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Studies on the occurrence of thiol related aromas in wine

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FEDERICO PIANO

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Abstract

Thiol related aromas play a key role in sensory profile of certain wines. In particular, 3-mercaptohexan-1-ol, 3-mercaptohexyl acetate and 4-mercapto-4-methypentan-2-one were firstly identified in *Sauvignon blanc* wines. Same molecules were then evaluated to contribute to several wine made from different grape varieties. The strong smelling properties of volatile thiols explain their importance, even if such molecules are present at extremely low concentration in wine (ng/L). This characteristic represents the main issue in their identification and quantification in wine.

Aromatic thiols are present in grape and juice as non-volatile compounds. In particular, glutathione and cysteine conjugates were identified to be precursors of such smelling molecules. During vinification process, yeast activity is responsible for their release from the cysteinilated precursors. The amount of thiol precursor in juice is influenced by oenological practices. Despite this, no direct correlation between the level of precursors in juice and concentration of thiol in wine has been observed, thus suggesting that alternative biogenetic pathway and/or more complex interaction are responsible for occurrence of aromatic thiol in wine.

The aims of this PhD were to evaluate new analytical approaches aimed to evaluate the occurrence of volatile thiols in wine, to study alternative biogenetic pathway leading to thiol realted aroma and to assess yeast strain effect on the amount in molecules responsible for thiol stability in wine.

The employmet of organo-mercury compounds represents the most effective method for thiol quantification in wine. The use of mercury constitutes hazard for health and environmental safety. Furthermore, such methods are extremely time-consuming. New analytical approaches were evaluated with the aim to develop an easy-to-apply method.

In gas chromatographic approaches, thiol group reactivity towards nucleophilic addition was used to make derivatised product.

Pentafluorobenzyl bromide has been reported by several authors. This derivatising agent has been employed by means of negative ions chemical ionization mass spectrometry. Chemical ionization is responsible for low fragmentation and shares with the electron capture detection a selective and sensitive response to electrophilic atoms (halogens), as fluorine is. Despite this, such techniques are not widespread. In this study electron impact ionization in positive mode mass spectrometry was then evaluated. The developed method allowed to identify characteristic fragments even if high amount of fragmentation was present. Derivatisation reaction was possible in different organic solvents.

Derivatisation of aromatic thiol can be carried out in aqueous media too. Thanks to their hydrophobicity, reaction products can be extracted by means of different sorbents. Despite this, the presence of alkali is required, thus leading to the hydrolysis of esters.

Solid phase extraction of volatile thiol from aqueous media is possible. Despite this, in-sorbent derivatization process, as well as derivatisation of volatile thiol in organic solvent, did not allow to quantify thiol related aroma at concentration close to their perception threshold.

The employment of an unsaturated alkene as derivatising agent was then studied. The key feature of ethyl propiolate employment in volatile thiols derivatization, is the thiol nucleophilic

addition to ynonates. The derivatised product can be identified by MS technique. The reaction take place in aqueous media. Neutral or mild basic conditions are required to carry out the derivatization procedure, where no ester hydrolysis was showed. The employment of this new derivatising agent allowed the assessment of volatile thiols in wine at concentration close to their perception threshold.

Thanks to the employment of fluorescent derivatising agent, quantification of volatile thiol in wine was demonstrated to be possible by means of liquid chromatographic approaches too. The key feature in this analytical approach was the optimization of derivatisation reaction and the individuation of chemical compounds responsible for volatile thiol loss.

Interaction between *trans*-2-hexenal and bisulfte was studied in order to evaluate alternative biogenetic pathways leading to 3-mercaptohexan-1-ol (3-MH) in wine. Biogeneic pathway of stable sulfonates is stricktly related to species whose structure is similar to 3-MH. The addition occours in aqueous acidic media, thus suggesting similar pathway in grape juice. The initial addition of bisulfite to the unsaturated aldehyde is at the aldehydic function. Despite this, the most stable product is a disulfonate which was demonstrated it is not a putative precursor of volatile thiol in wine. The formation of such stable product is correlated to a mono-sulfonate whose structure is similar to 3-mercaptohexan-1-ol. Pure species synthesis will allow to evaluate if this compound can be regarded as a precursor of the volatile thiol in wine.

Thiol nucleophilic addition to quinones is the major responsible for thiol related aroma loss in wine. The presence of compounds able to reduce such quinone is strictly correlated to thiol stability in wine. The tripeptide glutathione, naturally occurring in grape and wine, is responsible for this protective effect. Wine making technique can affect GSH content in wine. In particular, several yeast strains were tested during laboratory-scale alcoholic fermentation. As a result, yeast strain was evaluated to influence GSH content. Moreover, during aging on lees total glutathione content did not vary, thus production and liberation of this tripeptide is related to living cells.

Indagine sulla presenza di aromi a funzione tiolica nei vini

Gli aromi a funzione tiolica svolgono una funzione importante nella determinazione delle caratteristiche sensoriali di alcuni vini. In particolare, il 3-mercaptoesanolo, l'acetato di 3-mercaptoesile ed il 4-mercapto-4-metilpentan-2-one, identificati inizialmente in vini derivanti da uve Sauvignon blanc, sono stati riscontrati anche in altre varietà suggerendo che essi possano contribuire all'aroma di diversi vini. L'elevato impatto olfattivo di tali molecole ne spiega l'importanza nonostante esse siano presenti nei vini a concentrazioni estremamente basse (ng/L). Quest'ultima caratteristica rappresenta la difficoltà maggiore alla loro identificazione e quantificazione.

I tioli varietali sono presenti in mosti e uve come precursori non volatili. In particolare, coniugati del glutatione e della cisteina sono stati identificati come precursori. Nel corso del processo di vinificazione, l'attività del lievito è alla base della liberazione di questi aromi. Le pratiche enologiche possono influenzare il contenuto in tali precursori nei mosti anche se non è stata identificata una diretta correlazione tra contenuto in precursori nei mosti e tioli liberi nei vini, suggerendo che vie sintetiche alternative ed interazioni più complesse siano responsabili della presenza di tali aromi nei vini.

Gli scopi della ricerca sono stati la valutazione di nuovi approcci analitici volti ad evidenziare la presenza di aromi a funzione tiolica nei vini, indagini riguardanti possibili vie biosintetiche alternative e l'effetto del ceppo di lievito sul contenuto in molecole responsabili della stabilità degli aromi tiolici nei vini.

L'impiego di composti mercurati è il metodo più efficace ad oggi in uso per l'analisi dei tioli volatili. L'utilizzo di tali molecole rappresenta tuttavia un rischio per la salute e la sicurezza, inoltre le metodiche proposte sono estremamente complesse. Nuovi approcci analitici sono quindi stati valutati al fine di individuare una metodica più semplice e sicura.

Nel corso di approcci gas cromatografici la reattività del gruppo tiolico verso addizioni nucleofile è stata utilizzata per la formazione di derivatizzati.

Alcuni autori hanno proposto la derivatizzazione mediante pentafluorobenzil bromuro, solitamente correlata ad acquisizioni spettrometriche con ionizzazione chimica e selezione di ioni negativi. Si ottiene pertanto sensibilità favrita da una minor frammentazione delle molecole ed una maggior selettività per elettrofili, qual è il fluoro. Tuttavia queste tecniche sono poco diffuse. Si è pertanto testata la possibilità di utilizzare la ionizzazione ad impatto elettronico e selezione degli ioni positivi (più diffusa nei laboratori di ricerca enologica). La tecnica messa a punto ha permesso di individuare ioni caratteristici, nonostante una maggior frammentazione. La reazione di derivatizzazione è stata possibile in diversi solventi organici.

