while the sotolon levels were not significantly different at lower concentration of ethanal. The formation of sotolon was not influenced by the concentration of tartaric acid in fact all treatments showed the same amount of sotolon (10 µg/L).

Finally, the formation of sotolon, in the absence of oxygen and iron, was particularly influenced by the concentration of ethanol added to the synthetic wine. Its formation was stimulated at 12% of ethanol while it was inhibited at higher and lower concentrations of ethanol added (7% and 17%).

1.21 Conclusion

The samples of Franciacorta sparkling wines treated with SO_2 show the best protection against wine oxidation whereas the other commercial antioxidants tested caused detrimental effects due to the sotolon production. The phenolic composition of commercial antioxidants has influenced the production of sotolon indeed AO2 showed the highest ammount of gallic acid (21%) and procyanidins (380 mg of gallic acid/g of powder) which could stimulate the formation of sotolon as follows reported.

Danilewicz, (2012) reported that polyphenols and sulfur dioxide act as antioxidants in wine when they are simultaneously present, while if present alone they can promote the oxidation. The adding of AO2, which present the highest concentration of polyphenols, could stimulate the production of sotolon through the oxidation of polyphenols, which was stimulated by the absence of SO_2.

During storage sotolon formation is affected by the storage temperature indeed, at 25°C the sotolon was always detected in all treatment, whereas at 15°C sotolon was found only in samples treated with AO2 which showed the highest amount of phenols.

Moreover, Cutzach et al., (2000) reported that the high temperature (up to 33°C) has facilitated the formation of sotolon in Vins doux Naturels. The high storage temperatures may ease the Maillard reaction to produce sotolon. Glutathione concentration was higher in samples stored at 25°C than the samples stored at 15°C, probably such a behavior could be due to the higher oxygen solubility at low temperature that ease the oxidation reactions. As the storage temperature increases oxygen solubility and oxidations decrease but the Maillard reaction is promoted as well as the consequent formation of sotolon.

The synthesis of sotolon was strongly affected by the temperature, catechin, GSH, reducing sugars concentration, ethanol and redox condition. The kinetics of sotolon formation is highly dependent on sugar contents, and these results point toward a sugar-related anoxic pathway. It has been demonstrated that sugar degradation products due to Maillard reaction were the starting material for the formation of the furanone in oxidative conditions (Schneider et al., 1998; Cutzach et al., 2000; Hofmann et al., 1995), but the influence of reducing sugars in anoxic conditions is not yet clear. Camara et al, (2004) also showed a high dependence between sotolon levels, sugars wine content and maturation time in Madera wines.

The tests previously carried out showed which sotolon can be formed by several formation pathways indeed it was generated under both reducing and oxidative conditions.

Sotolon formation is enhanced by simultaneous presence of Fe^{++} and O_2, of amino groups (putrescine) and of phenols (catechin). On the contrary Cutzach et al., (1999) reported that the presence of antioxidant polyphenolic compounds slows down the sotolon formation in red wine, because the phenols are able to combine preferentially the acetaldehyde (in an acid environment), which is less avaliable to the sotolon formation.

Glutathione inhibited the formation of sotolon only when it was simultaneously added with amino groups and phenols in an oxidizing environment or when it was present in oxidative conditions. Dubordieu & Lavigne (2004) have also reported, that GSH prevents the formation of sotolon in dry white wine.

Under anoxic conditions it has been demonstrated that there is a high dependence between sotolon and reducing sugar contents, whereas tartaric acid and acetaldehyde didn't affect the formation of sotolon. Ethanal (boiling point 21°C), during the storage at 70°C, could have been evaporated in the headspace of transformation containers and then it was not available as a precursor of sotolon.

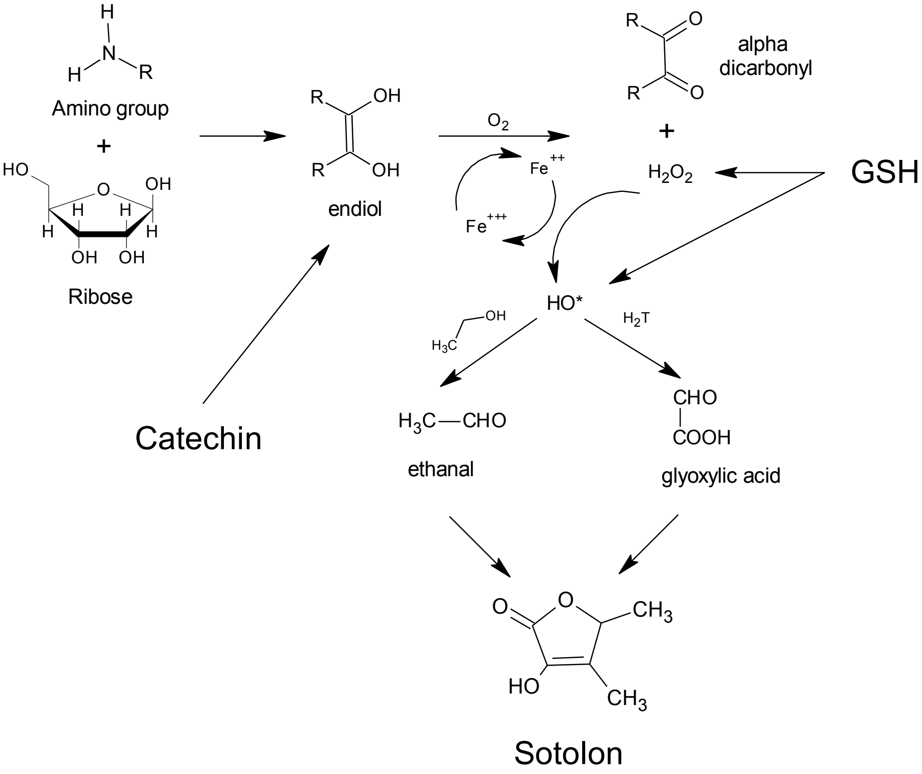

Figure 1.17 Hypothesis on sotolon formation in oxidative conditions.

Surprisingly ethanol has a positive effect on sotolon formation at 12% while at 17% and 7% of ethanol a negativffect was registered. These data were confirmed by Pham et al., (1995) which observed a negative effect of alcoholic content (15% v/v) on formation of sotolon in "Vin Jaune" wines.

The first hypothesis on the formation of sotolon under oxidative conditions has been proposed.

The formation of sotolon under oxidizing conditions could involve to Maillard reaction (Figure 1.17). The reaction occurs between reducing sugars and compounds containing a free amino group to produce endiol compound, which could be oxidized to alpha dicarbonyl. The oxidation of endiol occurs through the simultaneous reduction of Fe $^{+++}$ to Fe $^{++}$ and the consequent production of hydrogen peroxide and the alpha dicarbonyl.

Afterwards, hydrogen peroxide is reduced to hydroxide radical through the simultaneous oxidation of iron. Hydroxide radical is a chemistry species highly reactive which could be able to oxidize ethanol to ethanal and to catalyze the oxidative degradation of tartaric acid to glyoxylic acid. Clark et al., (1960) and Santos-Buelga et al., (1999) reported that glyoxylic acid and acetaldehyde are the precursor of sotolon in oxidative condition. The oxidation of catechin could increase the formation of sotolon because it might stimulate the production of hydrogen peroxide and hydroxide radical, which are responsible for precursors production of sotolon. In contrast, the glutathione

could block the hydrogen peroxide or hydroxide radical, preventing the production of sotolon.
The low amount of sotolon (between 0 to 2.7 µg/L) detected in samples stored at room temperature (Table 1.20) didn't permit an evaluation of the mechanisms of sotolon formation at 30° C. In future tests will be increased the storage time to be able to better understand the influence of polyphenols and glutathione on formation of sotolon at room temperature.
The formation of sotolon in anoxic environment has not yet been clarified and further tests will be conduct to understand the role of tartaric acid, ethanol, ethanal and ribose on sotolon production in synthetic wine.

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Development and validation of a reversed-phase UHPLC-UV method for the quantification of biogenic amines in red wine from different malolactic activity conditions.

2 Introduction

2.1 Biogenic amines

Biogenic amines (BAs) are basic nitrogenous low-molecular-mass compounds produced by decarboxylation of amino acids (Table 2.1) or by amination and transamination of amino aldehydes and ketons.

α-amino acid	**Biogenic amine**	
arginine	agmatine →	spermidine ↑ putrescine ↓ spermine
cysteine	mercapto-ethylamine	
histidine	histamine	
phenylalanine	phenylethylamine	
serine	ethanolamine	
ornithine	putrescine	
5-hydroxytryptophan	serotonin	
lisine	cadaverine	
tyrosine	tyramine	

Table 2.1 Biogenic amines present in wine and their corresponding amino acids (Karovicova & Kohajdova, 2005).

Their chemical structure can be either alyphatic (i.e. cadaverine, putrescine, spermine, spermindine) or aromatic (i.e. tyramine, phenylethylamine, histamine, tryptamine) (Karovicova & Kohajdova, 2005) Figure 2.1. Biogenic amines are naturally present in both raw and processed foods such as fruit, meat, chocolate, vegetable, fish and beer. Their levels can increase during spontaneous or controlled spontaneous microbial fermentation or in the course of food spoilage. The presence of BAs in food was suggested as an indicator of poor hygienic conditions of production chain or storage (Kirshbaunm et al., 2000). Biogenic amines are produced by lactic acid bacteria (LAB) during the malolatic fermentation in the wine making process. In high amounts, in beverage or food, the biogenic amines result in direct or indirect toxicity, whereas when they are present in low concentrations are essential for many physiological functions of animals, plants and microorganisms. (Ertan & Bayram, 2009). Biogenic amines play several important physiological functions in human metabolism, such as: nitrogen source and precursor of hormones, proteins, nucleic acid, alkaloids (Shalaby et al., 1996) and they affect certain physiological functions including the regulation of body temperature and blood pressure. The uptake of milligram amounts of histamine by humans, depending on individuals, could cause hypo- or hypertension, headache, or anaphylactic shock syndrome. Biogenic amines in wine could also cause commercial import and export difficulties. Certain countries will reject wines that contain more than a certain concentration of histamine. The upper limits for histamine in wine in some European countries are (mg/L histamine): Germany (2), Holland (3), Finland (5), Belgium (5 to 6), France (8), Switzerland and Austria (10) (Lehtonen, 1996).

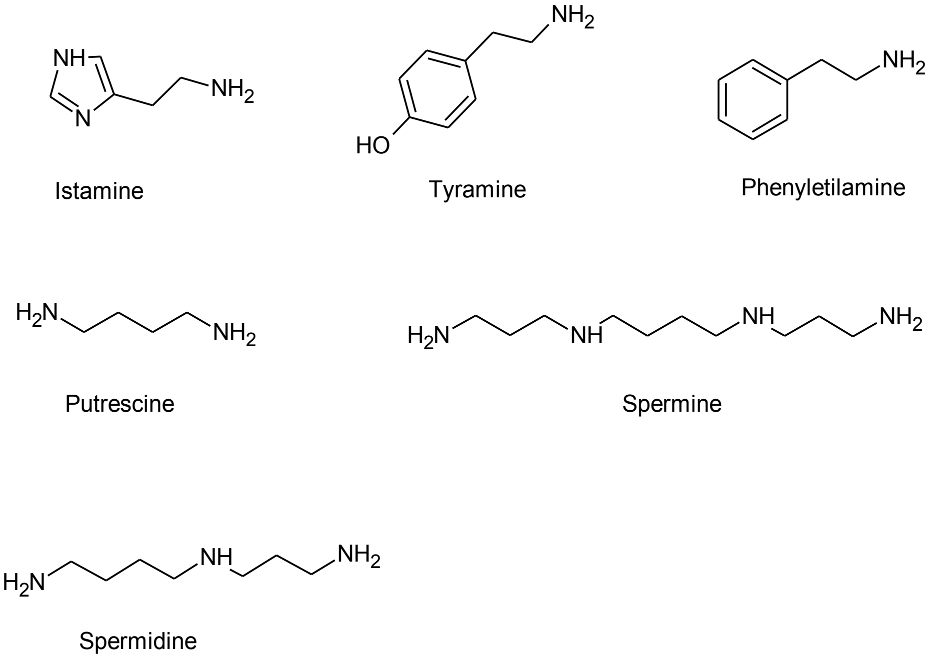

Figure 2.1 Chemical structure of the most common BAs in wine.

The poisoning from histamine (10 mg/l in wine for sensitively people) appears with different symptoms including rash, nausea vomiting, headache, palpitation and hypo-hypertension, stimulation of the heart as result of adrenaline liberation, control of gastric secretion and excites smooth muscles of the intestine and of the respiratory tract (Shalaby, 1996). Moreover, the aromatic amines, as phenyethylamine and tyramine, are vaso-active amines (Ertan & Bayram, 2009) which cause, symptoms in humans as migraine, tachycardia and increase of blood pressure. Tyramine is slightly toxic by itself and its toxicity has to be attributed to its absorption coupled with alcohol. Dangerous amounts of phenylethylamine in alcoholic beverages are 3 mg/L whereas tyramine levels as low as 25 mg/L are reported as hazardous (Moreno-Arribas et al. 2000).

Polyamines, putrescine, spermine, spermidine and cadaverine, are ubiquitous components occurring in animals plants and microorganism (Kalac & Krausova, 2005). Furthermore putrescine, spermine and spermidine can affect the processes of DNA and RNA replication and also the proteins synthesis of tissues in rapid growth. Tyramine and the last three (putrescine, spermine and spermidine) biogenic amines are capable of complexing the radicals. Putrescine (PUT) and cadaverine (CAD) seem to be less toxic than histamine (HIS) and tyramine (TYR); Halasz et al. (2002) reported that PUT and CAD can cause hypotension and bradycardia and, they may have a synergistic effect with histamine and tyramine, increasing their harmful effect.In addiction PUT and CAD can react with nitrites to form nitrosamines, which are reported to be the carcinogenic compounds (Karovicova & Kohajdova, 2005). Healthy individuals can degrade oxidatively BAs, without having any poisoning symptoms, owing to an enzymatic pool present in human gut. The enzymes responsible for their digestion are, monoaminooxydase (MAO), diaminonoxydase (DAO), polyamine oxidase (PAO) and phenolsulfotransferase M (PST M) (Jansen et al. 2003).The degradation of BAs involves oxidative deamination to aldehydes, hydrogen peroxide and ammonia (Smit et al. 2008). Several factors can affect their regular activity, for example people with gastro-intestinal disease are more at risk of BAs poisoning than healthy individuals, because the activity of oxidases in their intestines is usually lower (Bardocz 1995). Additionally, some generic medicines such as paracetamol, and the ethanol levels decrease their detoxification potential, in alcoholic beverages.Littlewood et al. (1988) showed that many alcoholic beverages contain powerful inhibitors of the amino oxidase activity especially, enzymatic activity of PST M. Red wine exerts inhibition properties higher than all the other alcoholic beverages due to its high procianidine content, that are substrates as well as inhibitors of PST M enzyme. Therefore the ingestion of high amount of BAs, as it occurs eating same blue cheeses, which can contain up to hundreds milligrams per kilogram of BAs, might not cause any poisoning symptoms, whereas the ingestion of cheese with red wine could potentially cause the health disturbs above described.

2.2 Biogenic amines and sensitivity factors for humans

The genetic or hereditary deficiency of the enzyme systems of degradation of BAs, as MAO (monoamine-oxidase) and DAO (diamine-oxidase), cause an increase the sensitivity of man to the pathological effects of biogenic amine. It is known that, individuals affected by allergies food showed the lower intestinal DAO activity, than healthy individuals (Raithel et al., 1999). Moreover, according to Jarisch et al., (2004), also in subjects intolerant to histamine manifested a scarce DAO activity. Similarly to the previous cases, also subjects suffering from diseases of the gastrointestinal tract and respiratory system appeared more sensitive to the pathological effects of biogenic amines than healthy individuals. Moreover, it has been recently observed that cigarette smoke reduces the efficiency enzymatic of MAO, until 40% (Broadley, 2010). Finally, Hui & Taylor, (1985) demonstrated that, also ethanol, phenols and acetaldehyde can negatively affect the detoxifying ability of enzymes.