La formazione dei prodotti di derivatizzazione è possibile in ambiente acquoso dal quale possono esser estratti selettivamente utilizzando diverse fasi stazionarie, grazie alle loro caratteristiche di idrofobicità, anche se l'ambiente fortemente basico determina l'idrolisi di gruppi esteri.

L'estrazione di tioli aromatici da soluzioni acquose mediante fasi solide è possibile. Ciononostante, la derivatizzazione applicata a molecole tioliche trattenute in fase solida od eluite mediante solvente organico non ha permesso l'identificazione di aromi a funzione tiolica ad un livello sufficiente di sensibilità.

Si è pertanto studiato l'impiego di un estere alchinico come derivatizzante capace di addizione ai gruppi tiolici. Il prodotto di addizione tra etil propiolato e tioli volatili può essere identificato mediante tecniche di spettrometria di massa. La reazione, che avviene in ambiente acquoso, è favorita da condizioni basiche non così drastiche da condurre all'drolisi di gruppi esteri. Il prodotto ottenuto da tale addizione nucleofila può esser selettivamente estratto da soluzioni acquose. Una metodica analitica innovativa basata su tale reazione ha dunque permesso l'identificazione di tioli aromatici in vino a concentrazioni prossime alla loro soglia di percezione.

La quantificazione di aromi tiolici in vino anche mediante metodiche di cromatografia liquida è possibile grazie all'impiego di derivatizzanti con caratteristiche fluorescenti. L'individuazione delle condizioni ottimali di derivatizzazione e di composti responsabili della perdita di tioli volatili hanno giocato un ruolo chiave nel successo di tale approccio analitico.

L'interazione tra ione bisolfito ed esenale è stata indagata nel corso della valutazione di vie biosintetiche alternative responsabili della presenza di 3-mercaptoesan-1-olo (3-MH) nei vini.

La formazione di sulfonati stabili è strettamente correlata a specie la cui struttura è simile al 3-MH. Tali reazioni avvengono in ambiente acido, suggerendo un simile meccanismo anche nei mosti. La prima addizione dello ione bisolfito è al gruppo carbonilico dell'aldeide insatura. Ciononostante il prodotto più stabile è rappresentato da un disulfonato che si è rivelato non essere un precursore dell'aroma tiolico. La formazione di tale sulfonato è correlata ad un monosulfonato di struttura simile al 3-mercaptoesan-1-olo. La sintesi della specie pura permetterà di valutare se possa esserne considerato un precursore.

L'addizione nucleofila di tioli a chinoni è la maggior responsabile della perdita di tali aromi nei vini. La presenza di composti che riducano tali chinoni è strettamente correlata alla stabilità degli aromi tiolici. Il glutatione, naturalmente presente nelle uve e nei vini, è in grado di svolgere tale funzione. Il contenuto di GSH nei vini è influenzato dalle operazioni di vinificazione. In particolare, sono stati testati diversi ceppi di lievito nel corso della fermentazione alcolica e si è verificato che la scelta del ceppo di lievito può influenzare il contenuto in tale tripeptide. Inoltre nel corso della fase sur lies non è stato riscontrata una variazione del contenuto totale di glutatione, ascrivendo il rilascio del tripeptide alle fasi di attività metabolica.

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1.Thiol related aromas in wines: state of the art

1.1. Volatile thiols in wine

Sulfur aromas can be present in wine as result of different causes. They can come from grapes as non-volatile precursors, from microbial fermentation or from chemical reaction taking place during storage. The extraction of thiol compounds from wood is another cause of sulpur related aroma in wines (Landaud *et al*., 2000).

Some thiol aromas can be generated starting from sulfur-containing amino acids, fermentation and metabolism products from the sulfur-containing pesticides. Thermal and chemical reaction of sulfur compounds during winemaking and storage are responsible foe thiol aromas in wines (Mestres *et al*., 2000).

Many volatile sulpur compounds, such as carbon sulfide, ethanethiol, methanethiol, and hydrogen sulfide, which are mainly produced at high levels during alcoholic fermentation are responsible for olfactory defect (Bartowsky & Pretorius, 2009). Those compounds are responsible for notes as cabbage, onion, rotten egg, garlic and rubber (Vermeulen *et al*., 2005). Among negative sulfur compounds, hydrogen sulfide is characterized by high volatility and high reactivity. Dimethyl sulfide (DMS) is another sulfur-containing volatile which was found in wines, with sub-part per billion to sub-part per million levels (Acinobar Beloqui *et al*., 1996). S-methylmethionine has been reported as possible precursor (Segurel *et al.*, 2005). These compounds are responsible for reduced notes in wines.

On the opposite, some long-chain volatile sulphur compounds supply the typical pleasant aromatic profile of certain wines. In particular, 3-mercaptohexan-1-ol (3-MH), 3mercaptohexylacetate (3-MHA) and 4-mercapto-4-methylpentan-2one (4-MMP) are regarded as the most important pleasant volatile thiols in wines. 3-MH and 3-MHA are responsible for passion fruit, grapefruit notes and their perception threshold is 4 ng L^{-1} and 60 ng L^{-1}, respectively for 3-MHA and 3-MH. The 4-MMP aroma is described as box tree, black currant, or cat urine at high concentration and its perception threshold in wine is 0.8 ng L^{-1} (Tominaga *et al*., 1998a). 3-MH and 3-MHA can impart sweaty aromas at excessive concentrations.

4-mercapto-4-methylpentan-2-ol (4-MMPOH) is reminiscent of citrus zest and grapefruit. The perception threshold for this compound is 55 ng L^{-1} in acqueous alcoholic solution (Tominaga *et al*., 1998b).

The long-chain sulfur compounds mentioned above characterize the typical varietal aroma of Sauvignon blanc wine (Tominaga T *et al*., 1998a). 3-MH and 3-MHA have been identified in

certain red wine varieties, such as Merlot and Cabernet Sauvignon (Bouchilloux *et al*., 1998). These volatile thiols, together with 4-MMP, contribute to the aromas of white wine made from different *Vitis vinifera* grape varieties, such as Gewurtztraminer, Muscat, Riesling, Sylvaner, Pinot gris, Pinot blanc, Colombard, Petit Maseng, botrytized Semillon and Grenache (Tominaga *et al*., 2000a, Ferreira *et al*., 2002).

3-MH and 3-MHA are present in wines as two different stereoisomers. The R and S forms of these compounds are equivalent in the case of 3-MH (Tominaga *et al*., 2006). The perception thresholds for the R and S enantiomers are similar for 3-MH (50 and 60 ng L^{-1}, respectively for S and R forms), but they are responsible for different aromas: the R form is described as grapefruit, while the S form evokes passion fruit. The ratio between the two enantiomeric forms is close to 1 in dry white wine; on the other hand, for sweet wines made from botryzized grapes the proportion of R to S forms is measured as 30:70 (Tominaga *et al*., 2006).

3-MHA S enantiomer is more abundant than R form. Moreover, the two enantiomers of 3-MHA show different aromas and perception thresholds (Tominaga *et al*., 2006). The less odoriferous R form, which threshold is 9 ng L^{-1}, is characterized by passion fruit descriptor. The S form is reminiscent of box tree and its perception threshold is much lower (2.5 ng L^{-1}). In dry Sauvignon blanc and Semillon wines the R to S enantiomeric ratios have been reported to be 30:70, thus the most powerful isomer is even the most abundant (Tominaga *et al*., 2006).