2.3 Microorganisms producing biogenic amines in wine

To avoid biogenic amine formation it is suitable knowing which bacteria are present in wine and which is able to produce biogenic amines. Biogenic amines can be produced by contaminating microorganism (Landete et al., 2005a/b). In fermented food, the decarboxylase activity is expressed mainly by bacteria such as, *O.oeni, Pediococcus spp, Lactobacillus hillgardi, Lactobacillus curvatus* (Spano et al., 2010). Histamine is produced by Gram positive and negative bacteria contaminated. Kanki et al., (2007) reported that the histidine decarboxylase activity of bacteria is expressed during their lifetime, but also after their autolysis. Tyramine is mainly produced by Gram positive bacterial (*Enterococcus spp* and *Lactobacillus spp*) in cheese and fermented sauces, whereas putrescine and cadaverine are mainly produced by Gram negative bacterial (Enterobacteriacee and Psuedomonadacee). Landete et al., (2007) showed that *Lactobacillus brevis* was able to produce tyramine and phenylethylamine whereas Arena &

Manca De Narda, (2001) reported that *L. hilgardii* is the main putrescine producer in a synthetic growth medium.

According to Manfroi et al., (2009), also yeast can produce high levels of polyamines in wine, as spermidine which is involved in physiological cell processes. Moreover, also cadaverine is involved in cell elongation process and putrescine is an intermediate compound of the formation of spermidine, therefore polyamines can be released by yeast cell lysis at the end of alcoholic fermentation. Nevertheless the most of the studies available in literature are aimed to the identification of histamine producers (*L. hilgardii, L. buchneri, L. reuteri, and L. sakei*) and on the decarboxylase activity of O. oeni, the main malolactic bacteria (Costantini et al., 2009).

The results reported by Guerrini et al., (2002) showed that *O. oeni*, isolated from different Italian wine productions, was able to contribute to the overall level of BAs of wine, particularly in putrescine. Moreover, was reported in same study that in a culture medium, 27 out of 44 of *O. oeni* strains were producers of histamine in a range between 3 to 10 mg/L. Rosi et al., (2009) investigated winemaking conditions at laboratory scale and reported *O. oeni* able to produce histamine and tyramine; the amount produced strongly affected by bacteria-yeast contact time after alcoholic fermentation. Lopez et al., (2009) has detected up to 10 mg/L of histamine in wine carried out with a spontaneous malolactic fermentation in which a strain of *O. oeni* was isolated; the above results strongly suggest that, this bacteria can have histamine decarboxylase activity. On the contrary to the findings as above, others researches showed the inability of *O. oeni* to produce histamine. Gardini et al., (2005) isolated strains of *O. oeni* from italian red wine, which were not histidine-decarboxylase positive. Moreno-Arribas et al., (2003) carried a similar experiment isolating *O. oeni* strains from Spanish wine, including commercial starter cultures, and according to its result in vitro, there were no histamine productions in the bacteria analyzed. These results for biogenic amines production in synthetic growth media do not imply similar behavior in wine or in food product. It should be considered that wines are complex matrix with a wide number of chemical and physical factors influencing microbial growth and decarboxylase activity; therefore analytical errors may affect the published results, as suggested by the review of Garcia-Moruno & Munoz, (2012). Furthermore, Guerrini et al., (2002) and Moreno-Arribas et al., (2003) used the same strains of *O. oeni* to study the ability to produce histamine; the findings showed all the bacterial strains tested were not able to produce histamine.

The final results of Costantini et al., (2009), reported commercial preparations in a dry active form can be a substrate for bacterial contamination. O'Brien et al., (2004) reported the presence of *Enterococcus, coliforms* and *Escherichia col*i in the manufacturing process of commercial yeast. Manufacturers of starters inform about the risk of contamination during storage, and they recommend to open the packages just before use; but the results of the present study indicate that commercial yeast starters can be contaminated with lactic acid bacteria in unopened packages. The problem arises during production of these starters, where it is very difficult to maintain sterile conditions, and the results obtained in the present study confirm the presence of contamination. Moreover, Garcia-Moruno & Munoz, (2012) showed the BAs content strongly depends by the contaminating bacteria other than *O. oeni* in wine produced with spontaneous malolactic fermentation.

2.4 Biogenic amines: from grapes to wine

The BAs are mainly generated in wine by contaminating microorganisms with positive decarboxylase activity (Halasz et al., 1994). Manfroi et al., (2009) reported that some biogenic amines are the normal constituents of berries; moreover the spermidine was detected in higher amount than putrescine, cadaverine, spermine, histamine, tyramine and phenylethylamine. The amount of biogenic amines in grapes is affected by cultivar, soil composition, climatic condition, grapes maturity and fertilization (Ferreira & Pinho, 2006). Furthermore, pesticide treatments influence the BAs concentration in grapes. Marques et al (2008) reported that wines produced by healthy grapes showed lowest concentrations of biogenic amine than wines produced by grapes affected by fungi. However, the higher amounts of BAs are generated during the winemaking. In wine, their production is influenced by the availability of free amino acid, presence of microorganisms with positive decarboxylase activity, autolytic phenomena and bacterial strains. Furthermore, all the variables affecting microorganisms growth can influence the formation of BAs in food such as pH, presence of fermentable carbohydrate, oxygen supply, red-ox potential, temperature and the presence of sodium chloride. Biogenic amines can be formed in an acid environment since they are alkali compounds used by microorganisms also as protective mechanism against the acidity, particularly at pH range 4.0 - 5.5. The microorganisms growth and amino acid decarboxylase activity in bacteria are affected by D-glucose concentration. D-glucose content in the range 0.5-2.0 % was reported to be optimal, whereas levels exceeding 3% inhibited enzymes formation. In growth medium, bacteria species as *Enterobacter cloacae* produce about half the quantity of putrescine in anaerobic conditions compared with aerobic conditions, and *Klebsiella pneumoniae* synthesize significantly less cadaverine but acquires the ability to produce putrescine under anaerobic conditions (Halasaz et al. 1994). A low red-oxx potential (reductive conditions) increases histamine production; histidine decarboxylase activity seems to be inactivated or destroyed in the presence of oxygen. Low temperatures can limit the bacterial growth and their decarboxylases activity, whereas temperature in the range 20°C to 37°C is optimal for the growth of the most bacteria containing decarboxylases activity. Sodium chloride stimulate tyrosine decarboxylase activity, whereas it inhibits histidine decarboxylase activity, an example is *Lactobacillus buchneri* which is no able to produce histamine when sodium chloride rich 5% (Karovicova & Kohajdova, 2005).

Putrescine, histamine and tyramine, are mainly produced during the malolattic fermentation (Lonvaud-Funel, 2001; Marcobal et al., 2005). Several microbiological studies, showed that biogenic amines are mainly generated by *O.oeni* strains (Guerrini et al., 2002; Landete et al., 2005; Lonvaud-Funel et al., 1994), whereas Costantini et al (2006) reported that only *Lactobacillus ssp.* was able to produce the biogenic amines. *O. oeni* is considered, among the lactic acid bacteria, the main responsible for malolactic fermentation and production of histamine, although in low concentrations. Landete et al., (2005) showed that also *Pediococcus parvulus and Lactobacillus hildegardi* are able to produce the highest amounts of biogenic amines. It is reported that the concentration of histamine and tyramine, during wine aging and storage, is stable or slowly decreases (Jimenez-Moreno et al., 2004). The degradation of BAs could be related with the presence in wine of enzymes with amino oxidase activity (Ancin-Azpilicueta et al 2008). On the contrary, wines which are left in contact with their lees show an increasing concentration of BAs (Perez-Serradilla et al., 2008) because the autolytic process of yeasts can give free amino acids, which are degraded to the corresponding biogenic amines (Marques et al., 2008; Jimenez-Moreno et al., 2003). The microbiological studies of Leuschner et al., (1998); Gardini et al., (2002) reported the ability of different microbiological species, such as *Micrococcus varians and Staphilococcus xylosus*, to degrade the biogenic amines in wine. The concentration of biogenic amines is positively affected by contact time between the skins and must, availability of free α-amino acids and contact time with yeast lees (Vidal-Carou et al., 1990). It is known that phenolic compounds are able to reduce the formation of BAs especially putrescine (Maria et al., 2006). Furthermore, cadaverine and putrescine have been suggested as indicator of hygienic conditions of cellar and of the sanitary conditions of grapes.

Caruso et al., (2002) showed that also the yeasts such as, *Brettanomyces bruxellensis* (average 15 mg/L) and *Saccharomices cerevisiae* (average: 12 mg/L), are able to produce biogenic amines during the alcoholic fermentation.

2.4.1 Biogenic amines in wine

The main biogenic amines associated with wine are putrescine, histamine, tyramine and cadaverine. These are mainly the products of microbial decarboxylation of ornithine, histidine, tyrosine and lysine, respectively (Smit et al. 2008), although putrescine can also be formed via the arginine deiminase pathway from arginine Figure 2.3 (Arena & Manca de Nadra, 2001; Mangani et al. 2005).

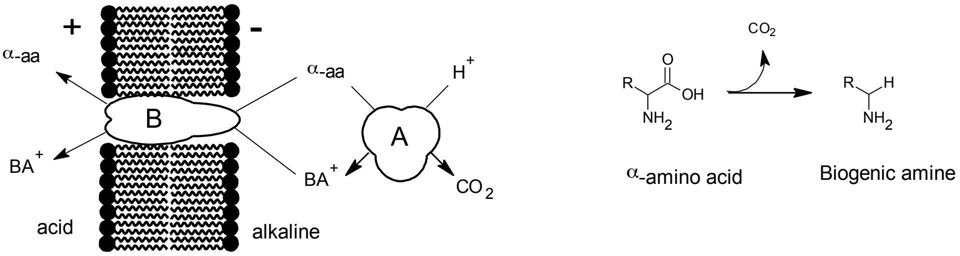

Figure 2.2 Microbial decarboxylation of α-amino acid to biogenic amine.

It is reported by Moreno-Arribas et al., (2003) that the decarboxylase activity is strain dependent, and is also affected by nutritional condition of the matrix and especially by the concentration of free amino acids. Microbiological studies of Leitao et al., (2000) confermed, that strains of *O. oeni* are able to express enzymatic activities such as, proteolytic decarboxylase activities. The microbiological formation pathway of biogenic amines involves two enzymatic steps Figure 2.2; the first (A) one step involves to the decarboxylation from amino acid to biogenic amine and carbon dioxide. The second enzyme step (B) involves to the simultaneous, extrusion and intrusion of biogenic amine and amino acid respectively (Bonnin-Jusserand et al., 2011). The formation pathway previously reported, in addition to being capable of producing biogenic amines, it had also produced energy as ATP through a system antiport between amino acid and biogenic amine, generating proton power Figure 2.2 (Molenaar et al., 1993).

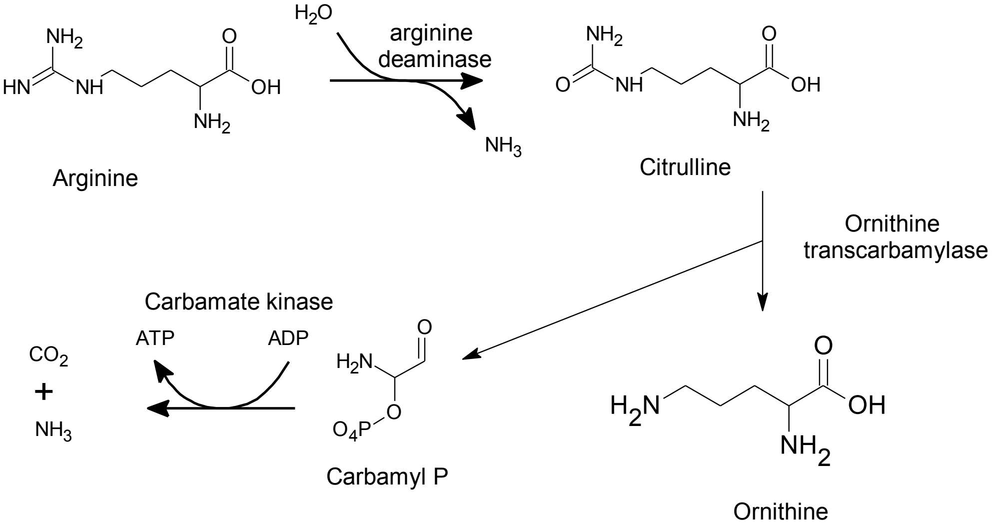

Figure 2.3 Bacterial degradation of amino acids to form biogenic amine and ATP.

Marcobal et al., (2004) reported that in genome of *O. oeni* was found the ornithine decarboxylase, which is responsable for the production of putrescine. However, Magnani et al., (2005) showed that putrescine could also be produced by the deamination of arginine.

The gene responsable for the histidine production (istidine decarboxylase) is localized in an unstable plasmid and the lactic acid bacteria, grown in synthetic growth media, could lose the ability to produce histamine (Lucas, et al., 2007). The strains of the species *L. brevis* have expressed tyramine decarboxylase activity for the production of tyramine, contrary to *O. oeni*. The concentration of tyramine is affected by the levels of glucose and tyrosine in wine (Moreno-Arribas et al., 2000).

2.5 Malolactic fermentation

Following to alcoholic fermentation (AF) lactic acid bacteria of the genera *Oenococcus, Lactobacillus, Pediococcus and Leuconostoc* are able to transform L-malic acid to L-lactic acid Figure 2.4 The lactic acid bacteria (LAB) are rod-shaped bacilli or cocci and characterized by an increased tolerance to a lower pH range. Malolactic fermentation (MLF) is considered to be essential for red wine and some white wine. Lactic acid bacteria are the main responsable for the production of biogenic amines, due to the optimal conditions for their formation during the winemaking, especially for an in increase in concentration of putrescine, histamine amd tyramine (Zotou et al., 2003).

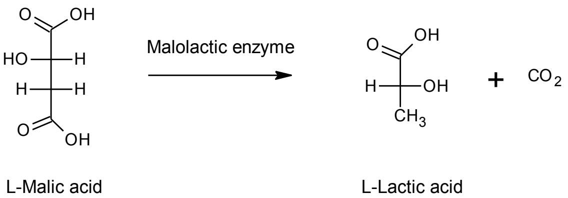

Figure 2.4. Equation of the malo-lactic reaction.

Malolactic reaction therefore involves a decarboxylation without an intermediary product capable of following another metabolic pathway (Peynaud et al., 1968). Even if another metabolic pathway existence cannot be ruled out, malolactic transformation is the only reaction that exists in the lactic acid bacteria involved in winemaking. Therefore, the hypothesis of the existence of an enzyme catalyzing the direct decarboxylation of L-malic acid into L-lactic acid was made. The enzyme, called the malolactic enzyme, was isolated for the first time in *Lactobacillus plantarum* (Schutz & Radler, 1973).The malolactic enzyme is a dimeric or tetrameric protein formed by the association of a 60 kDa polypeptide. It functions only in the presence of the NAD^+ cofactor and bivalent ions, Mn^{2+} being the most effective, and uses a sequential mechanism. The Mn^{2+} and the NAD^+ fix themselves on the protein before the L-malate.The malolactic fermentation can be carried out with four different ways being: by spontaneous induction; by inoculation during or after completion of alcoholic fermentation and by simultaneous inoculation for yeast and LABs also called co-inoculation (Liu, 2002).Spontaneous MLF is due to indigenous LAB genera, such as *Oenococcus, Lactobacillus, Pediococcus and Leuconostoc*; their concentration on grape skin is low, generally less than 10^3 CFU/g of grape. Their concentration ranges between 10^3-10^4 CFU/ml after crushing and immediately before AF; during alcoholic fermentation they decrease in number due to the previously added SO_2 and to the increase of ethanol concentration.

At AF completion the surviving LAB cells, above all *O.oeni* strains, start multiplying, and when their population exceeds 10^6 CFU/mL malolactic fermentation begins. (Lerm et al., 2010). When malolactic fermentation is over, the remaining LAB population may be still able to grow, producing undesirable off-flavors, degrading free amino acids and citric acid therefore increasing biogenic amines concentration (Agouridis et al., 2005). The ability of indigenous LAB to produce BAs can't be foreseen; furthermore their growth is negatively affected by several factors as low pH and temperature, high level of ethanol, SO_2 and procyanidins content (Vincenzini et al., 2005). Therefore, to avoid risks associated with spontaneous MLF, wine-makers can use selected starter cultures of LAB promoting the rapid start and completion of MFL. The choice of the strains has to be based on their fermentation capabilities and growth characteristics such as ability to survive at the vinification process, the lack of off-flavor and off-odor production and the inability to produce BAs. Even with the use of controlled starter cultures, a successful MLF is not guaranteed; wine-makers have to strictly follow the procedures reported by the manufactures (Lerm et al., 2010). Among MLF inducing technique, co-inoculation of yeast and LAB in must is not a common practice due to a lack of knowledge about it, the absence of many research data and the scare of the loose of wine quality. By co-inoculation approach a higher amount of LAB occurs in the favourable growth conditions (higher amount of nutrients and low ethanol concentration) in the must (Vila-Crespo et al., 2010; Cañas et al., 2012). Moreover, during the alcoholic fermentation of α-amino acids are metabolized by yeast almost entirely in the first two days of the AF. The study of Soufleros et al., (1998) has reported that the abundance of amino acids has an overall positive effect on the content of biogenic amines. Therefore, the malolattic fermentation performed by simultaneous inoculation for yeast and LABs (Liu, 2002) could puduced lower amount of BAs than MLF carried out by spontaneous and by inoculation techniques. On the contrary, **(Lerm et al., 2010)** reported that presence of SO_2 and antimicrobal compounds produced by the yeast due to high ethanol concentration, might decrease LAB population during MLF. The study of **Cañas et al., (2012)**, showed that BAs production as cadaverine and tyramine seems to decrease under co-inoculation conditions.

2.6 Determination of biogenic amines in wine

The quantification of BAs in wines can be carried out by different analytical techniques as reviewed by Molnar-Perl, (2003).
The monitoring of BAs, during winemaking is important owing to toxicological risk of BAs also considering the ability of phenol compounds and ethanol to reduce amine-oxydase activity (DAO and MAO) and the amount of BAs can be related to grapes and vinification conditions, therefore their concentration in wine, as in food, deserves careful investigation (Kirshbaunm et al., 2000). In literature, several analytical methods have been reported for the assessment of BAs in wine, which can be determined either by qualitative or quantitative methods.

2.6.1 Qualitative analytical methods

Qualitative determinations usually consist in the use of a simple microbiological screening method that involves different agar with a pH indicator where a change of color is the result of an acidity decrease due to the presence of BAs (Marcobal et al., 2006). In alternative an enzymatic test can be applied, using two enzymes, an amino oxydase, specific for each BAs and a peroxidase to produce a color change. (Smit et al.,2008). These kinds of tests have demonstrated to be specific enough to accurately determine BAs, especially histamine in wines, however the detection limit is poor which make quantitative analytical methods preferable for BAs assessment (Garcia-Villar et al., 2006). On the other hand, Landete et al., (2007) assessed the ability of lactic acid bacteria to express the decarboxylase activity through the application of Polymerase Chian Reaction (PCR). However, the PCR technique is capable of indicating the potential to produce BAs, but does not provide qualitative information of BAs.

2.6.2 Quantitative methods

Quantitative methods usually require a purification step and a separation process owing to the matrix complexity. BAs concentration is low (up to 130 mg/L) and they do not exhibit natural UV adsorption or fluorescence therefore BAs cannot be easily detected by spectrophotometric or fluorimetric detector without a chemical derivatization with fluorophors (Beard Nigel et al., 2004).
Biogenic amines are usually determined by separation technique as capillary electrophoresis gas and liquid chromatography. Among them capillary electrophoresis is interesting because of its short time of analysis and high resolution but, on the other hand, it lacks of sensitivity due to the short pathlenght of light through the window capillary in the UV detector. This fault can be overcome by coupling the CE to mass spectrometry detection or laser-induced fluorescence detector as reported in the study of Santos et al., (2004).
Due to the high BAs boiling points, liquid chromatography is the most widespread analytical method adopted; this approach requires a pre or post- separation column derivatization often performed by o-ptalaldehyde (OPA) or dansylchloride (Dns-CL).
In recent years many analytical methods were proposed with the use of these compounds as labeling agent. Anli et al., (2004); Pineda et al., (2012), are examples of recent HPLC analysis made with Dns-Cl. In both methods, after samples purification, to achieve a successful derivatization, long times and high temperatures are required, moreover Dns-Cl is not particularly sensitive and it produces complex chromatograms as it gives more than one derivatisation product (Busto et al., 1996).
Another approach uses OPA which has great sensitivity and speed of reaction but has a limit of derivatives stability as reported by Pereira Montero & Bertrand, (1994).

To avoid the problem derivatization in the sample injection loop is needed, as suggested by Pereira et al., (2008).

Many other labeling agents are reported in literature as diethyl ethoxymethylenemalonate (DEEMM). (Gomez-Alonso et all., 2007), Dabsyl chloride (Romero et al., 2000) or 1, 2-naphthoquinone-4-sulfonate NQS (Garcia-Villar et al. 2006).

Nevertheless, the methods reported above, are affected by a high solvents consuming due to the long chromatographic separation time. The yeld of derivatization of Dabsyl-Cl is affected by the derivatization temperature and the pH of derivatization reaction (De Meya et al., 2012). Moreover, De Meya et al., (2012) reported that the dabsyl- labeled biogenic amines were unstable at room temperature, when kept for 24 h.

Additionally, the basic nature of the BAs often requires to use poorly soluble salts as eluent modifiers which endanger the efficiency of the chromatographic apparatus.

2.7 Reference

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2.8 Aim of work

Biogenic amines in wine, as in other food and beverages, are important marker of quality. Even if the authorities have not defined a maximum level of BAs concentration, in the oenological field there is a constant effort in keeping their content as low as possible. To this purpose it is important to have new analytical methods.

The aim of the work was to develop a fast, sensitive and easy to apply analytical method (UHPLC-UV) for the BAs assessment in wine. In addition, the method described below does not require particularly expensive analytical procedure, which, in the current period of economic uncertainties is a desirable plus.

The BAs were derivatized as previously described by Kirshbaunm et al. (2000), using dinitrobenzoyl-chloride (DNBZ-Cl) as labeling agent. The analytical procedure should allow to sensitively decrease the processing time of wine samples, derivatization and chromatographic separation, to increase the analytical response, as well as reveal the BAs UV detection, which is commonly used in several laboratories. Method was then applied to the BAs determination in red wines obtained with different wine making steps, differing for the malolactic fermentation condition. This investigation was also aimed to gain preliminary results on BAs production in wine related to the adoption of the co-inoculum technique (Saccharomyces cerevisiae- malolactic bacteria).

2.9 Reference

Kirshbaunm J et al., 2000, Liquid chromatographic determination of biogenic amines in fermented foods after derivatization with 3,5dinitrobenzoyl chloride. Journal of Chromatography A 881:517-530.

2.10 Materials and Methods

2.10.1 Chemicals and reagents

Labeling agent 3,5-Dinitrobenzoyl chloride (DNBZ-Cl) (purity ≥98%), isopropanol (2-prOH) (≥99.9%), acetonitrile (≥99.9%), citric acid (≥99,9%) and methanol (≥99.9%) (metOH) were purchased from Sigma Aldrich. Oenological Polyvinylpolypyrrolidone (PVPP) was purchased from Fluka and frits (diameters 7mm) for self-made cartridges were from Sigma Aldrich. Sodium hydroxide (≥99.9%) was from Merck and hydrochloric acid (≥98%) from Tracepur. Acetic acid glacial (≥96%) was from Carlo Erba.Biogenic amines: histamine (≥99.9%), tyramine (≥99%), cadaverine (≥98%), phenylethylamine (≥99.9%), isopentylamine (≥98%), spermine (≥99%), spermidine (≥99.5%), heptylamine (≥99.5%), putrescine (≥98%), were purchased from Fluka, Sinefrine (99.5%) was from Sigma Aldrich and tryptamine (98%) from Merck.
All reagents used were of analytical grade, at least. HPLC grade water was obtained by a Milli-Q system (Millipore Filter Corp., Bedford, MA).

2.10.2 Standard solutions

Biogenic amines were dissolved in hydrochloric acid 0.1 M and methanol (50:50) to obtain a concentration of 500 mg/L. Appropriate dilutions were prepared for calibration curves.

2.10.3 Samples

Thirteen samples of red wine of the same vintage (2012) were obtained from the oenological region of Langhe (Piedmont, Italy) and from Sardinia. Eight of them were produced by co-inoculum of *S. cerevisiae* strains and malolactic bacteria, for two samples the inoculum of malolactic bacteria was carried out at the end of alcoholic fermentation. Three red wine samples were produced by spontaneous MLF (Talble 2.2).

Winery	n.	Region	Cultivar	MLF
a	1	Sardinia	Carignano	Spontaneous
	2	Sardinia	Carignano	Inoculum
b	3	Piedmont	Dolcetto	Co-inoculum
	4	Piedmont	Barbera	Co-inoculum
c	5	Piedmont	Barbera	Co-inoculum
	6	Piedmont	Merlot	Co-inoculum
d	7	Piedmont	Barbera	Co-inoculum
e	8	Piedmont	Dolcetto	Spontaneous
	9	Piedmont	Dolcetto	Spontaneous
	10	Piedmont	Dolcetto	Inoculum
	11	Piedmont	Barbera	Co-inoculum
	12	Piedmont	Barbera	Co-inoculum
	13	Piedmont	Nebbiolo	Co-inoculum

Table 2.2 wine samples description.

2.10.4 Sample preparation.

Eighty-one milliliters of wine sample were diluted up to 100 ml with methanol (19%) in a volumetric flask, to obtain a final concentration of total alcohols up to 30%. 2 ml of diluted wine were purified by solid phase extraction by a 300 mg PVPP cartridge recovering the loaded fraction. Elution was carried out with 2 ml of 20% methanol solution. The loaded sample was submitted to the derivatization procedure.

2.10.5 Derivatization procedure

80 µl of purified samples, 50 µl of NaOH 2M, 70 µl of 2-prOH, and 210 µl of DNBZ-Cl 50 mM (dissolved in acetonitrile), were added in a reaction vial and shaken by a Vortex mixer. After 10 minutes the reaction was stopped by the addition of 100 µl of HCl 1 M. The mixture was filtered in a hydrophilic PVDF 0.22 µm purchased from Millipore prior to be injected.

2.10.6 Ultra-high-performance UHPLC-UV analysis

UHPLC-MS separations were performed with a Waters Acquity H-Class UHPLC coupled with a Photo Diode array detector (DAD). The column used was core-shell reversed-phase separation column (KinetexTM, Phenomenex) RP18, 2.6 µm particle size, 100 Å pore size, 150mm x 3 mm ID flowing a ternary gradient (Table 2.3). The biogenic amines were detected at 260 nm. The mobile phases was (A) pH 7.0 sodium citrate 30 mM (B) pH 4.3 sodium acetate 20 mM and (C) acetonitrile the flow rate 1.0 mL/min. The injection volume was 10 µL and the column temperature was at 25° C.

	Eluents %		
Time (min)	**A**	**B**	**C**
0.0	85.0	0.0	15
1.22	80.0	0.0	20
1.63	75.5	0.0	24.5
1.90	71.0	0.0	24.5
2.31	69.0	0.0	29
2.72	72.0	0.0	31
2.85	69.0	0.0	28
3.67	67.0	0.0	31
3.80	65.5	0.0	33
4.35	65.5	0.0	34.5
4.62	64.0	0.0	34.5
5.30	0.0	0.0	36
5.44	0.0	64.4	36
6.12	0.0	50.0	50
6.80	0.0	48.0	52
7.61	0.0	40.0	60
7.88	0.0	30.0	70
8.02	0.0	0.0	100
8.56	0.0	0.0	100
8.70	85.0	0.0	15
12.00	85.0	0.0	15

Table 2.3 Gradient profiles for characterization of the BAs by UPLC. The mobile phases was (A) pH 7.0 sodium citrate 30 mM (B) pH 4.3 sodium acetate 20 mM and (C) acetonitrile the flow rate 1.0 mL/min.

2.10.7 Validation procedure

The validation of the method was carried out with respect to qualitative (selectivity) and quantitative (linearity, LOD, LOQ, repeatability and recovery) parameters.

2.10.7.1 Selectivity

Selectivity was tested by spiking model wine and red wine with 20 mg/L of biogenic amines, performing the sample preparation procedure and the separation, and comparing the chromatograms (Figure 2.5). The selectivity of the method was evaluated by comparing the results obtained for the detection of biogenic amines in the absence and presence of possible interferences originating from the white wine matrix.

2.10.7.2 Linearity

Calibration graphs were obtained by spiking known amounts of biogenic amine to water (5 mg/L – 50 mg/L) and red wine (2.50 mg/L - 20.0 mg/ L). The limit of detection (LOD) and limit of quantitation (LOQ) were calculated as the lowest concentration of analyte in a sample that resulted in a signal-to-noise ratio of 3 and 10, respectively. The baseline noise was calculated by the software.

2.10.7.3 Recovery

The recovery of the method was assessed by comparing the peak areas obtained from the aqueous solutions purified, derivatized (5 mg/ L, 10 mg/ L) and directly injected, with the spiked wine samples (5 mg/ L, 10 mg/ L) prepared as above. The recovery was evaluated through triplicated determinations performed in three different days.

2.10.8 Quantification of biogenic amines

BAs were quantified in water solutions and white wine samples by the external standard method. Chromatographic data acquisition and processing were performed by Empower 2 software (Waters).

2.11 Results and Discussion

2.11.1 Improvement of the derivatization

As first step in developing the method, we tested the derivatization conditions described by Kirshbaunm et al. (2000) to assess biogenic amines in red wine. As reported by Kirshbaunm et al. (2000) the same conditions are suitable for the evaluation of amino acid. The first derivatization step of BAs had the following conditions:

- 40 µl Sample +
- 120 µl NaOH 1M +
- 70 µl 2-pr-OH +
- 210 µl DNBZ-Cl 50 mM +
- 5 minutes shaking +
- HCl 2 M.

The chromatographic separation showed the separation of five out of nine biogenic amines added in the standard solution. The derivatization conditions were tested first in order to increase the sensitivity of the method. In the original derivatization procedure 40 µl of sample were diluted to about 500 µl so decreasing more than one order of magnitude the amount of sample injected into the chromatographic system. Changes aimed to increase the amount of sample in the mixture did not allowed to achieve the pH values needed to complete and stop the reaction. Therefore, volume and concentration of the other reactants were modified. Eventually, the following conditions produced the best analytical response and separation conditions.

- 80 µl Sample +
- 50 µl NaOH 2M +
- 70 µl 2-pr-OH +
- 210 µl DNBZ-Cl 50 mM +
- 10 minutes shaking +
- 100 µl HCl 1M
- Final pH 7.0.

The best chromatogram was obtained with the reaction ended at pH 7, but the peak of DNBZ-Cl showed a wide area and since not all the biogenic amines were still discriminated we thought that some of them could be eluted with its peak, therefore we tried to modify the labeling agent concentration. The reaction was tested with DNBZ-Cl at 20, 30, 40 and 50 mM, these trials did not bring the hoped results, in fact to have the best derivatization yield 50 mM DNBZ-Cl was needed.
In conclusion, optimized reaction conditions for biogenic amines, as well as for aminoacids, are:

- 80 µl Sample +
- 50 µl NaOH 2M +
- 70 µl 2-pr-OH +
- 210 µl DNBZ-Cl 50mM +
- 10 minutes shaking +
- 100 µl HCl 1M.

2.11.2 Optimization of the chromatography separation

After testing the performance of the reaction we began to improve the chromatographic separation on biogenic amines in red wine. Kirshbaunm et al., (2000) use the following chromatographic conditions (Table 2.4):

Chromatography condition	
Column	ODS-3 CP 120 RP 18, 250x3 mm, 5 µm
Wavelength (nm)	260 nm
Injection volume (µl)	10 µl
Flow rate (ml/mim)	1 ml/min.
Conditioning	85% A; 15%C
Eluents	(A) 97% 0.1 M NaOAc (pH 7.0) and 3% THF (v / v); (B) NaOAc 100 mM pH 4,3; (C) ACN

Table 2.4 Chromatography conditions developed by (Kirshbaunm et al. 2000).