Benzenemethanethiol, 2-furanmethanethiol and 3-mercaptopropionate represent another group of volatile thiols responsible for pleasant notes in aged wines (Tominaga *et al*., 2003a). 2-furanmethanethiol is a particularly strong-smelling compound reminiscent of roasted coffe and its perception threshold in a hydroalcoholic solution is extremely low (0.4 ng L^{-1}). This compound has been identified in sweet white wines made from Petit maseng grape, and in certain red Bordeaux wines made from Merlot, Cabernet franc and Cabernet sauvignon grape varieties (Tominaga *et al*., 2000b). Benzenmethanethiol is a volatile thiol with a strong empyreumatic aroma reminiscent smoke, identified in boxwood (*Buxus sempervirens* L.) (Tominaga & Dubourdieu, 1997) and in both in red and white *Vitis vinifera* wines (i.e. Sauvignon blanc, Semillon, Chardonnay) which contain several dozen nanograms per liter, which represent 100 folds higher than its perception threshold (0.3 ng L^{-1} in model hydroalcoholic solution) (Tominaga *et al*., 2003b). Moreover, both this compound, and 2-furanmethanethiol and 3-mercaptopropionate, have been identified in aged Champagne wines (Tominaga *et al*, 2003a). Nonetheless, 3-methyl-3-mercaptobutanal and 2-methylfuran-3-thiol, together with 3-mercaptopropyl acetate, 3-MH and 3-mercaptoheptanal, play a key role in

Sautern wine (Bailly *et al*., 2006. Bailly *et al*., 2009). In a similar way, wines made from *Botrytis*-infected grapes are characterized by the presence of thiol related aromas as 3-mercaptopentan-1-ol, 3-mercaptoheptan-1-ol and 2-methyl-3-mercaptobutan-1-ol. The first two have citrus and grapefruit aromas whereas the third compound is reminiscent of raw onion. The concentration of such aromas in commercial botrytized wines ranges from tens to thousands ng L^{-1}. Despite their perception threshold is similar to the measured quantity in wines, their olfactory impact on the overall aroma of botrytized wines is confirmed (Sarrazin *et al*., 2007).

As described above, several odoriferous thiols have been identified in *Vitis vinifera* white and red wines (figure 1.1). Nonetheless, the most important Sauvignon blanc varietal thiols are 4-MMP, 3-MHA and 3-MH.

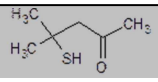
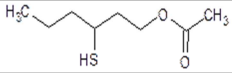
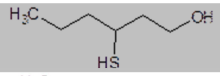
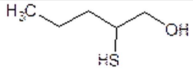
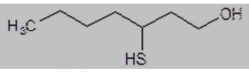
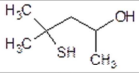
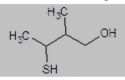
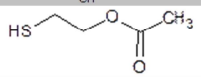
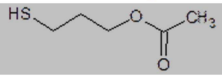
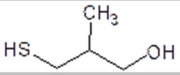
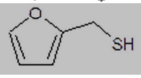
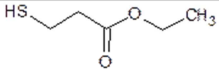
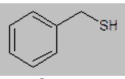
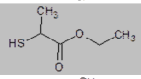
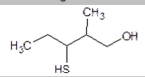
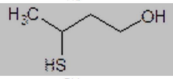
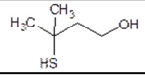
MOLECULES	NAMES	ODORS	PERCEPTION THRESHOLDS IN MODEL SOLUTION (ng L^{-1})	CONCENTRATION RANGE REPORTED IN LITERATURE (ng L^{-1})
	4-methyl-4-mercaptopentan-2-one (4-MMP)	Box-tree, blackcurrant bud	0.8	until 400
	3-mercaptohexyl acetate (3-MHA)	Box-tree	4.2 in racemic mixture	until 2500
	3-mercaptohexan-1-ol (3-MH)	Grape fruit, passion fruit	60 in racemic mixture	until 19000
	3-mercaptopentan-1-ol	Grape fruit	950	90-300
	3-mercaptoheptan-1-ol	Grape fruit	35	25-75
	4-methyl-4-mercaptopentan-2-ol (4MMPOH)	Citrus zest	55	until 90
	2-methyl-3-mercaptobutan-1-ol (4MMPOH)	Raw onion	nr	80-150
	3-mercaptoethyl acetate	Meaty	nr	na
	3-mercaptopropyl acetate	Meaty	nr	na
	3-mercapto-2-methylpropan-1-ol	Broth, sweat	3000 (in water)	25-10000
	2-furanmethanethiol	Coffee	0.4	0.4-62
	Ethyl-3-mercaptopropionate	Meaty	200	40-1200 (in sparkling wines)
	Benzenemethanethiol	Smoky	0.3	10-40
	2-methyl-3-furanthiol	Meaty	0.4-1.0 (in water)	> 100
	Ethyl-2-mercaptopropionate	Fruity	500	na
	2-methyl-3-mercaptopentan-1-ol	Raw onion	nr	na
	3-mercaptobutan-1-ol	Onion, leek	nr	na
	3-methyl-3-mercaptobutan-1-ol	Cooked leek	1500	na

Figure 1.1. Volatile thiols identified in *Vitis vinifera* wines. (Roland *et al*., 2011).

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1.2. Sulphur aroma precursors

3-mercaptohexan-1-ol is present in grape and juice as non-volatile precursor. In particular, it is present as cysteinyl-conjugates (Tominaga *et al*., 1995), as glutathione conjugates (Peyrot des Gachons et al., 2002a) and as cysteinylglycin-conjugates (Capone *et al*., 2011a). 4-mercapto-4-methylpentan-2-one has been identified in grape and must as cysteine conjugate and glutathione conjugate (Fedrizzi *et al*., 2009). As a result, free varietal thiols are practically absent in grape juice and are released during the alcoholic fermentation by the wine yeast, *Saccharomyces cerevisiae*, from odourless precursors.

Cysteinylated precursors are described as S-cysteine conjugates: S-4-(4-methylpenta-2-one)-L-cysteine (Cys-4-MMP) and S-3-(hexan-1-ol)-L-cysteine (Cys-3-MH), for 4-MMP and 3-MH respectively (Tominaga *et al*., 1995, Thibon *et al*., 2008).

In model solution, cell-free enzyme extract of *Eubacterium limosum* (containing β-carbon-sulfur lyase enzymes) allows the release of 4-MMP from synthetic Cys-4MMP (Tominaga *et al*., 1998).

Furthermore, non-volatile crude extracts obtained from Sauvignon blanc must were shown to contain Cys-3-MH and Cys-4-MMP (Tominaga *et al*., 1998). Finally, action of the same cell-free enzyme extract of *E. limosumon* in Sauvignon blanc must extract allowed the release of 3-MH and 4-MMP, 3-MH release being correlated with Cys-3-MH decrease (Tominaga *et al*., 1998). This enzyme catalyses the breakage of the thioether bond following the reaction reported in figure 2.1.

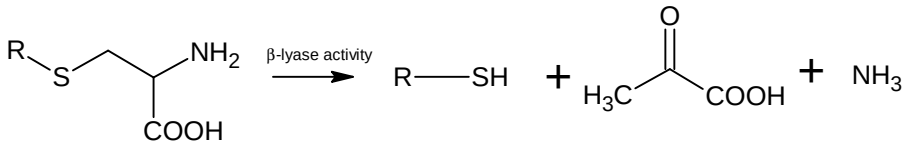

Figure 2.1. Reaction of breakage of cysteinylated precursors in the presence of enzyme β-lyase.

Consequently, in wine, the volatile thiols release from their corresponding precursors involves similar wine yeast enzymes with carbon-sulfur lyase activities, proposing a β-elimination reaction catalyzed by a cysteine-S-conjugate β-lyase activity [EC 4.4.1.13] of *S. cerevisiae*. Studies on putative yeast carbon-sulfur lyases have been performed by Howell *et al*. (2005). Moreover, a stereoselectivity of 3-MH liberation from S-(R/S)-3-(hexan-1-ol)-L-cysteine by

oenological yeasts have been demonstrated. (Grant-Preece *et al*., 2010, Pardon K H *et al*., 2008).