We used a new type of column, Kinetex^{TM}, Phenomenex RP18 150 x 3mm 2,6 µm 150 Å , with a lower flow rate, 0,6 ml/min instead of 1 ml/min because the efficiency of this core-shell packed bed column is poorly affected by flows exceeding 0.5 – 0.6 ml/min. The eluents were: A) citric acid (H_3C) 20 mM pH 7; B) NaOAc 20mM pH 4, 3; and C) ACN.
The chromatographic conditions allowed to discriminate all the biogenic amines: histamine, tyramine, cadaverine, putrescine, phenylethylamine, tryptamine, spermine, spermidine, heptylamine, isopentylamine with the exception for sinefrine which is probably co-eluting with-DNBZ-Cl. Since the separation was achieved we aimed our experiments to improve the signal. Most of the BAs aliquoted in the stock solution do not have an high solubility which might disturb the quantification. For this reason we tested stock solutions prepared in different medium: HCl 0.1 M:MetOH 50:50, HCl 0.1 M:MetOH 70:30, MetOH only. The former showed the highest analytical response.We evaluated the effect of increased injection volumes from 10-15-20-25 µl, but the higher was the volume injected the lower was the peak area, therefore 10 µl is the recommended injection volume for this analysis.In spite of the high efficiency obtained for all of the peaks separated histamine was eluted as a tailored peak which decreased the sensitivity for such an important compound. Since histamine was eluted when high concentration of eluent A was flowed (A 65.5%, C 34.5%) different eluting conditions were tested. Citric acid solution was tested with THF concentrations increasing from 1 to 7 %. The best shaped histamine peak was obtained with 2% of THF but spermidine and heptylamine were no longer separated and so the best agreement, between histamine resolution and spermidine-heptylamine separation, was found with no addition of THF in eluent A. We also investigated the effect of increasing ionic strength on the resolution. Buffer solutions pH 7 with sodium citrate concentration in the range 20-80 mM were evaluated and the best resolution was obtained with H_3C 30 mM. Moreover, high salts concentration was responsible for in-column salt precipitation following to mixing with acetonitrile, so compromising the column performances.

Chromatography condition	
Column	Kinetex^{TM}, Phenomenex RP18 150 x 3mm 2,6 µm 150 Å
Wavelength (nm)	260 nm
Injection volume (µl)	10 µl
Flow rate (ml/mim)	0,6 ml/min.
Conditioning	95% A; 5%C
Eluents	(A) H_3C 30 mM pH 7;(B) NaOAc 30 mM pH 4,3; (C) ACN

Table 2.5 Final chromatographic conditions for the separation of biogenic amines in red wine.

With the following chromatographic condition (Table 2.5) we were able to separate, in order of increasing elution time: histamine, tyramine, putrescine, tryptamine, phenylethylamine, cadaverine, isopentylamine, spermindine, spermine, heptylamine. The gradient is reported in Table 2.6.

	Eluents %		
Time (min)	**A**	**B**	**C**
0.0	85.0	0.0	15
1.22	80.0	0.0	20
1.63	75.5	0.0	24.5
1.90	71.0	0.0	24.5
2.31	69.0	0.0	29
2.72	72.0	0.0	31
2.85	69.0	0.0	28
3.67	67.0	0.0	31
3.80	65.5	0.0	33
4.35	65.5	0.0	34.5
4.62	64.0	0.0	34.5
5.30	0.0	0.0	36
5.44	0.0	64.4	36
6.12	0.0	50.0	50
6.80	0.0	48.0	52
7.61	0.0	40.0	60
7.88	0.0	30.0	70
8.02	0.0	0.0	100
8.56	0.0	0.0	100
8.70	85.0	0.0	15
12.00	85.0	0.0	15

Table 2.6 Gradient profiles for characterization of the BAs by UHPLC.

2.11.3 Wine purification

Phenolic compounds, occurring mainly in red wine, interfere in the determination of derivatized amines, therefore in the analytical method developed a sample treatment to remove polyphenols from wine was necessary prior derivatization. Phenolic compounds can be adsorbed in the SPE cartridge therefore, C_{18} 500 mg, C_{18} 300 mg and PVPP 300 mg SPE cartridges were tested with a solution of synthetic-wine.

At the beginning the purification procedure was done in three steps;

- Conditioning: 5 ml metOH;
  10 ml H2O;
- Loading: 2 ml of samples, followed by derivatization;
- Elution: 2 ml of H20 + 20 % of metOH, followed by derivatization.

The best recoveries were obtained with PVPP 300 mg as stationary phase (lab made SPE cartridges) which are also the cheapest cartridges. To improve the recovery, wine added with different amounts of methanol was tested for the recovery. We discovered that diluting wine with methanol ,

to a final concentration of total alcohols up to 30%, prior SPE extraction, allowed a better BAs recovery. A further advantage was the easy sample preparation and the shorter analysis time since just one derivatization reaction is needed per sample. The samples preparation chapter shows the final purification procedure.
Applying the chromatographic condition previously reported, to wine sample we were able to separate the following biogenic amines: histamine, tyramine, putrescine, tryptamine, cadaverine, phenylethylamine, isopentylamine, spermine, spermidine, heptilamine, in wine.

2.11.4Analytical method development

Selectivity was evaluated by comparing the chromatographic peaks of the biogenic amines in the presence and in the absence of interferences from the matrix. The UHPLC-UV chromatograms of samples resulting from the sample prep of spiked water and red wine are reported in Figure 5.

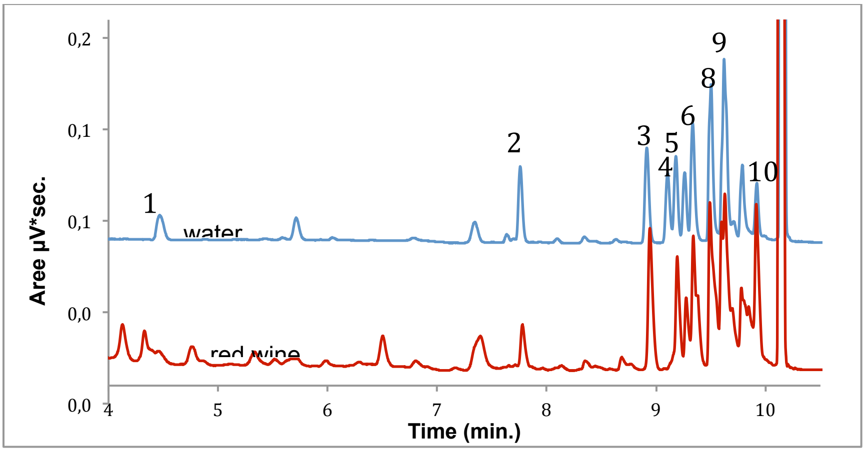
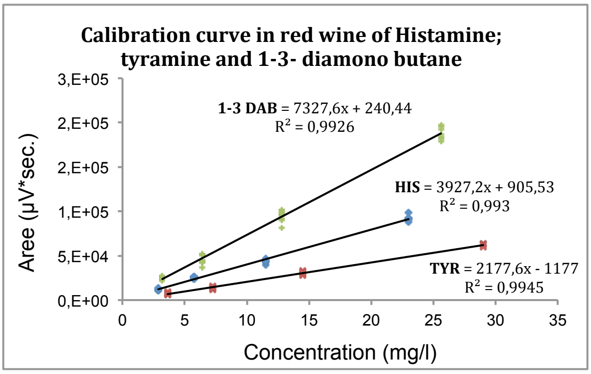
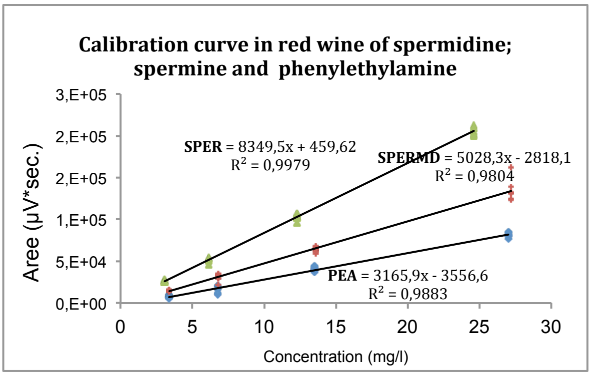


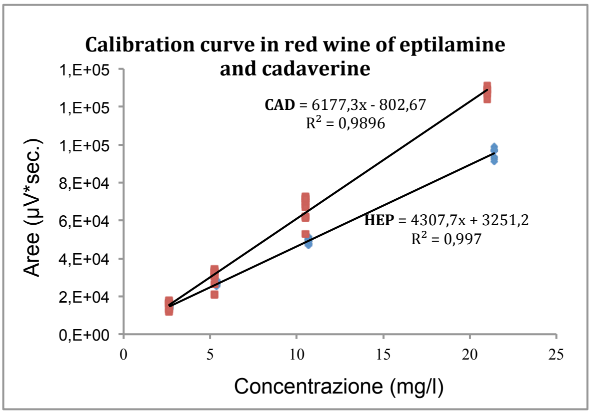
Figure 2.5 UHPLC separation of BAs standard water solution and in red wine (20mg/L): 1, histamine; 2, tyramine; 3, putrescine; 4, tryptamine; 5, Cadaverine; 6, phenylethylamine; 7, 1-3 diaminobutane; 8, spermine; 9, spermidine; 10, heptylamine.

The precision parameters were evaluated for the biogenic amines concentrations (up to 20 mg/L) usually reported in the literature for the red wine (Busto et al., 1996). Therefore, BAs levels up to 23 mg/ L were evaluated in both water aqueous solution and red wine. The analytical method showed linear response (R^2 in the range 0.999 - 0.988) in the whole range of concentration tested with both the matrices (Figure 2.6). Good correlation coefficients (R^2 = 0.988-0.999) were obtained in red wine for the following amines: histamine, tyramine, cadaverine, phenyletylamine, 1-3 diaminobutane, spermine, spermidine, heptylamine. Tryptamine was separate but its quantification was not allowed owing to poorly repeatable recoveries, probably due to its high affinity with PVPP.

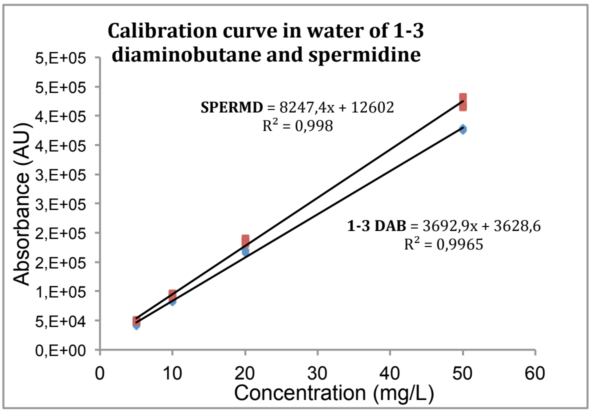
Calibration curve in red wine of BAs:

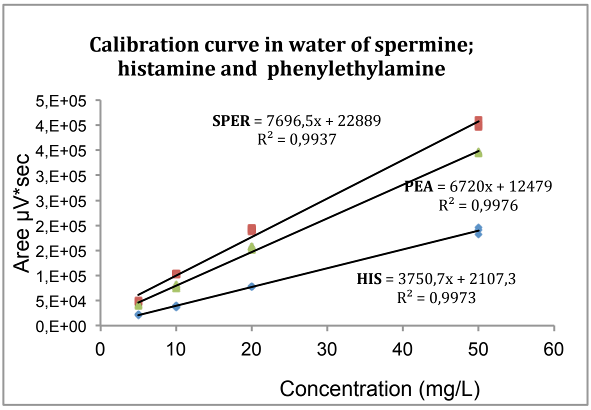






Calibration curve in water of Bas:





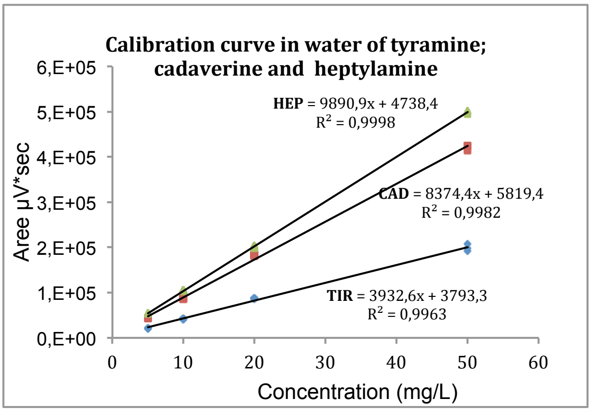


Figure 2.6. Calibration curve of BAs in red wine and water.

The method showed good linear response (Figure 2.6) and this linear range is in accordance with those previously reported in wine (Kirshbaunm et al., 2000).
The relative intermediate repeatability (RDS%) of biogenic amines in water was lower than 2.30% for the concentration level 2.5 mg/L, whereas the RDS% was between 1.24% and 2.29% for higher concentration values. (Table 2.77). However the relative intermediate repeatability values of BAs in red wine for peak areas were between 10.12% and 3.78% for low and high concentrations of BAs, respectively (Table 2.7). The RDS% averages of analytical method in water and red wine were 1.83% and 7.03% respectively.

	Water				**Red wine**			
Concentration (mg/L)	**5**	**10**	**20**	**50**	**5**	**10**	**20**	**50**
Average SD repeatability (μV*sec)	693	1502	2312	3174	1324	2532	3338	4750
Average Repeatability limit (μV*sec)	961	2082	3206	4400	1835	3509	4626	6583
Average RSD (%)	2.01%	2.29%	1.81%	1.24%	10.12%	8.83%	5.40%	3.78%
Average RSD (%)	1.83%				7.03%			

Table 2.7 Precision parameters of biogenic amine quantification in water solution and red wine. Legend: SD: mean standard deviation of BAs (uV*sec.); RSD: mean relative standard deviation of BAs (%%).

The limits of quantification of biogenic amines in red wine were between 0.56 e 0.09 mg/L whereas LODs showed values between 1.87 -0.37 mg/L. Hystamine showed the highest values of LOD and LOQ (0.56 and 1.87 mg/L respectively), while spermine has the lowest values of LOD and LOQ (0.09-0.30 mg/L respectively). The LOD calculated is lower than, or comparable to, the LOD values reported by other researchers (Kirshbaunm et al., 2000 and Pereira, et al., 2008). The data of LOD and LOQ for each BAs are reported in Table 2.8.

Biogenic amines	**LOQ (mg/L)**	**LOD (mg/L)**
Histamine	1.87	0.56
Tyramine	1.23	0.37
Cadaverine	0.37	0.11
Phenylethylamine	0.77	0.23
Isopentylamine	0.38	0.11
Spermine	0.30	0.09
Spermidine	0.37	0.11
Heptylamine	0.51	0.15

Table 2.8. Quantification and Detection Limits.

The recovery values, of the several biogenic amines, obtained were between 47% and 118% for the spiked red wine at concentration levels 5 mg/L and 10 mg/L. The recovery data of BAs are reported in Table 2.9. Hystamine showed the best values of recovery (109% and 118%) at both the concentration levels investigated, whereas phenylethylamine presented lowest recovery values (47% and 58%). Such values were higher than those previously reported in previous method using HPLC-Fluorescence detection (Pereira, et al., 2008).

	Recovery (%)	
Biogenic amines	**5 mg/L of BAs**	**10 mg/L of BAs**
Histamine	109	118
Tyramine	63	71
Cadaverine	75	78
Phenylethylamine	47	58
Isopentylamine	99	105
Spermine	106	94
Spermidine	64	65
Heptylamine	111	96
Average recovery (%)	84	87

Table 2.9. Recovery of BAs in red wine at two concentration levels (10 mg/L and 5 mg/L).

2.11.5 Quantification of biogenic amine in wines submitted to malolattic fermentation

Biogenic amines were quantified by the method described above, in the wine samples considered in the study (paragraph sample). The results obtained are reported in Table 2.10.

			BAs (mg/L)								
Sample (N°)	***MLF***	**Winery**	**His**	**Tyr**	**Cad**	**Pea**	**1-3 Dab**	**Spe**	**Spd**	**Hep**	**tot.**
1	*Spontaneous*	a	0.0	0.0	0.4	2.1	2.0	5.3	20	5.5	35.3
2	*Inoculum*		0.0	0.0	0.4	6.2	0.7	0.0	7.4	1.4	16.1
3	*Co-inoculum*	b	0.4	0.0	0.7	7.1	1.9	2.2	9.0	13.7	35.0
4	*Co-inoculum*		0.0	0.0	2.8	2.8	2.9	0.0	7.2	8.0	23.6
5	*Co-inoculum*	c	3.1	0.0	0.5	1.7	1.7	4.5	5.5	11.8	28.8
6	*Co-inoculum*		0.0	0.0	0.6	6.5	1.0	2.0	9.9	13.2	33.2
7	*Co-inoculum*	d	0.0	0.0	0.8	2.1	0.3	0.0	2.0	1.6	6.7
8	*Spontaneous*	e	0.0	0.0	0.7	3.4	1.9	0.0	7.5	0.0	13.4
9	*Spontaneous*		0.0	0.0	0.7	6.7	0.8	2.2	8.0	0.0	18.3
10	*Inoculum*		0.0	0.0	0.6	5.6	1.0	1.3	7.1	0.0	15.6
11	*Co-inoculum*		0.0	0.0	0.0	5.5	1.1	1.7	9.2	0.0	17.5
12	*Co-inoculum*		0.0	0.0	0.0	0.0	0.0	0.0	1.3	0.0	1.3
13	*Co-inoculum*		0.0	0.0	0.7	1.0	2.3	5.8	5.7	8.6	24.1

Table 2.10. Quantification of biogenic amines in wine samples from different malolactic fermentation induction methods (mg/L).

Biogenic amines contents were generally low for all the samples over all if compared with wines of previous vintages, as in 2012 grape ripened under hot dry climate which reduced amino acids content in musts, precursors of biogenic amines (Riberau- Gayon, et al. 2006). Histamine was detected only in the wine treated with the co-inoculum technique (samples n° 3 and 5). Tyramine doesn't detected in all samples.

Wines treated with the co-inoculum technique, they were also able to produce a higher amount of BAs than wines processed with the spontaneous malolactic fermentation and inoculation technique. The concentration of polyamines (such as: putrescine, spermidine and spermine) detected was higher than the concentration of pressor amines such as histamine and tyramine.

2.12 Conclusion

The derivatization reaction developed for the BAs detention increases the sensitivity of the analytical method proposed by Kirshbaunm et al., (2000). The purification step by adding methanol up to 30 % allows the direct quantification of BAs and higher recoveries. The samples purification developed was faster and easier to apply than other analytical methods proposed by (Pineda, et al., 2012; Gomez-Alonso, et al., 2007)

At the same time, UHPLC separation permits a sensitive reduction of the time analysis as well as of the volumes of chemicals waste compared to HPLC technique (Pineda, et al., 2012; Kirshbaunm et al., 2000).

The BAs of oenological importance were separated and quantified, with the exception of tyramine, by the chromatographic conditions applied.The peak of histamine results splitted, due to a reason not completely clear, but carrying out further experiments its separation can be improved.

Good linear response and good correlation index have been achieved; detection and quantification limits have been lower than the recommended limits of biogenic amines in wine, also in view of possible limits imposed by law.

All this factors make the proposed analytical method reliable, fast and accurate but mostly applicable by oenological laboratories as does not require the use of expensive materials and equipment which making it a suitable vehicle for the easy BAs assessment in wine.

Additionally the method can be easily applied to amino acids assessment by minor modification of the chromatographic gradient separation (data not shown).

The preliminary results concerning the different malolactic fermentation conditions, suggest that co-inoculum technique does not seem to prevent BAs formation in wine.

2.13 Reference

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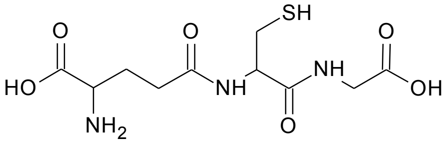
Assessment of intra and extra-cell glutathione levels in grape ferments (South African's Sauvignon blanc), supplemented with glutathione-enriched inactive dry yeast preparations (GSH-IDYs) using an UPLC-MS/MS method.

3 Introduction

3.1 Glutathione

The tripeptide, L-g glutamyl-L-cysteinyl-glycine Figure 3.1, or GSH, (mol.wt. 307), is the major low-molecular-mass thiol compound in plants and animals (Meister & Anderson, 1984). The biologic importance of glutathione is due to the nucleophilic and reducing properties of the thiol group in the cysteine residue (Penninckx, 2000). GSH lacks the toxicity associated with cysteine (Vina et al., 1983), making this compound suitable as a cellular thiol "redox buffer" to maintain a given thiol/disulfide redox potential. GSH was detected in different microorganisms such as Gram positive and negative bacteria, and yeasts as *Saccharomyces cerevisiae*. The thiol tripeptide is widely present in microbial eucaryotes, where it may have been distributed by endosymbiotic events (Penninckx & Elskens, 1993). GSH may account for 0.5 to 1% of the dry weight in the yeast *S. cerevisiae*, depending on the growth conditions. Elskens et al., (1991) shows that GSH accounts for more than 95% of the amount of non-protein thiols present in a wild-type strain *S. cerevisiae*. In bacteria GSH occurs primarily in Gram-negative aerobic species but very rarely in anaerobes and Gram-positive strains (Fahey, 2001). GSH is used as nutrient source of nitrogen or sulfur by *Saccharomyces cerevisiaeis* and it may be mobilized to ensure cellular maintenance. Glutathione S-transferases may be also involved in the detoxification of electrophilic xenobiotics; furthermore, the vacuolar transport of metal derivatives of GSH ensures resistance to metal stress.



L-g-glutamyl-cysteinil-glycine
(GSH)

Figure 3.1 Molecular structure of glutathione.

In the plant kingdom, GSH is involved in the metabolism of sulfur in response to biotic and abiotic stresses and in the biosynthesis of aromatic thiol compounds (Kobayashi et al., 2010.). In addition to the key role within living cells, GSH is involved in the technological process of wine making, starting from the grape, through the yeast and lactic acid bacteria. Its production and release from microorganisms is an important feature to be monitored during the wine making, given its role in must and wine.

Considerable amount of data obtained in vitro and in vivo experiments have shown which phenolic compounds in wine have great antioxidant activities, such as complexing of reactive oxygen species (ROS) (Giugliano, 2000; Makris et al., 2006). However, many constituents of wine, including phenolic compounds, tyrosine, aldehydes and certain metals are susceptible to oxidation during the winemaking process and lead to browning.

Browning is an oxidative process involving sugars, lipids, amino acids or phenols in food. It is one of the main problems encountered during wine making as it adversely affects the sensory properties of wine (loss of colour, flavour and aroma, and increase of astringency) (Escudero et al., 2002; Ferreira et al., 1997) and results in the loss of nutritional value of wine (Sioumis et al., 2005). The inhibitory action of GSH on browning is caused by combination with the catalyzing quinone. Moreover, GSH conducts an important action in the composition of aromatic potential, not only in grapes, but also in must. Glutathione is able to react with hexenal, which is produced from unsaturated fatty acids by lipoxygenase enzymes present in the must, to produce the precursors of aromatic molecules; a well-known example is the glutathionylated precursor of the 3-glutathionilate mercaptohxan-1-ol (G3MH) (Roland et al., 2010). In addition to its protective role against browning of must, it was reported by Lavigne & Dubordieu (2004) that GSH may also play a protective role against oxidative loss of the thiol varietal aromas during the wine-making and aging. It is known that the only GSH reduced formare able to exert the antioxidant properties.

3.2 Formation of GSH in plant

The plants produce GSH through the the enzymes (Figure 3.2) γ-glutamyl-cysteine ligase or γ-glutamyl-cysteine synthase (γ-ECS or GSH1) and glutathione synthase (GS or GSH2) (Noctor et al., 1997; Leustek et al,. 2000).

- γ -glutamyl-cysteine ligase or γ-glutamyl-cysteine synthase (γ-ECS or GSH1);
  The enzyme γ-ECS catalyzes, in chloroplasts, the formation of the bond between L-glutamate and L-cysteine with the use of ATP. The reaction involves the condensation of the γ -carboxyl group of L-glutamate with the α-amino group of L-cysteine, to form γ -glutamylcysteine (γ -Glu-Cys or γ -EC) (Noctor et al., 1997).
- glutathione synthase (GS or GSH2).
  GS enzyme catalyzes the formation of GSHnwhich is ATP-dependent. The reaction involves to the formation of a bond between α-carboxyl group of cysteine and α-amino group of glycine to form GSH. The latter reaction occurs both in the cytosol and in chloroplast, depending on the strain of yeast (Leustek et al,. 2000).

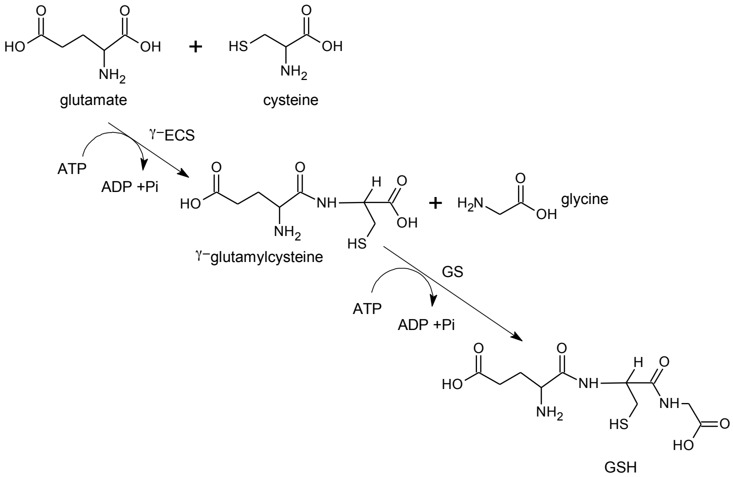


Figure 3.2 GSH biosynthesis in plants.

Although intracellular GSH is used in its reduced form in many physiological functions, it can be converted in its oxidized form (GSSG). The regeneration of reduced GSH is performed by glutathione reductase (GR), which is a dimeric flavoproteins NADH-dependent. The reduction reaction involves to the reduction of disulfide group of GR and at a later stage occurs an exchange reaction dithiol-disulfide between GR and GSSH and two molecules of GSH are released.

3.3 Glutathione: presence in grape

GSH is normally the non-protein molecule containing a thiol group which is present in most plant tissues; usually, its concentration is higher than that its precursor (cysteine) (Adams & Liyanage, 1991). GSH conducts different biological functions in plant tissues such as the protection of cells from reactive oxygen compounds and it plays a central role in the response to environmental stress as a precursor of phytochelatins which are the class of peptides that binds toxic heavy metals such as cadmium and lead (Kobayashi et al., 2010). Moreover, Adams & Liyanage, (1993) reported that GSH plays an important detoxifying function from xenobiontic which are the substrates of the glutathione-S-transferase. The concentration of glutathione in grapes depends on the cultivar and the climatic conditions. Doubourdieu & Lavigne, (2002) reported that the amount of GSH was between 17 mg/kg and 114 mg/kg of grape (56 μmoli/kg and 372 μmoli/kg) in different white grapes. Chardonnay and Riesling showed the highest concentration of GSH whereas Chenin blanc and Grenache blanc which presented the lowest amounts of glutathione. Furthermore, the relationship between the concentration of hydroxycinnamic acids and GSH is variable in the range between 1.3 and 12.7, which is also linked to the cultivar (Cheynier et al., 1989). However, the level of GSH is also influenced by the availability of nitrogen and water in the soil (Doubourdieu & Lavigne, 2002). These important differences in the levels of GSH in grapes, may explain the variable susceptibility to the browning of musts. The cultivars highly susceptible to oxidation/ browning of must show the lowest concentration of GSH compared to the total amount of hydroxycinnamic detected.

The glutathione accumulation occurs at the beginning of ripening, in correspondence to the veraison phase of grape. GSH is trasported through the phloem from leaves to the sink as reduced glutathione; this transport mechanism is confirmed by Lappartient & Touraine, (1997) that observed an increase of glutathione in the berries with a corresponding decrease in the leaves during ripening. GSH is trasported to leaves, as it represents the transport form of sulfur within the plant, in order to ensure the presence of GSH in plant tissues (Adams & Liyanage, (1993)). GSH performs an important role in the biosynthesis of many aromatic precursors present in berries of several cultivars, characterizing the varietal aromas. An example is the Sauvignon blanc cultivar in which the volatile thiol compounds characterize its typical aroma of flower boxwood, blackcurrant (Mercapto-4-methyl pentan-2-one 4MMP) and citrus (3-mercapto-hexan-1-ol, 3MH) which are present in grape in conjugated form to GSH and cysteine. The chemical bond between precursors and GSH is cleaved by yeast during the alcoholic fermentation with the release of aromatic molecule and GSH Figure 3.3 (Roland et al., 2010).

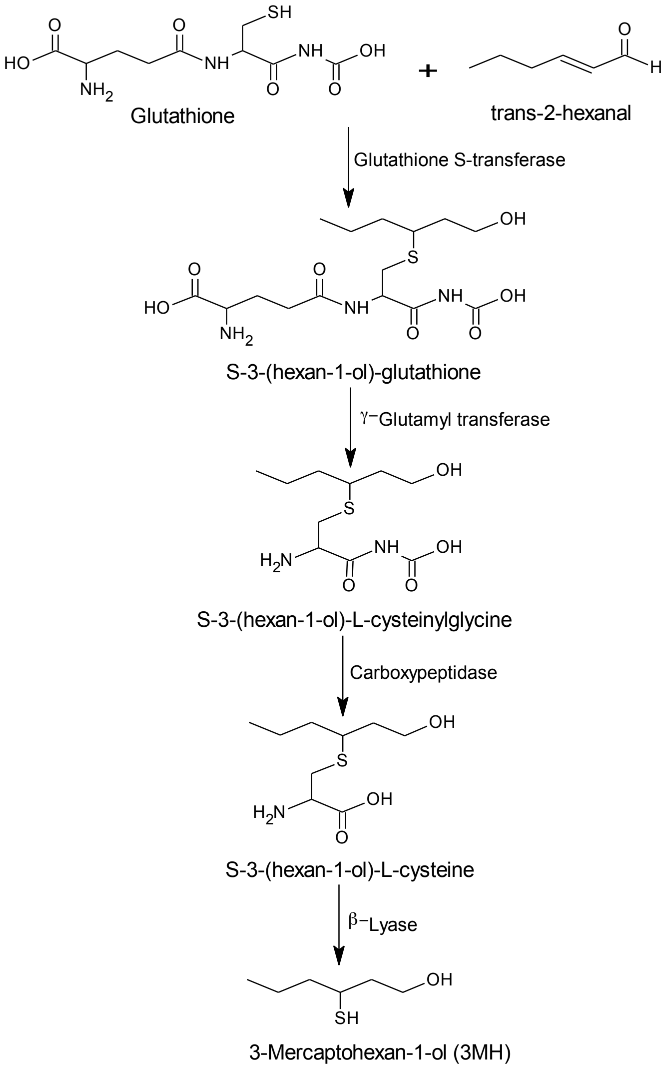


Figure 3.3 Formation pathway of 3MH during alcoholic fermentation.

The biosynthesis and accumulation of glutathione and cysteine in grape are affected by agronomic practices. The nitrogen nutrition of plant affects the availability of GSH in must; Masneuf, (2000) reported that the amount of glutathione was greater in musts with high nitrogen availability that those deficient in nitrogen. Also, the availability of water appears to influence the content of cysteine in the berry; Adams & Liyanage, (1993) showed that a moderate water stress seems to be favorable to its accumulation.

3.4 Glutathione: presence in must and wine

The GSH content in grape juice ranges from 10 to 100 mg/L (Cheynier et al., 1989) and factors such as exposure to oxygen, tyrosinase activity and grape skin maceration during pre-fermentation can affect its concentration (du Toit et al., 2007; Maggu et al., 2007). The concentration of GSH in wine is lower than in juice and grapes and it ranges from 1 to 20 mg/L (du Toit et al., 2007). In instances where the concentration of GSH in white wine exceeds 6–10 mg/L, both colour and aroma were better preserved during ageing and storage (Lavigne & Dubourdieu, 2004). *Saccharomyces cerevisiae* can also affect the GSH content in wine during alcoholic fermentation as well as during the ageing on the lees (Lavigne et al., 2007). According to du Toit et al., (2007) the concentration of glutathione during crushing and pressing is affected by the oxygen level, mechanical harvest and the pressing degree of grape. The crushing under anoxic conditions of grapes (< 0.3 mg/L of dissolved oxygen) showed the higher levels of GSH in must and wine than the oxidative conditions (3.5-4 mg/L of dissolved oxygen) in which was detected the lower amount of glutathione. Maggu et al., (2007) reported that lower amount of GSH was detected in must obtained from grapes picked with mechanical harvesting as well as moderate amount of 2-S-glutathionyl-caftaric acid (9-15 mg/L). Moreover the first-press juice is characterized by higher amount of glutathione than the juice obtained by the further pressing cycles (Maggu et al., 2007).

Several authors reported conflicting results on the trend of glutathione during alcoholic fermentation. Andujar-Ortiz et al., (2012) demonstrated an increase in the level of GSH, whereas du Toit et al, (2007) showed a decrease of GSH during alcoholic fermentation.

In living tissues, GSH assumes pivotal roles in bioreduction, protection against oxidative stress, detoxification of xenobiotics and endoge-nous toxic metabolites, transport, enzyme activity and sulfur and nitrogen metabolism. (Penninckx, 2002).