Correlation between Cys-3-MH content of the must and 3-MH concentration in wine has been pointed out (Murat *et al*., 2001b). Moreover, Dubourdieu *et al*. (2006) have been also demonstrated that Cys-3-MH concentration decreases in must during alcoholic fermentation and, at the same time, 3-MH amount increases.

In grape, Cys-4-MMP content was shown to be equivalent in the juice and skin while the Cys-3-MH concentration was higher in the skin (Peyrot des Gachons *et al*., 2002).

3-MHA is also generated from Cys-3-MH, but by an indirect pathway. Infact, it is produced through the yeast metabolism during alcoholic fermentation due to the of ester-forming alcohol acetyltransferase activity [EC 2.3.1.84], encoded by the ATF1 gene (Swiegers & Pretorius, 2007).

Anyway, the molar conversion yields of cysteinylated precursors into their corresponding thiols are low: the yield ranges from 0.1 to 12% and from 0.06% to 10% for Cys-3-MH/3-MH (Dubourdieu *et al*., 2006; Masneuf-Pomarede *et al*., 2006; Murat *et al*., 2001b) and Cys-4-MMP/4-MMP (Masneuf-Pomarede *et al*., 2006; Murat *et al*., 2001c) respectively.

The glutathione derivate precursor of 3-MH has been identified in grapes and juice (Peyrot des Gachons *et al.*, 2002), postulating that this conjugate is catabolized to S-3-(hexan-1-ol)-L-cysteine in grapes by the action of γ-glutamyltranspeptidase and carboxypeptidase activity. Because cysteinylated precursor of 3-MH is found to be alongside the metabolic pathway of the degradation of the corresponding glutathione conjugate, S-3-(hexan-1ol)-gluathione may be considered the biogenetic precursor of S-3-(hexan-1-ol)-L-cysteine, and subsequently 3-mercaptohexan-1-ol.

An alternative pathway for the biogenesis of 3-mercaptohexan-1-ol in wine has been proposed starting from *trans*-2-hexenal (Schneider *et al*., 2006). This unsaturated aldehyde is present in grape juices due to the oxidative breakdown of unsaturated fatty-acids (Joslin & Ough, 1978). This pathway is based on the addition of the sulfydryl group of a thiol compound to the unsaturated carbonyl molecule. Hydrogen sulfide and L-cysteine were proposed firstly as they are present in grape juices and must during alcoholic fermentation. 3-MH would directly lead by-product of the yeast sulfur metabolism when the sulfur addition is from hydrogen sulfide. On the contrary, 3-MH would be indirectly obtained after carbon-sulfur lyase activity by yeast via cysteine addition.

However, S-3-(hexan-1-ol)-L-cysteine and *trans*-2-hexenal cannot be longer considered as the major 3-MH precursors (Subileau *et al*., 2008). Indeed, the deletion of the OPT1 gene, encoding for glutathione transporter, is responsible for a lower 3-MH and 3-MHA content, thus suggesting that the major precursor is a S-glutathionyl conjugate like S-3-(hexan-1ol)-glutathione.

Even for 4-MMP, the identification of a glutathionylated conjugate (S-4-methylpentan-2-one)-glutahione in Sauvignon blanc juice (Fedrizzi *et al*., 2009) suggests that glutathione conjugates can be considered as major precursors.

The liberation of 3-MH from glutathione conjugates, alongside cysteine conjugates, has been shown (Grant-Preece *et al*., 2010). Moreover, the employment of isotopically labeled synthetic compounds gives evidence that S-3-(hexan-1-ol)-glutathione is an absolute precursor of 3-MH (Roland *et al*., 2010b). Despite this, the molar conversion is only 3%.

Ratio between the amount of glutathione and cysteine conjugates, and between their isomeric forms, has to be elucidate. In fact, S-3-(hexan-1-ol)-L-Cysteine has been detected in higher concentration than the corresponding glutathione conjugate (Roland *et al*., 2010b). However, more recent researchers suggest that both R and S enantiomers of glutathione conjugate are more abundant than both R and S enantiomers of cysteine conjugates (Capone *et al*., 2010). Moreover, these authors showed the S isomer concentration was higher than the R isomer for both conjugates.

The relation between cysteine and glutathione cojugates, itself generated after conjugation of glutathione on *trans*-2-hexenal, has been recently elucidated (Thibon *et al*., 2011). The conjugate between glutathione and 3-MH can be considered as a pro-precursor of 3-MH finally released in wines.

Despite this, the employment of labeled 3-MH and 4-MMP glutathione conjugates suggests that the conversion yeld after alcoholic fermentation in wines is close to 0.3%, which can represent the starting point for additional studies on varietal thiol biogenesis (Roland *et al*., 2010).

The identification of intermediates involved in the biosynthetic pathway of 3-mercaptohexan-1-ol conjugates in yellow passion fruit (*Passiflora edulis f. flavicarpa*) (Fedrizzi *et al*., 2012) suggesting a similar pathway in other plants, such as grapevine. In both grape and yellow passion fruit, 3-MH may originate from non-volatile precursors linked to the GSH metabolism which can begin from the conjugation of GSH with an electrophile under the catalysis of glutathione-S-transferase (Dixon *et al*., 2002, Edwards *et al*., 2000, Lamoureux & Rusness, 1987, Ohkama-Ohtsu *et al*., 2007, Rennenberg, 1982) (figure 2.2) . The building blocks of this

molecule suggest a 1,4-Michael addition between (E)-2-hexenal and GSH followed by a carbonyl reduction which is carried out by an aldo-keto reductase. This pathway occours in several living organisms in response to oxidative stress.

Three precursors (i.e. S-glutathionylated (1), S-glycyl cysteinylated (2) and S-cysteinylated (4)) related to 3-MH have been identified.