Doubordieu & Lavigne, (2004) reported that yeast metabolizes GSH in must during the growth stage and it is released at the end of the alcoholic fermentation during autolysis. According to Lavigne at al., (2007) the GSH released is affected by yeast strain, whereas Fracassetti, (2010) reported that the amount of GSH increased during the alcholic fermentation and during the cell lysis of yeast, whereas GSH is not metabolized by the yeast at the beginning of alcholic fermentation. The concentration of GSH in wine (from 3 to 20 mg/L) is lower than in juice and grapes (Cassol & Adams, 1995; du Toit et al., 2007) and it can increase through the choice of an adequate yeast strain (Rauhut, 2009). No GSH is detected from yeast under nitrogen starvation during the alcoholic fermentation (Lavigne & Dubourdieu, 2004). Furthermore, the yeast can release compounds other than GSH after lysis, such as amino acid, peptides, fatty acid, polysaccharides and glyciprotein, all of them performing different function. It is reported by Ugliano et al., (2011);Fracasetti et al., (2010) that during the aging of wine the decreases the amount of glutathione.

3.5 Glutathione and yeast

GSH is present in high amount (up to 10 mM) in the yeast cells. Glutathione is involved in many vital function of *Saccharomyces cerevissiae*, such as maintaining the integrity of mitochondrial membranes, response to oxidative stress, xenobiotics and endogenous toxic metabolite detoxification and response to deficiencies of sulfur and nitrogen (Penninckx, 2002). Mutant strains of *Saccharomyces cerevisiae and Schizosaccharomyces pombe*, that expressed a low capacity to produce GSH, showed a greater sensitivity to heavy metals and to xenobiotics, and to oxidative stress (Grant et al., 1996). The GSH synthesis in yeast occurs as in plants. Penninckx, (2002) reported that GSH in addition to being synthesized by yeast, it can also be imported from the outside environment. Two enzymatic different transport systems (GSH-P1 and GSH-P2) of GSH were described by Penninckx, (2002) in *Saccharomyces cerevisiae*.

3.6 Relations between GSH and yeast

3.6.1 Glutathione and nutrient starvation of yeast

Saccharomyces cerevisiae can degrade GSH in response to the nitrogen and sulfur starvation as source of these elements (Elskens et al., 1991). GSH can be taken up by yeast cell through two transport system (GSH-P1 and GSH-P2). The enzyme GSH-P1 is a system ATP-dependent high-affinity which transports GSH in response to sulfur deficiency, whereas enzyme GSH-P2 is characterized by low-affinity to GSH and unregulated system. About 50% of the yeast glutathione is stored in the cytoplasm and the remaining is stored in the vacuole during the growth, when nitrogen is avaliable.

The cytoplasmic GSH is used as endogenous sulfur source (Elskens et al., 1991) and in case of total sulfur deficiency, it reaches a residual concentration until 10% of its normal value. If the yeast is in nitrogen starvation more than 90% of GSH is transported to the central vacuole in which it is splitted in the rispective amino acid by the γ-glutamyl transpeptidase (Mehdi & Penninckx , **1997**).

3.6.2 Glutathione and oxidative stress of yeast

Several studies demonstrated GSH carries out an important role in yeast against the oxidative stress. Yeast produces toxic oxidative compounds during its metabolism such as hydrogen peroxide, hydroperoxide lipid (LOOH), superoxide anion and alkylhydroperoxide (ROOH). These radicals can degradate in very reactive compounds which can cause the cell death and metabolic imbalances. The glutathione peroxidase (GPx) is key enzyme in the defence mechanisms against hydroperoxide lipid radicals. The GPx protein catalyses the reduction of hydrogen peroxide to water and the organic peroxide to the corrisponding stable alcohols. The glutathione peroxidase performs the biological reduction through the use of GSH as equivalents source, according to the reaction as follows:

$$2\ \mathrm{GSH} + \mathrm{ROOH} \longrightarrow \mathrm{GSSG} + \mathrm{H_2O} + \mathrm{ROH}$$

This enzyme is induced by the oxidative condition in *Saccharomices cerevisiae* and it is localized in cytoplasm and mitochondria, where high amounts of peroxides are produced (Spector et al., 2001).

3.5.3 Detoxification of heavy metals and xenobiotics

Moreover, unfavorable yeast growth condition and environmental stress cause an accumulation of toxic heavy metals and xenobiotics. GSH carries out an important role in cellular defense against reactive electrophiles. The reaction of chelation is catalyzed by the glutathione S-transferase enzyme, according to the reaction as follows:

$$\text{GSH} + \text{RX} \longrightarrow \text{GSX} + \text{RH}$$

Glutathione can chelate the heavy metals present in cytoplasm (i.e. copper, silver and lead) and the complexes formed are stored in the central vacuole and then eliminated (Pennickx, 2000; Ortiz et al., 1992).

3.7 Adding of glutathione in wine: GSH-IDYs

Elevated wine GSH levels can indirectly contribute to wine quality. Nevertheless, the addition of food-grade GSH to must or wine is not allowed by current wine regulation (Ugliano et al. 2011). There are, however, other permitted additives available on the market that claim to increase wine GSH levels and have positive effects on wine quality, especially in terms of aroma and colour preservation (Pozo-Bayo´n et al. 2009a). These products, referred to as GSH-enriched inactive dry yeast preparations (GSH-IDYs), are incorporated during alcoholic fermentation and claim to increase wine GSH content either by the liberation of GSH into the wine or by allowing the yeast to assimilate GSH precursors for GSH synthesis during alcoholic fermentation. GSH, which presents more than 95% of the non-protein thiols in *S. cerevisiae* (Elskens et al. 1991), may account for 0.5–1% of the cell's dry weight, depending on the growth conditions (Penninckx. 2000). GSH-IDYs are manufactured from the thermal inactivation of *S. cerevisiae* cultivated under specific conditions that stimulate the intracellular accumula-tion of GSH.

3.8 Browning of must and wine and the role of glutathione

The browning is an oxidative process that occurs during wine making and it negatively affects to the color and the characteristic aromas of wine (Li et al., 2008). The oxidative deterioration of white wine is mainly caused by exposure to the oxygen (Singleton, 1987). Oxidation can be performed through non-enzymatic or enzymatic reactions. The enzymatic oxidation is carried out in must and it is correlated to the amount of hydroxyl-cinnamic acids (caftaric acid and coutaric acid) and flavans-3-ol (Li et al., 2008). Singleton et al., (1985) reported that the average amount of caftaric acid in juice is approximately 100 mg / L and it is affected by cultivar and by environmental conditions. The phenols, in particular the ortho-phenols, are mainly responsable for the oxidative browning of must and wine (Betes-Saura et al., 1996). The oxidation of phenols is carried out by tyrosinase or polyphenol oxidase of grapes (PPO), which may show two types of enzymatic activities such as cresolase and catecholase (Sanchez-Ferrer et al., 1995). On the other hand the non-enzymatic oxidation, also known as chemical oxidation of wine, occurs in wine. The reaction involves to the oxidation of catechols to form unstable polymers of quinones that are responsable for browning of wine (Oliveira et al., 2011). This reaction is affected by the presence of oxygen and by the availability of phenols and transition metals (Danilewic et al 2008).

3.9 Enzymatic oxidation

Oxygen consumption rate varies from 0.5 to 5 mg/l/min, depending on must origin and on the phenols matrix. Tyrosinase enzyme is normally present in healthy grapes, whereas laccase is present in grapes affected by *Botrytis cinerea* (Dubernet & Ribereau-Gayon, 1973-1974). The PPOs present two different enzymatic activities such as, cresolase and catecholase (Figure 3.4).

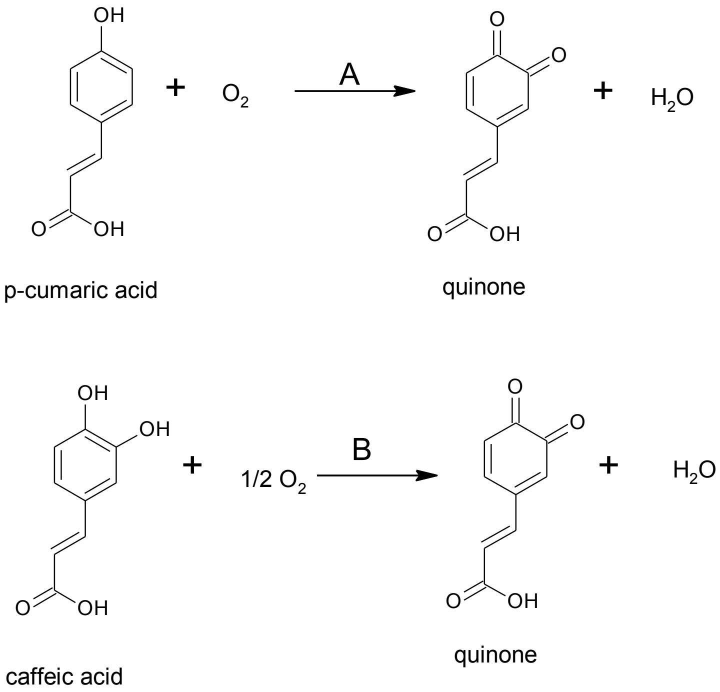


Figure 3.4 Mode of action of grape tyrosinase on hydroxycinnamic acids (Mayer & Harel, 1991). (A) Cresolase activity and (B) Catecholase activity.

The cresolase activity is able to form a o-quinone by the oxidation of a p-monophenol to catechol (A), whereas the catecolasica (B) activity produces a quinone by the oxidation of catechol Figure 3.4 (Sanchez-Ferrer et al., 1995). The must browning is affected by flavonoid concentration and consequently by mechanical treatments during wine making (e.g. maceration). These operations are also involved in the solubilization of the tyrosinase bound to the cell wall of berry cells. In grape must, this enzymatic activity (tyrosinase) preferentially oxidizes tartaric derivatives of hydroxyl-cinnamic acids (1), to form quinones (2), which are unstable and likely to involve into two different reactions Figure 3.5. The quinones produced are very reactive and can condense with other phenolic compounds (flavonoids), forming polymerized products. Their color evolves from yellow to brown according to the degree of condensation (Singleton, 1987). The quinones can also react with a strongly reductive molecule such as glutathione. The reaction between quinone and GSH produces a colorless derivative, S-glutathionyl-2-trans-cafeoyltartaric acid, known as the Grape Reaction Product (GRP) (Salgues et al., 1986). GRP (3) derivative is not oxidizable by tyrosinase and thus does not modify the color of the must.

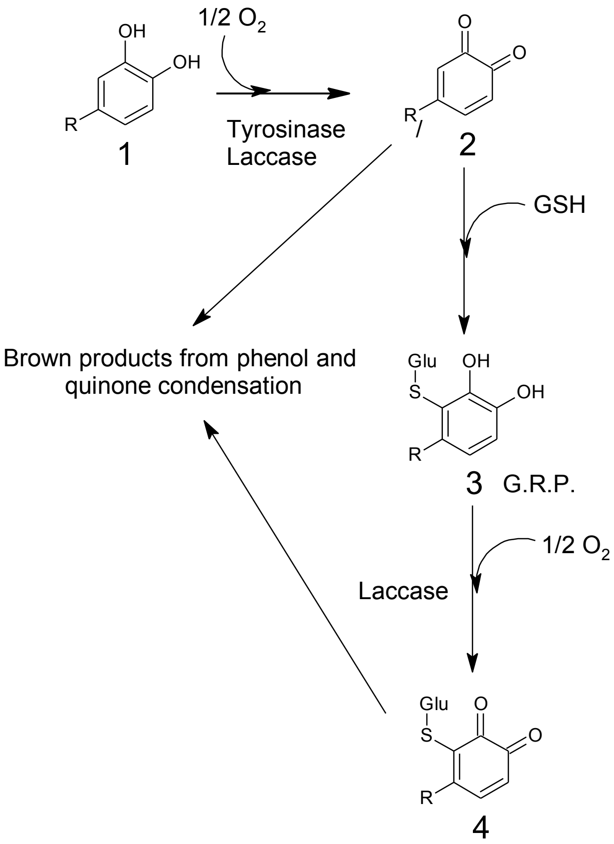


Figure 3.5 Oxidation mechanisms of healthy grape must by tyrosinase and botrytized grape must by laccase (Salgues et al., 1986).

Glutathione limits the oxidation phenomena, through the trapping of quinone and the must browning is therefore also dependent on the glutathione concentration.

The tyrosinase activity in must is affected by the pH, temperature and amount of sulfur dioxide. This enzyme is active but unstable at the pH of must (optimum activity at pH 4.75) and temperatures above 55° C or the addition of more than 50 mg/L of sulfur dioxide are necessary to denature this enzymatic activity (Cheynier et al., 1989; Ribereau-Gayon et al., 2004).

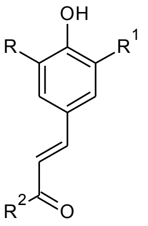
Sulfur dioxide concentrations lower than 50 mg/L only modify oxidation rates. In fact, the bisulfite ions regenerate the potential enzyme substrates by reducing the quinones formed. Finally, treating must with bentonite reduces the soluble fraction of tyrosinase.

Phenols oxidation is much more dangerous when the grapes are infected by *Botrytis cinerea*. Botrytized grapes contain a PPO known as laccase (Mayer & Harel, 1991). Contrary to tyrosinase, this enzyme is stable at the pH of must and it is much more resistant to sulfur dioxide and it is also able to oxidize a greater number of phenolic substrates and other compounds.

Laccase is thus able to oxidize the GRP (3) to quinone (4). The glutathione, therefore, can no longer trap quinone (Salgues et al., 1986). The brown condensation products, which are formed from the same initial phenolic substrates by laccase, are more brown than those generated during the oxidation by tyrosinase. If the glutathione concentration in must is elevated, the quinone (4) can be partially reduced to phenol with the chemical bond of a second glutathione molecule. Moreover, this new complex can't be further oxidized by laccase (Salgues et al., 1986). Oxidation reactions and the corresponding browning are thus limited. This second reaction is not promoted in botrytized grape musts (Salgues et al., 1986). The rate of oxygen consumption by botrytized grape musts is lower than in healthy grape must, but the action of sulfur dioxide is slower. The contaminated grape contains many other oxidase enzymes that also consume oxygen to oxidize other compounds, such as glucose, amino and, etc.

3.10 Non-enzymatic oxidation

The polyphenols presents in must, especially tartaric derivatives of hydroxyl-cinnamic acids (Figure 3.6), are the major substrate for the oxygen. Moreover, polyphenols are released into the wine, during wine making, (eg, catechins, anthocyanins, procyanidins and etc.) can be oxidized more easily, than the polyphenols present in must (Danilewicz, 2012). The oxygen is responsible for oxidation chain reaction to form free radical.



R	R^1	R^2	Compounds
H	H	OH	p-coumaric acid
OH	H	OH	caffeic acid
OCH_3	H	OH	ferulic acid
H	H	H_2T	coutaric acid
OH	H	H_2T	caftaric acid
OCH_3	H	H_2T	fertaric acid

Figure 3.6 Hydroxycinnamic acids present in grapes and grape must.

The oxygen amount consumed is related to the amount of quinone produced. The formation of a quinone from phenol (Figure 3.7) need two atoms of oxygen for each mole of catechol oxidized. This reaction also produces a mole of hydrogen peroxide (3) and a semi-quinone unstable. The oxidative chain mechanism can continue until the semiquinone is oxidized to the corresponding quinone. (Singleton, 1987).

Autoxidation of wines's catechol (H_2Q)

$$\begin{array}{lcl}
1.\ O_2 + HQ^- & \rightleftharpoons & O_2^{-\bullet} + HQ^- \\
2.\ O_2^{-\bullet} + H_2Q & \rightleftharpoons & HQ^- + HO_2^{\bullet} \\
\quad HO_2^{\bullet} & \rightleftharpoons & H^+ + O_2^{-\bullet} \\
3.\ O_2^{-\bullet} + H_2Q + H^+ & \longrightarrow & HQ^{\bullet} + H_2O_2 \\
4.\ O_2 + HQ^{\bullet} & \longrightarrow & Q + O_2^{-\bullet}\, H^+ \\
5.\ O_2^{-\bullet} + HQ^{\bullet} + H^+ & \longrightarrow & Q + H_2O_2 \\
\hline
O_2 + H_2Q & \longrightarrow & Q + H_2O_2
\end{array}$$

Figure 3.7 Autoxidation reactions of catechols in wine (**Danilewicz, 2012)**.