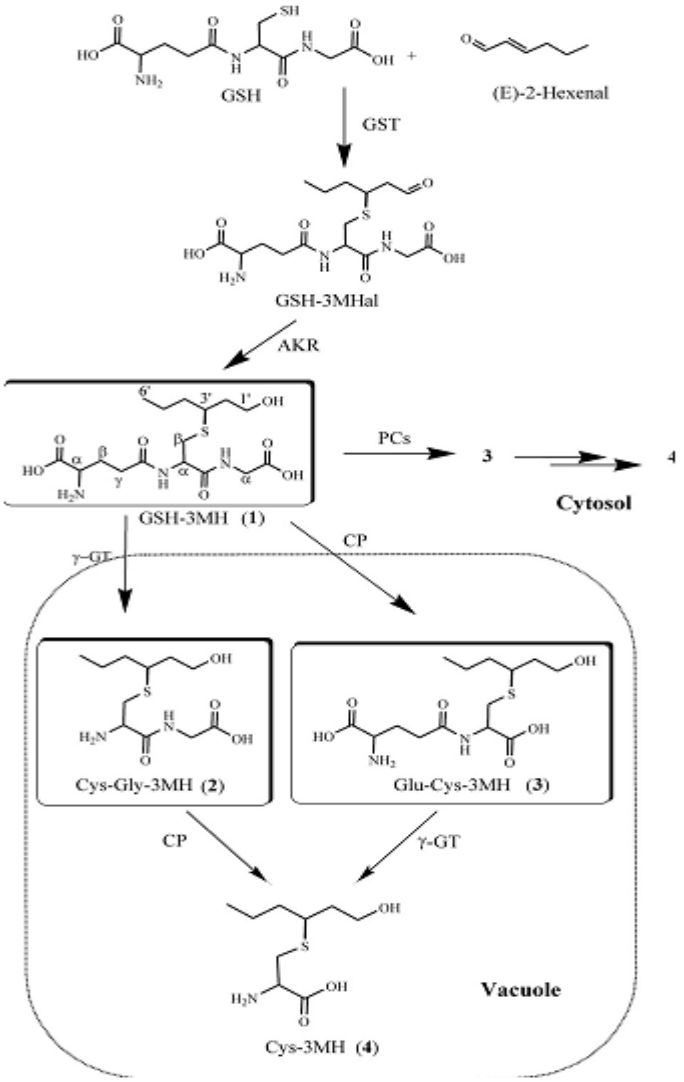

Figure 2.2. Hypotetical pathway for the biosynthesis of 3-mercaptohexan-1-ol conjugates in yellow passion fruit (Fedrizzi *et al*., 2012). Legend: glutathione-S-tranferase (GST); Aldo-keto reductase (AKR); γ-glutamyl transpeptidase (γ-GT); carboxypeptidase (CP) and phytochelatine syntases (PCS).

The formation of 3-mercaptohexan-1-ol precursor is a dynamic process and it can be influenced by vineyard (Peyrot des Gachons *et al*., 2005) and winery processing operations (Capone et al., 2012, Allen *et al*., 2011).

Severe water stress and nitrogen deficits limited the formation of S-cysteinil conjugates as a first impact of viticulture on these aromas (Peyrot des Gachons *et al*., 2005). Moreover the presence of moulds can influence thiol concentration since *Botrytis cinerea* stimulated the synthesis of the 3-MH cysteine precursor (Sarrazin *et al*., 2007). This observation would explain the high concentrations of 3-MH in sweet wines made from botrytized grape (Sarrazin *et al*., 2007, Tominaga *et al*., 2006, Tominaga *et al*., 2000). Moreover, as a result of *B. cinerea* infection, the ratio R:S isomers was modified as well as the R:S ratio of 3-MH in resulting wines (Tominaga *et al*., 2006).

The grape treatment during harvesting can improve thiol precursors: mechanical harvesting determines a higher concentration of thiol precursors (Capone *et al*., 2011b). Moreover, machine harvested grapes transportation leads to a higher content in thiol precursors (Capone *et al*., 2011b). Despite this, the highest content in thiol precursors (cysteine conjugates, glutathione conjugates and cysteinylglycin-conjugates) does not correspond to the highest content in thiols in corresponding wine (Allen *et al*., 2011). In a recent study, the lack of correlation among putative thiol precursors in juice and final thiol concentration in wine is described, suggesting that 3-MH in wine may derives from alternative pathways (Pinu *et al*., 2012).

The wine making can influence the concentration of thiol-related aromas in wines. As more than half of the total cysteine conjugates is located in garape skin (Murat *et al*., 2001b, Peyrot des Gachons *et al*., 2002), an increased skin contact time augments S-3-(hexan-1-ol)-L-cysteine in grape musts (Maggu *et al*., 2007). In the same way, stronger pressing condition can lead to higher concentration of thiol precursors in juice. Nevertheless, higher concentration in such precursors in juice does not correspond to higher concentration in volatile thiol in corresponding wine (Patel *et al*., 2010). In fact, pressed juices are characterized by lower concentration in 3-MH and 3-MHA in comparison to free run juices: the higher is the pressure, the higher the concentrationin thiol precursor and the higher the oxidative potential in the juice (and subsequently the corresponding wine).

The liberation of volatile thiols from their precursors depends on several fermentation conditions: temperature (Masneuf-Pomarède *et al*., 2006), yeast species (Masneuf-Pomarède *et*

al., 2002) and yeast strain (Howell *et al*., 2004, Murat et al., 2001c, Swiegers *et al*., 2009) affect release yields.

Fermentation temperature of juices can modify thiol concentration in resulting wines: in the range of alcoholic fermentation for white wines, at higher fermentation temperature, higher concentration of thiol-related aromas are released. Among commercialy available *S. cerevisiae* strains commonly used in wine making, VL3 and EG8 release more volatile thiols, in comparison to VL1 and 522 (Murat *et al*., 2001c). Furthermore, *S. bayanus* var. *uvarum* strains and hybrids between this yeast and *S. cerevisiae* showed a higher ability to release 3-MH and 4-MMP if compared to *S. cerevisiae* strain (Masneuf-Pomarède *et al*., 2002).

Although such studies on different yeast species and strains, the employment of diammoniummphosphate as a nitrogen source in juices suppresses 3-MH production (Subileau *et al*., 2008b).

Although the quantity of 3-MH cysteine conjugate in must and 3-MH in corresponding wines is proportional (Tominaga *et al*., 1998), only a small amount of cysteine precursor is converted to the active aroma during alcoholic fermentation (Murat *et al*., 2001b).

As for 3-MH concentration, yeast can influence the amount of 3-MHA in wines. In particular, the ability of commercial wine yeast to convert 3-MH into 3-MHA has been shown to vary (Swiegers *et al*., 2009). The yeast strains with the lowest 3-MH release capacity exhibit the highest capacity in conversion 3-MH into 3-MHA. Some non-*Saccharomyces* yeasts (i.e. *Pichia kluyveri*) are low in capability to release 3-MH from the corresponding precursor but they show high conversion rate from 3-MH to 3-MHA (Anfang *et al*., 2009).

Therefore, the co-fermentation using both yeast strains heving high 3-MH released and 3-MH/ 3-MHA conversion can positively affect the volatile thiols concentration in wine. Indeed, the co-inoculation of must using *S. cerevisiae* yeast strains with the two characterisctics as described as above leads to higher level of 3-MHA in wine if compared to the employment of a single yeast during alcoholic fermentation (King *et al*., 2008). In a similar way, a mix of *Saccharomyces* and non-*Saccharomyces* such as *P. kluyveri* enhances the 3-MHA compared to single *S. cerevisiae* inoculum in Sauvignon blanc wine (Anfang *et al*., 2009).

Cysteine conjugates have been identified also for 3-mercaptopentan-1-ol, 3-mercaptoheptan-1-ol and 3-mercapto-2-methylbutan-1-ol. These compounds have been pointed out in Sauternes wine (Sarrazin *et al*., 2007) and in musts from different grape varieties either infected by noble rot or not (Thibon *et al*., 2010).

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1.3. Factors affecting volatile thiols stability in wine

As many aromatic wine components, the concentration of thiol related aromas change during wine ageing. A rapid decline of 3-MHA is observed after three months of bottle storage, while a much slower decline in 3-MH can be noticed. After one year storage the ester is completely disappeared, while 3-MH is still present but its content is halved. Different degradation kinetics are linked to different degradation mechanism.

Copper can react with thiols leading to loss of such compounds (Darriet *et al*., 2001). The wine oxidation is also detrimental to the concentration of those key fruity aroma compounds (Blanchard *et al.*, 2004).

Wine is exposed to oxygen at various stages of production and the amount of solubilized oxygen is affected by the temperature (Singleton, 1987). Even if thiols do not react directly with oxygen, their decrease is linked to other compounds (e.i. phenols) which react readily with oxygen (Blanchard *et al*., 2004). Among the wine compounds potentially oxidable, polyphenols represent the initial substrate of oxidative mechanisms. It was showed that polyphenol autoxidation products, namely *ortho*-quinones and hydrogen peroxide, react with varietal thiols (figure 1.3.1). As supported as described above, Danilewicz *et al*. (2008) reported an oxidative degradation mechanism of 3-MH mediated by polyphenols (e.i. (+)-catechin).

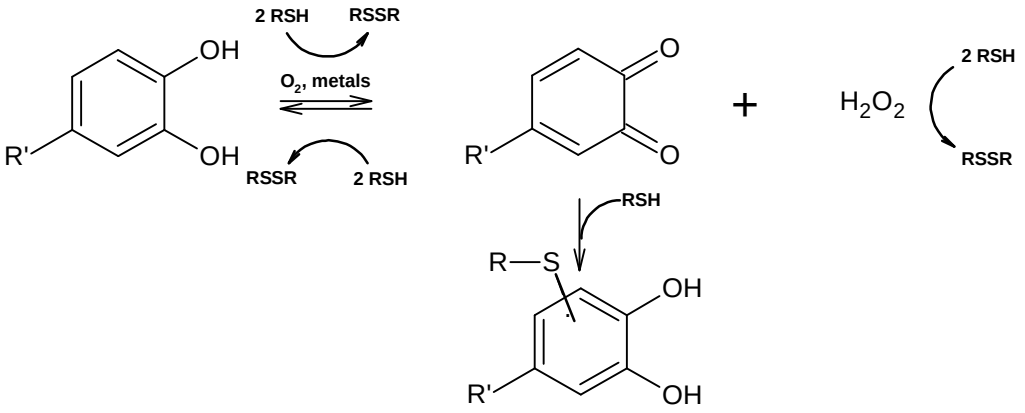

Figure 1.3.1. Oxidative degradation of volatile thiols (RSH) in wines.

The quinones formation in wines is due to the Fenton reaction, wherein not only the oxygen, but also iron and copper play a key role.

Phenols can react directly with oxygen only under basic pH condition. In fact, the weakly acidic character of phenolic compounds (pK_a 9 to 10) leads the formation of the phenolate anion which can react with oxygen. Despite this, since wine has acid pH, and phenols have a high dissociation constant, only a small fraction of wine phenols can be deprotonated. Consequently, direct oxidation pathway of phenolic compounds is no longer possible (Danilewicz, 2003).

Because of the poor direct reactivity of oxygen with such wine molecules, the oxidizing potential of molecular oxygen is improved by the generation of reactive oxygen species. The initial transfer of an electron leads to the formation of superoxide ion which exists as hydroperoxide radical at wine pH. This step requires a catalyst: a transition state metal, such as iron (Waterhouse & Laurie, 2005). The transfer of a second electron would then produce hydrogen peroxide. The reduction of the latter compound leads to an hydroxyl radical which is more reactive than the previous one. The reduction pathway is the Fenton reaction occurring between hydrogen peroxide and ferrous iron salt (Fe^{2+}) (Danilewicz, 2003). A molecule of water is the final product of oxygen reduction.

Nevertheless, hydroperoxide radical is able to oxidize wine phenols which can readily reduce the ferric ion to ferrous form originating a quinone (figure 1.3.2).

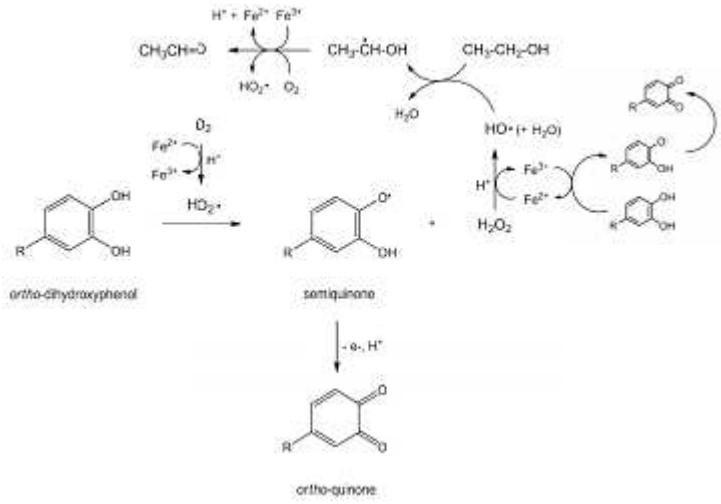

Figure 1.3.2. Oxidative process of phenolics in wines.

Thiols can be bound to oxidized phenolic compounds under wine oxidation conditions. During barrel and bottle aging of red and white wines, a decrease of volatile thiols, such as 3-mercaptohexan-1-ol, has been showed due to the oxygen dissolved (Blanchard *et al*., 2004, Lopes *et al*., 2009).

Thiols exhibit strong antioxidative activity, owed to their property reducing *ortho*-quinones and H_2O_2. As a result they will are converted to the corresponding disulfide reacting with H_2O_2 (Vermeulen *et al*., 2005). Thiols can easily form a disulfide bound when a metal ion catalyst (particularly cupric ions- Cu^{2+}) and oxygen are dissolved; this reaction occours faster by temperature increasing (Jocelyn, 1972).

Anyway, a clear difference exists among the reactivity of phenols (Nikolantonaki *et al*., 2010). *Ortho*-trihydroxyphenols (pyrogallol derivatives), such as gallic acid, and *ortho*-dihydroxyphenols (catechol derivatives), including caffeic acid, (+)-catechin, (-)-epicatechin and quercetin are the compounds most susceptible to oxidation (Kilmartin *et al*., 2001). The stability of quinone formed by the polyphenol oxidation is another important aspect. In fact, some quinones are more stable and they can be reduced back to the original polyphenol by such antioxidants (e.g. SO_2 and glutathione). Contrarily, other quinone products break down rapidly originating irreversibily oxidative compounds, such as the quinone deriving from quercetin (Makhotkina *et al*., 2009).

The composition of white wine polyphenols depends on grape variety, vineyard conditions and treatments, climate, grape maturity, vinification procedures as well as on chemical reactions taking place during the wine ageing. Wine making practices, such as skin contact and hard pressing, lead to higher polyphenolic content in wines, in particular the flavonoids content is increased (Cheynier *et al*., 1989).

The thiols reactivity is due to nucleophilic addition reactions with certain electrophiles. As volatile thiols are nucleophiles, they can thus add to the electrophilic sites of quinones in a conjugate addition according to a Michael-type addition (Cheynier *et al*., 1986, Patel *et al*., 2010).

The efficacy of this addition reaction depends upon the nucleophilic strength of the thiol and the oxidation rate of each phenolic substrate. The nucleophilicity of thiols is mainly modulated by their steric hindrance making primary thiols more reactive than tertiary thiols (Charles-Bernard *et al*., 2005). Among thiol related aromas, 4-mercapro-4methylpentan-2-one shows a lower reactivity towards oxidation-quinones, if compared to 3-mercaptohexan-1-ol or 2-furanmethanethiol. Sulfur dioxide and reduced glutathione are characterized by higher

reactivity than aromatic thiols toward quinones thus limiting thiol related aroma loss and preventing oxidative spoilage (Nikolantonaki & Waterhouse, 2012a).

The rate of these reactions is pH-dependent, since at wine pH the concentration of thiolate anion (RS^-), which is more reactive than its protonated form (RSH), is low. In fact, pK_a value for thiols ranges from 9 to 12 (Vermuelen *et al*., 2005). The greater proportion of thiolate anions at a higher pH, together with an higher concentration of quinones, may explain the lower levels in 3-MH in wines with higher pH (4.0 instead of 3.5) (Blanchard *et al*., 2004). It is well known that greater involvement of the phenolate anion form in wine oxidation is related to an higher amount of quinones in wines (Sioumis *et al*., 2005). As already mentioned, polyphenols are weak acids with a pK_a value ranging between 9 and 10, allowing them to exist as phenolate anions, which are capable of reacting directly with molecular oxygen, thus explaining the fast autoxidation of polyphenols under alkaline conditions (Cilliers *et al*., 1989). Under higher pH conditions, oxygen uptake in wine is much faster, due to a greater conversion of phenols to quinones with limited regenerative polymerization. However, only a small amount of polyphenols is deprotonated at wine pH but these compounds can still autoxidize to semiquinone and quinone, although at a slower rate.