In addition to tartaric derivatives of hydroxyl-cinnamic acids, the oxidation reaction may also involve other phenolic compounds such as catechin, epicatechin, gallo-catechin, gallic acid and the gallic acid esters (Kilmartin et al., 2001). Phenolic compounds are oxidized to the corresponding semiquinone radicals, whereas the oxygen is reduced to hydrogen peroxide through the redox cycle of iron and copper (Danilewicz et al., 2008). Oxygen is able to oxidize the phenolic compounds only in the presence of metal ions (Cacho et al., 1995). The quinones are unstable, under in wine, and they can spontaneously combine with other wine components such as: phenols and amines to form browning. Moreover, quinone can also react with other costituents of wine as ethanal and glyoxylic acid to form dark pigments (Boulton et al., 2001 and Zhai et al., 2001). The consume of oxygen and the formation of oxidized compounds in white wine are affected by the concentration of caftaric acid, GSH, flavon-3-ol and the presence of ascorbic acid.

3.11 Glutathione and its interaction with aromatic compounds of wine

3.11.1 Esters

Esters and higheralcohols in wine are mainly formed during fermentation and aging and they affect positively the fruity aroma of wine (Diaz-Maroto et al., 2005; Rapp et al 1985; Etievant, 1991).

3.11.2 Volatile thiols: protective role of glutathione

The varietal aroma of Sauvignon blanc wine is characterized by the presence of thiol compounds such as: 4-mercapto-4-methylpentane-2-one (4MMP), 3-mercaptoesan-1-olm (3MH) and 3-mercapto-hexan-1-ol (3MHA) (Tominaga et al., 1998; Darriet et al., 1995). Moreover, these aromatic compounds are also detected in wines produced from other varieties such as, Riesling, Merlot, Cabernet Sauvignon, Grenache, Colombard and Semillon (Tominaga et al., 2000; Murat et al., 2001).

The 4MMP is characterized by the aromas of boxwood flowers and its perception threshold is 0.8 ng/L in synthetic wine (Tominaga et al., 1998; Darriet et al., 1995), whereas 3MH (p.t. 50/60 ng/L) and 3MHA (p.t. 4 ng/L) are characterised by the aroma of passion fruit and aromatic mixture of boxwood flowers/passion fruit, respectively (Tominaga et al., 2006).
The volatile thiols are particularly susceptible to oxidation during storage (Darriet et al., 2002; Blanchard et al., 2004); furthermore was reported by Dubourdieu & Lavigne, (2004) that GSH is able to protect the volatile thiol compounds from oxidation during aging in bottle.
As previously reported, the hydroxyl-cinnamic acids can be oxidized to quinone, which can react with the thiol group by an addition reaction. Moreover the oxidation reaction of phenols also produces hydrogen peroxide which can oxidize the thiols compounds. GSH can protect the thiols against oxidation by complexing the quinones, which are not available to oxidize the volatile thiols.

3.12 Aging on lees, atypical aging and glutathione

The presence of lees during the storage and aging of wine prevents the loss of fruity character and the formation of reduction note (Dubourdieu & Levigne, 2004). In addition to the positive effect on the aromatic evolution of wine, the aging on lees can prevents the formation of atypical aging and oxidative off-flavour. Aroma compounds mainly responsible for atypical aging and oxidative off-flavour are sotolon and 2-aminoacetophenone.
Sotolon (3-hydroxy-4,5-dimethyl-2(5H)-furanone) is a powerful flavour compound with an intense spicy /curry odor (Girardon et al., 1986) and its perception threshold is 8 ug/L in wine.
2-aminoacetophenone (2-AAP) is a flavour compound with an intense wet paperboard odor and its perception threshold is 1 ug/L in wine. 2-AAP is mainly generated during the storage of wine. (Christoph et al., 1999; Hoenicke et al., 2002). (Christoph et al., 1999; Hoenicke et al., 2002).
It is demonstrated by (Lavigne & Dubourdieu, 2004) that the presence of the lees is able to limit the formation of sotolon and 2-aminoacetophenone and therefore preserves the aroma of white wines. Cysteine residues are included in many proteins and peptides in the lees (Lavigne & Dubourdieu, 2004). The reducing proteins and cysteine residues can be released in wine by cell lysis of yeast. The mercaptans, responsible for the reduction odors, can be complexed by quinones or by cysteine residues, released from the cell wall of the yeast, to form odorless disulfides (Lavigne & Dubourdieu, 1996). The addition of GSH (10 mg / L) at bottling significantly limits the appearance of yellow pigments in wine; the fruity aroma is better preserved (evaluated as the presence of 3MH) and the appearance of sotolon and 2-minoacetophenone was delayed (Lavigne & Dubourdieu, 1996). Recent studies concerned the quantification of cysteine residues in reduced protein (CPR) in commercial samples of active dry yeast, yeast hulls, lysates of yeast mannoproteins which were industrially produced (Tirelli et al., 2010a). The presence of these cysteine residues was confirmed but, due to the industrial production, these additives may not be useful in the protection of the wine from oxidation. Future work will investigate the best conditions of production and purification of yeast extracts, making possible their addition in the wine.

3.13 Analytical methods

In literature are reported several analytical methods effective to assess the amount of GSH in wine. Ellman, (1959) reported a spectrophotometric method able to quantify the total thiol compounds in biological matrix. The Ellman method involves the condensation between R-SH group and dithio-bis-nitrobenzoic acid (DTNB), to form a chromophore detectable at 412 nm.

GSH content can be also evaluated in must and wine by separation techniques as HPLC fitted to fluorescence detector. The thiols compound is derivatized with o-phtaaldeide (OPA) by a pre-column derivatization system (Park et al., 2000).

Moreover, other authors reported that capillary electrophoresis technique coupled to spectrophluorimetric detection allowed the detection of GSH in musts and wines. This method involves the derivatization of thiol compounds with monobromobimano (MBB) which generates a complex (GSH-MBB) with a high emission, detectable by spectrophluorimeter (Lavigne et al., 2007). Moreover, Tietze, (1969) developed an enzymatic method to assess the amount of GSH. The analytical method allowed the detection of GSH in musts and wines through the enzymatic reaction between quinone and thiol groups catalized by polyphenoloxidase.

The thiol compounds also have a strong reactivity against heavy metals. Bramanti et al., (2008) assessed GSH in wine through the derivatization of thiol group with mercury (Hg) and a subsequent reduction reaction which was detectable with atomic absorption spectrometer. The GSH is also quantified through a derivatization with p-benzoquinone to form 2-S-glutationil-p-hydroquinone. Separation of derivatized GSH was achieved by high-performance liquid chromatography (HPLC) coupled with UV detection at 303 nm (Tirelli et al., 2010) or mass spectrometry (Du Toit et al., 2007). Finally, GSH was determined in must and wine by HPLC separation technique coupled with an API Quattro microtriple quadropole mass spectrometer for the analysis of reduced and oxidized glutathione (du Toit, et al., 2007).

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3.15 Aim of the study

Glutathione is important thiol compound which is involved to regulatory systems in yeast growth (Penninckx, 2002) and oxidation-reduction reaction of the must and wine (Cilliers & Singleton, 1990). In addition, GSH also plays an important role in the protection and formation of aromas in the wine during the wine making.

The aim of this work was to study the influence of different levels of glutathione (Sigma Aldrich) and GSH-IDYs (Lallemand) on the release of intra-and extra cellular by yeast cells and grapes, respectively. Moreover, it was studied the influence of the treatments with GSH and GSH-IDYs on the concentrations of volatile thiols, alcohols higher, esters and fatty acids, produced during the alcholic fermentation.

The volatile thiols are particularly susceptible to oxidation during storage (Darriet et al., 2002; Blanchard, et al., 2004); furthermore was reported by Dubourdieu & Lavigne, (2004) that GSH is able to protect the volatile thiols compounds from oxidation during aging in bottle.

Sauvignon blanc cultivar from Stellenbosch (South Africa) was studied because its varietal aroma which is characterized by the presence of thiol compounds such as: 4-mercapto-4-methylpentane-2-one (4MMP), 3-mercaptoesan-1-olm (3MH) and 3-mercapto-hexan-1-ol (3MHA) (Tominaga et al., 1998; Darriet et al., 1995)..

Secondly, residual sugars, total acidity and alcohol were monitored during alcoholic fermentation,

The GSH quantification was performed with a Waters Acquity HClass UPLC system equipped with a mass spectrometer, API and microtriple quadropole.

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3.17 Materials and Methods

3.17.1 Chemicals and reagents

The organic yeast rehydration nutrient (GO FERM protect), strain yeast QA23 and GSH-enriched inactive dry yeast preparation (GSH-IDYs) were purchased from Lallemand Blagnac, France. Ascorbic acid, sulfur dioxide ($K_2S_2O_5$), methanol, reduced glutathione, formic acid, acetonitrile, Tris-EDTA pH 7.5, meta-phosphoric acid, 4-methyl-2-pentanol, ethanol, sodium sulphate anydrous, dichlorometane, diethyl ether, 2, 6-dimethyl-6-heptan-2-ol, ethyl acetate, ethyl butyrate, propanol, isobutyl alcohol, isoamyl acetate, butanol, 4-methyl-2-pentanol, isoamyl alcohol, ethyl hexanoate, pentanol, hexyl acetate, acetoin, 3-methyl-1-pentanol, ethyl lactate, hexanol, 3-ethoxy-1-propanol, acetic acid, ethyl octanoate, ethyl-3-hydroxybutanoate, propionic acid, isobutyric acid, butyric acid, isovaleric acid, diethyl succinate, valeric acid, ethyl phenylacetate, hexanoic acid, 2-phenylethanol, octanoic acid and decanoic acid were purchased from Sigma Aldrich. HPLC grade water was obtained by a Milli-Q system (Millipore Filter Corp., Bedford, MA). All the chemicals were of analytical grade, at least.

3.17.2 Juice and Winemaking Treatments

The intra- and extra cellular glutathione concentration was evaluated in juice and wine of Sauvignon blanc treated with GSH and a GSH-enriched inactive dry yeast preparation (GSH-IDYs Lallemand). The addition of GSH-IDYs and GSH was carried out to the different treatments as reported in Table 3.1 according to the supplier's recommendation. The juice was then divided into five treatments such as, control (C), juice added to 80 mg/L of GSH (GSH 80); juice added to 5.5 mg/L of GSH (GSH 5.5); juice added to 0.4 g/L of GSH-IDYs (YED) and juice added to 80 mg/L of GSH and 0.4 g/L of GSH-IDYs (YED 80). The sample GSH 80 presented the concentration of GSH (80 mg/L) which was a high amount detected in South African juice, while the treatment GSH 5.5 had the same concentration that was quantified in GSH-IDYs (0.4 g/L) with the analytical method described in (Kritzinger 2012). The treatments were performed in triple. A Sauvignon Blanc grape from Stellenbosch A.O. (Neil Ellis cellar from South Africa) was used in the experiments. The grapes were all grown according to standard viticultural practices and picked by hand when ripe. The grapes were conserved at 4 °C overnight. The grape bunches were mixed homogenously, and rotten berries were removed by hand. The pressing and destemming were carried out by a press and crusher custom-built small scales were used to extract the juice with minimum amount of oxygen. This was achieved by placing 15 kg of whole grape bunches around a central plastic membrane in the press. The press was filled with grapes, sealed, and filled with water. The water was displaced with CO_2 (Afrox SA) to achieve an inert atmosphere around the grapes before pressing. Around 10 L of the juice formed by this pressing action was accumulated in 12 L steel container that previously had been filled with water and displaced with CO_2.

After settling overnight, the juice was racked from the grape lees with CO_2. It was then added at 0.3 g/L of organic yeast rehydration nutrient (Lallemand Blagnac, France) and was subsequently inoculated with the yeast strain QA23 (Lallemand Blagnac, France) at 0.3 g/L according to the supplier's recommendations and fermented at 15 °C. At the end of fermentation, 50 mg/L SO_2 was added to all treatments, and the wine was stored at -4°C for ten days to carry out the tartaric stabilization. It was then racked with CO_2 from the yeast lees and bottled in green 750 mL wine bottles under CO_2 gas and sealed with screw tops.

Treatment	GSH (mg/L)	GSH-IDYs (g/L)
Control	-	-
GSH 5.5	5.5	-
GSH 80	80	-
YED	-	0.4
YED 80	80	0.4

Table 3.1 Additions of GSH and GSH-IDYs in juice of Sauvignon blanc.

3.17.3 Sampling Procedure

Samples destined for LC-MSMS analyses were collected just after the juice was collected in the 12 L stainless steel canisters. The sampling was carried out before and after the addition of GSH-IDYs and GSH; (T0-T1) 0%; 25% (T2); 50% (T3); 75% (T4) and at the end of alcoholic fermentation (T5). This was done by transferring 20 mL of juice from the 12 L stainless steel canisters into 20 mL glass vials containing 1000 mg/L SO_2 and 500 mg/L ascorbic acid. The 5% SO_2 solution was made from $K_2S_2O_5$ and 0.4 mL was added to 20 mL of juice to obtained the desired concentration. A 5% ascorbic acid solution (Sigma-Aldrich) was also used and prepared daily before starting the experiments. Such high SO_2 and ascorbic acid concentrations were used to completely inhibit any residual phenolic oxidase or laccase activity in the samples.

The glass vials were saturated with CO_2 before and after the juice was transferred into the glass vials. This was made to completely inhibit any residual oxidation enzyme activity. Additional carbon dioxide was blown on the headspace, and the vials were sealed hermitically. The vials were then frozen at -20 °C until they were analyzed.

3.17.4 Sample preparation of glutathione extra cellular

Samples were thawed and centrifuged (4 mL) (Centrifuge 5415 D, Eppendorf, Hamburg, Germany) at 14000 rpm for 5 min. Afterwards the samples were diluted five time with a solution of 1 g/L of SO_2 and 0.5 g/L of ascorbic acid, on the day of the LC-MSMS analyses. Finally the samples were filtered through a 0.45 µm syringe filter and injected in LC-MSMS instrument.

3.17.5 Sample preparation of glutathione intra cellular

The analytical method to assess the amount of intra-GSH cellular was previously reported by Kritzinger (2012). Two milliliters of sample were centrifuged in a glass vial, previously weighed, at 12000 RPM for 10 minutes at 20°C. The supernatant was removed with a syringe and glass vial with just the pellet was weighed again. The pellet was added to 2 ml of 1 ml Tris-EDTA pH 7.5 and stirred for 5 minutes. Subsequently, the sample was centrifuged at 12,000 RPM for 10" at 20°C, and then the supernatant was removed with a syringe. The pellet was added micro-balls (tip of a spoon), 0.300 ml of EDTA - Tris pH 7.5 and stirred for 20 minutes. The sample was subsequently centrifuged at 12000 RPM for 5 ' at 20 ° C and the micro-balls were eliminated.

The extract of intracellular GSH was added to 0,300 ml of 10% meta-phosphoric acid and stored at -20 ° C for 5 minutes. Finally the sample was centrifuged at 13000 RPM for 5 'at 20 ° C and the supernatant was filtered through a 0.45 µm syringe filter and injected in LC-MS/MS instrument.

3.17.6 Ultra performance liquid chromatography of GSH

A Waters API Quattro microtriple quadropole mass spectrometer with a 2690 Alliance HPLC system was used for LC-MSMS analysis. Separation was achieved on a Waters Atlantis C18, 3 µm, 2.1*150 mm column using 0.1% formic acid (solvent A) to an acetonitrile (solvent B) gradient at a flow rate of 0.2 mL/min. The solvent composition was kept at 100% solvent A for the first 0.5 min, followed by a linear gradient over 6.5 min to 80% solvent B and re-equilibration to 100% A for 7 min. Reduced glutathione eluted at 3.14 min and oxidized at 4.12 min. The MS method consisted of two multiple reaction monitoring (MRM) functions with electrospray ionization in the positive mode, a capillary voltage of 3.5 kV, and argon as the collision gas. The first MRM monitored the reduced glutathione with am/z 308>179.1 transition at a collision energy of 17 eV and cone voltage of 18 V. The second monitored the oxidized glutathione with am/z 613.1>355.1 transition at a collision energy of 20 eV and cone voltage of 30 V. The transition settings for the reduced glutathione were selected at a collision energy that was not optimum (the optimum collision energy was 20 eV and cone voltage was 30 V) but that allowed a linear calibration at higher concentrations.

3.17.7 Quantification of GSH

The compounds were quantified chromatographically by the external standard method in juice, synthetic wine solution and white wine. Data acquisition and processing were performed by Empower ^{2} software (Waters, Milford, MA).

3.17.8 Thiol Analysis

Chemicals, standards, and the model wine matrix have been described previously by Tominaga, et al., (1998). Analyses were undertaken on the wine samples to quantify five volatile thiols, 3-mercaptohexan-1-ol, 3-mercaptohexyl acetate, 4-mercapto-4-methylpentan-2-one, 4-mercapto-4-methylpentan-2-ol and 3-mercapto-3-methylbutan-1-ol. The analytical method to extract and quantify the volatile thiols was reported by Tominaga, et al., (1998).