Such nucleophilic additions between thiols and quinones have been reported using caftaric acid (Salgues *et al*., 1986), caffeic acid (Cilliers & Singleton, 1990), gallic acid (Quideau *et al*., 1995), (+)-catechin (Mordiani *et al*., 2001) and epicatechin (Tanaka *et al*., 2002). These reactions have been described in alkaline buffer, organic medium, acidic white grape must and wine model system.

In this scenario, wine composition and oxygen exposure are the major responsible for thiol related aroma loses both during winemaking and post-bottling (Brajkovich *et al*., 2005, Ugliano *et al*., 2011).

Since 3-MHA is not only a thiol but also an ester, hydrolysis is the predominant mechanism rather than oxidation leading to the degradation of this volatile thiol. Acetate esters are present in wine in excess in comparison to their equilibrium constant. They are produced enzymatically during fermentation by the action of yeasts: biogenetic pathway is linked to the combination of acetyl-CoA with an alcohol catalyzed by alcohol acetyltransferases. As most acetate esters, 3-MHA undergoes to hydrolysis at wine pH during ageing and the hydrolysis-esterification equilibrium is the key mechanism to explain its loss (Makhotkina & Kilmartin, 2012). For this compound temperature and wine pH are responsible for its decrease rather than oxidative condition.

The most common antioxidants present in wine are sulfur dioxide, ascorbic acid, and reduced glutathione. These antioxidants limit the polyphenol oxidation, either by removing oxygen from wine or by reversing and altering the oxidation process.

The equilibrium established upon SO_2 dissociation in wine, is pH dependent. Moreover, this equilibrium is affected by the presence of wine constitutents that bind bisulfite (HSO^{3-}) as well as by wine temperature (Usseglio-Tomasset, 1992). Sulfur dioxide exists in wine in both free and bound forms, their sum equaling total SO_2. Free sulfur dioxide is present as molecular SO_2, bisulfite (HSO_3^-) and sulfite (SO_3^{2-}): the latter can react directly with oxygen, but its concentration is extremely low at wine pH. Bisulfite is the predominant form of free SO_2 at wine pH and binds a wide range of wine compounds, thus producing bound sulfur dioxide. The importance of this antioxidant in preventing volatile thiol loses leads in bisulfite competition for *ortho*-quinones. In fact, it has been argued that thebisulfite form HSO_3^- converts *ortho*-quinones back to *ortho*-dihydroxyphenols and react directly with ortho-quinones (Makhotkina & Kilmartin, 2009) (figure 1.3.3). As a result, the presence of free sulfur dioxide slows down volatile thiols oxidation (Nikolantonaki *et al*., 2012b).

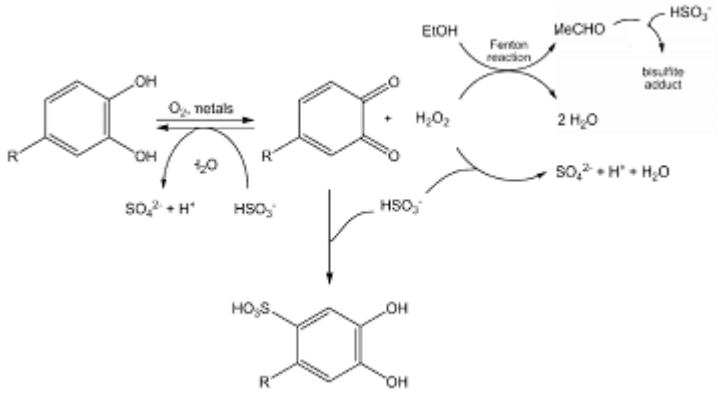

Figure 1.3.3. Antioxidative effect of sulfur dioxide (as HSO_3^-) in wines.

In a similar way, the antioxidative activity of glutathione has been ascribed to its nucleophilic addition quinones forming a reduced glutathione conjugate. As the thiols, glutathione is able to reduce quiones and hydrogen peroxide. Therefore, it can be hypothesized that glutathione competes in these reaction with aromatic thiols, thus preventing these aromatic molecules loss. Moreover, the decline of both glutathione and aromatic thiols is limited keeping the wine on

lees before bottling. The uptake of oxygen by lees may explain this phenomenon (Salmon *et al*., 2002).

The antioxidant effect of ascorbic acid is due to its rapid scavenging ability of molecular oxygen, thereby preventing the onset of oxidative mechanism. The potential of ascorbic acid to recycle *ortho*-quinones back to *ortho*-dihydroxyphenols has been suggested by various authors (Danilewicz *et al*., 2003, Isaacs *et al*., 1997, Singleton, 1987). More recently, complete reduction of quinones to phenols by ascorbic acid in wine acidic conditions has been studied (Nikolantonaki & Waterhouse, 2012). Even if the lack of rapid interaction between ascorbic acid and *ortho*-quinones has been noticed (Makhotkina & Kilmartin, 2009). However, under oxygen-rich conditions, ascorbic acid is readily oxidized, especially in presence of iron (Fe^{3+}) and copper (Cu^{2+}) (Danilewicz, 2003), leading to the formation of dehydroascorbic acid and hydrogen peroxide which are strong oxidative agent. The hydrogen peroxide shares into the oxidation of other compounds: among the wine components, it can oxidize volatile thiols. Therefore, since SO_2 is capable of quenching hydrogen peroxide, ascorbic acid covers a supplementary role scavenging directly the oxygen released by the SO_2 activity.

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2. Aims of the study

The aims of this PhD were to evaluate new analytical approaches aimed to evaluate the occurrence of volatile thiols in wine, to study alternative biogenetic pathway leading to thiol realted aroma and to assess yeast strain effect on the amount in molecules responsible for thiol stability in wine.

Despite their strong contribute to smelling properties of wine, aromatic thiols are present at trace concentration. In fact, their analytical quantification has always posed the most significant obstacle for their study. Moreover, thiols strong reactivity towards various molecules in wine makes them fairly susceptible to oxidation. As the presence of the thiol group represents the most important issue for the analytical quantification, several derivatizing agent were tested. Nowadays, the most effective methods apply organomercury molecules. These are the only methods capable of detecting thiols at level sufficiently low to be applicable in wine matrices. Despite this, the use of mercury constitutes a health and safety risk in the laboratory and additionally these methods are very time-consuming. Other very promising derivatising agents have been suggested in the literature. Nevertheless all these methods failed in achieving the quantification of volatile thiols at their perception threshold or were extremely time consuming, leaving the mercury methods the only way to quantify such strong smelling molecules. The development of a novel analytical method applicable to determine volatile thiols is then necessary in order to gain a useful tool to identify, quantify and evaluate occurrence of thiol related aromas in wines.

The volatile thiols determination will be approached by gaschromatographic and liquid chromatographic methods.

During gaschromatographic approaches, pentafluorobenzyl bromide will be first studied as derivatizing agent. This derivatising agent has been employed by means of negative ions chemical ionization mass spectrometry. Chemical ionization is responsible for low fragmentation and shares with the electron capture detection a selective and sensitive response to electrophilic atoms (halogens), as fluorine is. Despite this, such techniques are not widespread. In this study electron impact ionization in positive mode mass spectrometry was then evaluated. The applicability of derivatization step will be evaluated in both organic solvent and aqueous media. Knowledge coming from this preliminarly results will be employed to develop purification methods with the aim to reach enough sensitivity in aromatic thiol determination in wine. Applicability of new derivatizing agent will be also evaluated.