3.17.9 Ester, Alcohol and Fatty Acid Analyses

Chemicals, standards, and the model wine matrix have been described previously. (Louw, et al., 2009 and Hebditch, et al., 2007). Five millilitres of wine with internal standard, 4-methyl-2-pentanol (100 µL of 0.5 mg/L solution in a 12% ethanol–water mixture), was extracted with 1 ml of diethyl ether by sonicating the ether–wine mixture for 5 min. The wine–ether mixture was then centrifuged at 2800*g for 3 min. The ether layer was removed and dried on Na_2SO_4. Each extract was injected into the GC-FID in triple. Validation of the method, in terms of selectivity, linearity, limits of detection, limits of quantification, recovery, robustness and repeatability, has been described (Roussis et al., 2005).

3.17.9.1 Gas Chromatography-FID (GC-FID) separation

A Hewlett Packard 6890 Plus GC (Little Falls, DE, USA) equipped with a split/splitless injector and FID detector were used. Separation was achieved on a J&W DB-FFAP capillary GC column (Agilent, Little Falls, Wilmington, DE. USA) with dimensions 60 m*0.32 mm*0.5 µm. The initial oven temperature was 33° C for 17 min., after which the temperature was increased by 12° C/min. to 240° C, at which it was held for 5 min. Three microlitres of the diethyl ether extract was injected at 200° C. The split ratio was 15:1, and the split flow rate was 49.5 ml/min. The column flow rate was 3.3 ml/min. using hydrogen as a carrier gas. The detector temperature was 250° C, with a column flow of 6ml/min., which cleaned the column of high boiling contaminants. Quantification was performed by comparing the ratio of the peak area and internal standard peak area with calibration graphs constructed using pure standards. (Louw, et al., 2009; Hebditch, et al., 2007).

3.17.10 Oenological parameters

The following parameters were evaluated according to standard methods in the oenological sector: residual reducing sugar (Reference met. OIV 2009); total acidity (Reference met. OIV 2009); alcohol (Reference met. OIV 2009) and volatile acidity (Reference met. OIV 2009).

3.17.11 Statistical Analyses

The glutathione concentrations in the stability test were statistically analyzed with one way ANOVA analyses (with Tukey HSD post hoc tests) using STATISTICA software (20). In the cases where juice and wine values were compared across treatments, repeated measures of ANOVA were used.

3.18 Results and Discussion

3.18.1 Trend of intra and extra-cellular GSH during the alcoholic fermentation

The intracellular and extracellular glutathione presented a different trend during the alcoholic fermentation (A.F.) Table 3.2. The extracellular glutathione (Figure 3.8) showed the same trend in all treatments. The 5.5GSH and YDE treatments presented the same concentration of extra-GSH during alcoholic fermentation while YDE 80 demonstrated a higher concentration of extra-GSH at the end of A.F. The evolution of extra-GSH of each treatment, showed a decrease of up to 50% of alcoholic fermentation, however towards completion of A.F. the extra-GSH concentration increased. The trend of extra-GSH could be attributed to many stress response mechanisms of the yeast (sulfur and nitrogen starvation, oxidative stress, and the detoxification of heavy metals and xenobiotics) and then it is secreted through cellular transports at the end of alcoholic fermentation (Kritzinger E.C et al., 2013).

sample	**Extracellular glutathione (mg/L)**						**Intracellular glutathione (mg/mg of pellet)**			
	T_0	T_1	T_2	T_3	T_4	T_5	T_2	T_3	T_4	T_5
C	3.0	2.6	9.3	2.6	2.5	10.1	0.017	0.016	0.023	0.023
5.5 GSH	2.7	8.8	7.1	4.1	4.8	11.8	0.015	0.010	0.033	0.045
80 GSH	3.3	76.5	68.2	54.9	59.4	67.3	0.020	0.017	0.026	0.019
YED	2.8	8.3	6.8	3.9	4.3	13.2	0.017	0.013	0.026	0.013
YED 80	2.4	91.7	50.8	52.3	58.8	69.4	0.028	0.026	0.044	0.051

Table 3.2 Mean values of intra- and extra cellular glutathione evolution during alcoholic fermentation of Sauvignon b. juice treated with GSH and GSH-IDYs.

During the alcoholic fermentation intra-GSH showed (Figure 3.9) the same trend in all treatments to decrease up to 50% of alcoholic fermentation, with the exception of the treatment GSH 80 in which the concentration of intra-GSH was increased. On the contrary the amount of intracellular GSH decreased in treatments GSH 80 and YDE until the end of alcoholic fermentation, while the other treatments showed the increased of concentration of intracellular GSH.

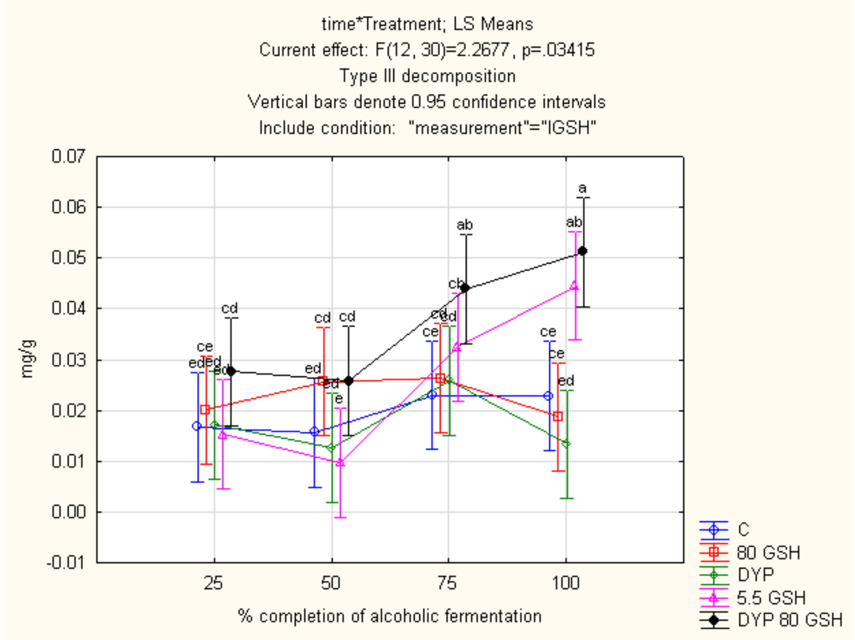

Figure 3.8 Trend of intracellular glutathione during alcoholic fermentation.

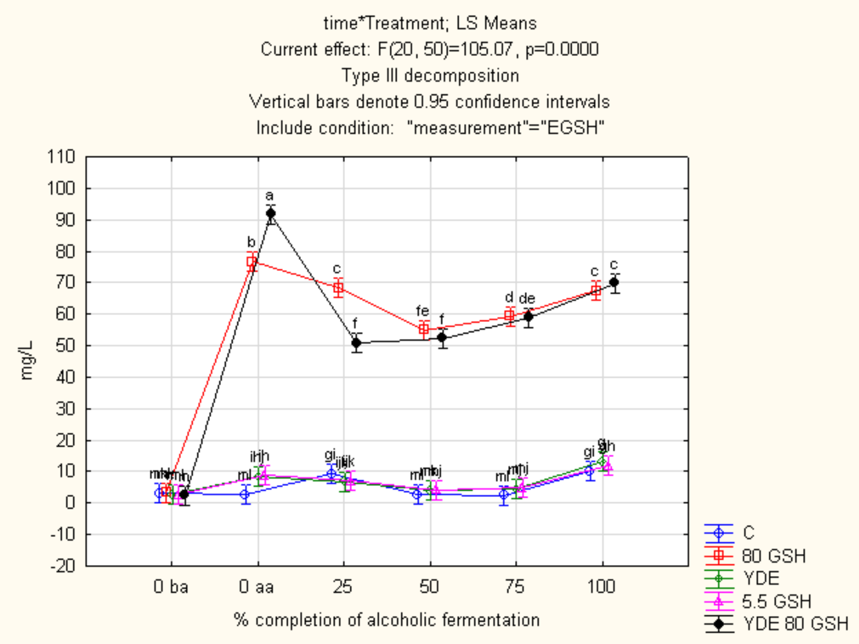

Figure 3.9. Trend of extracellular glutathione during alcoholic fermentation.

The results concerning the thiol aromas, alcohols higher, esters, and fatty acids are not been still processed. In the coming months it will be possible to assess definitively the work described above.

3.19 Reference

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Abbreviation and acronyms

S.T.: perception threshold.
TDN: 1,1,6-trimethyl-1,2-dihydronaphathalene.
FD: flavor dilution.
HIL: 4-Hydroxy-L-isoleucine.
GC-MS: Gas Gas chromatography fitted to mass spectrometer.
RP: Reverse Phase.
DOCG: Denominazione di Origine Garantita e Controllata (Italian Appellation)
DCM: dichloromethane.
SPE: Solid Phase Extraction.
pBQ: p-benzoquinone.
AA: ascorbic acid.
DHA: dehydroascorbic acid.
3MPA: 3-Mercaptopropionic.
TFA: trifluoroacetic acid.
OPDA: 1,2-phenylenediamine dihydrochloride.
Put: Putrescine.
AO: antioxidant compound.
Cys ab: absorbed cysteine.
T.P.: total polyphenols.
(+)-cat: catechine.
DFQ: furol [3,4-b]quinoxaline-1-one.
BAs: Biogenic amines.
LAB: lactic acid bacteria.
DNA: Deoxyribonucleic acid.
RNA: Ribonucleic acid.
PUT: Putrescine.
CAD: cadaverine.
HIS: histamine.
TYR: tyramine.
DAO: diaminonoxydase.
PAO: polyamine oxidase.
MAO: monoaminooxydase.
PST M: phenolsulfotransferase M.
MLF: Malolactic fermentation.
NAD^+: Nicotinamide adenine dinucleotide.
CFU: colony-forming unit.
Dns-CL: dansylchloride.
NQS: 1, 2-naphthoquinone-4-sulfonate.
DNBZ-Cl: dinitrobenzoyl-chloride.
UHPLC-UV: Ultra High Performance Liquid Chromatography fitted to ultraviolet detector.
2-prOH: isopropanol.
metOH: methanol.
PVPP: Oenological Polyvinylpolypyrrolidone.
ACN: acetonitrile.
DAD: Photo Diode array detector.
LOD: limit of detection.

LOQ: limit of quantitation.
THF: tetrahydrofuran.
NaOAc: sodium acetate.
H_3C: citric acid.
HEP: heptylamine..
SPER: spermine.
SPERMID: spermidine
1-3 DAB: 1-3 diaminobutane
PEA: , phenylethylamine.
SD: standard deviation.
RSD: relative standard deviation
GSH: L-g glutamyl-L-cysteinyl-glycine.
G3MH: 3-glutathionilate mercaptohxan-1-ol.
γ-ECS or GSH1: γ-glutamyl-cysteine ligase or γ-glutamyl-cysteine synthase.
GS or GSH2: glutathione synthase.
GR: glutathione reductase.
GSSG: glutathione oxidized.
4MMP: Mercapto-4-methyl pentan-2-one.
3MH: 3-mercapto-hexan-1-ol.
GSH-P1 and GSH-P2: glutathione transport systems.
GPx: glutathione peroxidase.
LOOH: hydroperoxide lipid.
ROOH: alkylhydroperoxide.
GSH-IDYs:
PPOs: polyphenol oxidase.
GRP: Grape Reaction Product.
3MHA: 3-mercapto-hexan-1-ol.
2-AAP: 2-aminoacetophenone.
3MHA: *3*-mercaptohexyl acetate.
OPA: o-phtaaldeide.
DTNB: dithio-bis-nitrobenzoic acid.
HPLC: high liquid performance chromatography.
MBB: monobromobimano.
GSH-MBB: complex between glutathione and monobromobimane.
API: atmospheric pressure ionization.
GO FERM protect: yeast rehydration nutrient.
QA23: strain yeast.
RPM: rotations per minute.
LC-MS/MS: liquid chromatography coupled to Quattro microtriple quadropole mass spectrometer**.**
GC-FID: gas chromatography coupled to a Flame Ionization Detector.
extra-GSH: extracellular glutathione.
Intra-GSH: intracellular glutathione.
A.F.: alcoholic fermentation.

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

Influenza dei composti fenolici e antiossidanti sulla formazione di sotolone

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Il sotolone (3-idrossi-4,5-dimetil-2(5)-furanone) è il composto volatile principalmente responsabile dell'invecchiamento atipico e dello sviluppo di alterazioni, descritte come "ossidato", nei vini. Questa molecola è stata identificata e quantificata in numerosi vini, soprattutto bianchi, dolci (es. *Port)* e barricati, e possiede una bassa soglia di percezione prossima a 7-8 µg/L. Il sotolone è chimicamente stabile e la sua formazione in vino è influenzata dalla concentrazione di ossigeno e zuccheri, dalla temperatura, dal tempo di invecchiamento e dalla presenza del lievito ed è legata all'acido ascorbico disciolto come pure alle reazioni di Maillard.

Al fine di chiarire quali fattori compositivi possano condizionare la sintesi di sotolone in un vino bianco, soluzioni modello contenenti pentosi e concentrazioni crescenti di catechina e glutatione sono mantenute ad elevate temperature (60-70°C) per tempi variabili.

Dalle prove preliminari è emerso che il sotolone è formato in elevate concentrazioni già dopo incubazione della soluzione modello a 70°C per 5 giorni, soprattutto in presenza di catechina (108.8 µg/L contro 81.5 µg/L in sua assenza), quantità che si sono più che raddoppiate dopo 12 giorni (230.7 µg/L e 266.3 µg/L, rispettivamente con e senza catechina).

I dati fino ad ora ottenuti hanno evidenziato la forte influenza della temperatura sulla produzione di sotolone che è ottenuto nelle condizioni sperimentali adottate anche in assenza di acido ascorbico, composto ritenuto il principale responsabile della sintesi di sotolone nel vino.

Determinazione del sotolone in vini addizionati di antiossidanti sostitutivi dell'anidride solforosa

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Il 3-idrossi-4,5-dimetil-2(5)-furanone, noto come sotolone, è un lattone chirale termolabile, stabile a pH acido ed in solvente organico polare. L'identificazione e la quantificazione di questo composto assume importanza dal punto di vista enologico dal momento che è il principale responsabile dell'invecchiamento atipico dei vini bianchi. I difetti derivanti da esso sono riconducibili ai descrittori di "ossidato" e "maderizzato" percepibili quando la concentrazione è compresa tra 0.02 μg/L in aria, 0,3 μg/L in soluzione acquosa e 7-8 μg/L in vino. In letteratura, numerosi studi riportano la presenza di sotolone nei vini e, proprio per questa ragione, è importante disporre di una metodica analitica semplice ed efficiente che consenta l'affidabile quantificazione di basse concentrazioni di questo composto unita a tempi brevi di analisi.

L'approccio analitico innovativo proposto prevede un'estrazione liquido/liquido del sotolone che è identificato e quantificato per UPLC/UV in 10 minuti di eluizione cromatografica.

Il livello di sotolone è stato misurato in vino spumante mantenuto a 15°C e 25°C per 6 mesi e addizionato di tre differenti antiossidanti (2 g/hL e 4 g/hL) alternativi all'anidride solforosa a base di estratti vegetali ellagici da *Vitis vinifera*, da gomma arabica e arricchiti in pareti cellulari di *Saccharomyces cerevisiae*.

La metodica validata presenta una risposta lineare per concentrazioni di sotolone comprese tra 2 μg/L e 20 μg/L; il limite di rilevabilità è pari a 1.4 μg/L e il limite di quantificazione corrisponde a 4.6 μg/L, ampiamente inferiori alla soglia di percezione in vino.

Il sotolone è presente nei vini addizionati di estratti da gomma arabica, in tracce per il dosaggio di 2 g/hL per entrambe le temperature, mentre i livelli più elevati sono rilevati per il dosaggio di 4 g/hL (6.4 μg/L e 13.4 μg/L rispettivamente a 15°C e 25°C). Tracce di sotolone sono misurate anche nei vini addizionati di estratti da *Vitis vinifera* (2 g/hL a 15°C; 4 g/hL a 15°C e 25°C) e arricchiti con pareti cellulari di *S. cerevisiae* (2 g/hL a 25°C; 4 g/hL a 25°C).

Il metodo di analisi descritto permette una rapida ed affidabile quantificazione del livello di sotolone e consente di studiare i fattori enologici che possono condizionare il danno ossidativo in vino.

Appendix 2 *List of Tables*

Appendix 3 *List of Figures*

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