During liquid chromatographic approaches, most promising derivatizing agents will be tested. Purification and concentration of aromatic thiols will be then studied to reach sensitivity and selectivity.

Occurrence of aromatic thiols in wine, expecially 3-mercaptohexan-1-ol, has been correlated to cysteine and glutathione conjugates precursors in grapes. Despite this, lack of correlation among thiol precursors in juice and final thiol concentration in wines, suggest that thiol precursors and aromatic thiol are related in a more complex way and/or the 3-MH in wine may derive from alternative pathways. Possible mechanisms for 3-MH occurrence in wine involve hexenal pathway, which includes direct addition of a sulfur donor to *trans*-2-hexenal. Among

sulfur donors, cysteine, glutathione and H_2S have been proposed. Sulfur in its bisulfite form can be considered a sulfur donor. Aimed to study alternative biogenetic pathway in 3-mercaptohexan-1-ol in wine, interaction between hexenal and bisulfite were then considered. In particular, addition products between the unsaturated aldehyde and bisulfite were evaluated as putative precursors of 3-MH in wine.

Loss of aromatic thiols in wine is due to oxidative processes. The presence of molecules which interefere in the process of polyphenol oxidation is then related to the stability of such smelling molecules in wines. The most common antioxidants present in wine are sulfur dioxide, ascorbic acid and glutathione in its reduced form. The latter molecules occours naturally in grape must, existing either in its reduced and oxidized form. Despite this, it diminishes during crushing due to its reaction with *ortho*-quinones and/or oxidation to the disulfide form. Moreover, it represents sulfur source for *S.cerevisiae* during alcoholic fermentation. As a result, at the beginning of the alcoholic fermentation GSH almost disappears and it increases during vinification process due to yeast activity. Wine making technique can affect GSH content in wine. In particular, several yeast strains were tested during laboratory-scale alcoholic fermentation. Glutathione in reduced and oxidized forms were evaluated in wine as a result of various *S. cerevisiae* yeast strains conducting the alcoholic fermentation.

3. Gaschromatographic approaches in volatile thiols determination in wines

Gaschromatography involves the analysis of volatile organic compounds, that is, molecules that exist in the vapour phase, at least at the typical GC operating temperatures between 40 and 300°C. Since aroma compounds must, by their nature, leave the wine matrix and travel through the air to be perceived, they are generally excellent candidates for analysis by GC.

3.1. Headspace sampling technique in volatile thiol determination

Although many volatile compounds may be solvent extracted, distilled, or otherwise isolated from the wine matrix, it is frequently preferable to take advantage of their volatility and rely instead on techniques of headspace analysis.

Static or dynamic headspace are two used methods in flavour analysis (Pawliszyn, 1999). In each case, however, sampling procedure is carried out in the atmosphere adiacent to the sample, leaving the actual sample material behind. In static headspace technique analytes are sampled at the equilibrium state between the liquid and the gas phases by using a fiber where analytes are adsorbed. Choices in fiber material, as well as sample temperature, and the presence of salt can improve extraction yield.

In a different way, dynamic headspace involves moving the analytes away from the sample matrix in the heaspace phase but instead of allowing the sample volatiles to come to equilibrium between the sample matrix and the surrounding headspace, the atmosphere around the sample material is constantly swept away from by a flow of carrier gas, taking volatile analytes with it. On the one hand, this technique prevents the establishment of an equilibrium state, thus, more of the volatile dispersed in the matrix will leave the sample and pass into the headspace. On the other hand, it increases the size of the headspace used in sampling phase. As a consequence the trapping stage of the analysis offers increased sensitivity (Lepine *et al*., 1997). Further, sorbents offer some selectivity within the range of collected volatiles. Choices in sorbent and temperature may permit collection and concentration of specific analytes while venting others. Despite this, the instrumentation requires more complexity. As a consequence, it is more expensive to purchase than other sampling techniques. Moreover, many sources of error in purge-and-trap instruments have been reviewed (Washall *et al*., 1990).

Sulphur compounds in wine are frequently divided into "light" (boiling point < 90°C) and "heavy" (boiling point > 90°C) compounds (Mestres *et al*., 2000) indicating difficulty of using a relevant common sampling/enrichment technique.

In volatile thilols analysis, static headspace analysis is applied in low boiling point sulfur compounds analysis in wine. In particular, the employment of Carboxen-polydimethylsiloxane fibre (CAR-PDMS) (Mestres *et al*., 1999) or Carboxen-polydimethylsiloxane-divinylbenzene (CAR-PDMS-DVB) (Fedrizzi *et al*., 2007, Fedrizzi *et al*., 2010) allows the determination of thiols, sulphides and disulphides in wines. Despite this, the volatiles analyzed by this technique are characterized by low boiling point (lower than 90°C) unlike 3-MH, 3-MHA and 4-MMP.

Among headspace sampling techniques, only purge and trap has been described to analyze 3-MH and 3-MHA in wines, reaching detection limits closer to their sensory threshold (Fedrizzi *et al*., 2008). Despite this, the other heavy volatile sulphur aromas were not identified at perception threshold, thus leaving derivatization procedures as the most promising techniques in thiol aromas extraction and analysis.

3.2. *p*-hydroxymercuribenzoate as derivatizing agent in volatile thiol extraction

Volatile thiols are present in wines at sub-ppb concentration levels. Their analysis requires to concentrate the analytes hundreds or thousands times to be able to identify the molecule of interest. Moreover wine represent a very complex matrix where such reactive compounds can decrease during wine aging but also during concentration step of analytes. In this scenario, concentration and purification of such molecules are key factors in order to reach appropriate methods.

For several methods, mercuric compounds are found to be an optimal choice in method development. In particular *p*-hydroxymercuribenzoate (pHMB) is the compound used in numerous methods. One of the properties of pHMB is to combine thiol-containing compounds function. Once derivatized, such compounds are no more reactive and can be extracted and purified as very polar compounds.

The first published method, employing such compound as derivatizing agent, used to extract volatile thiols by bubbling nitrogen gas through the wine. Extracted thiols were then forced to react with pHMB in water solution. Such method combines the extraction of wines volatiles constituents using a dynamic headspace technique and the selective reaction between thiols and

the mercuric compound (Bouchilloux *et al*., 1996). As the rection between thiol and mercuric salt is reversible, thiols are released by using an excess of cysteine (twenty times the molarity of pHMB). Once not derivatized, thiol polarity decreases and such compounds can be extracted in a liquid-liquid extraction using an organic solvent: dichloromethane and pentane mixture are proposed in this first method (Bouchilloux *et al*., 1996). Thanks to the low boiling point of organic solvents, extracted thiols can be concentrated without loses and, in the end submitted to gaschromatographic analysis. Despite analytes are concentrated 5000 times by using this method, a selective detector is necessary to identify thiol aromas (4-mercapto-4-methylpentan-2-one) in Sauvignon blanc wines. In particular, mass spectrometry and photometric flame detector are employed in this method (Bouchilloux *et al*., 1996).

Extraction of volatiles from wines was then modified using dichloromethane (Tominaga *et al*., 1996). Despite such organic solvent allowed to extract volatile thiols, many ineterferents were extracted. In fact, once extracted thiol in water solution, using pHMB, the aqueous layer was washed using dichloromethane after pH modification. This method, which allows a final concentration factor of 1500, allowed to identify 3-mercaptohexyl acetate by using FPD as detector (Tominaga *et al*., 1996). The same extraction method, applied to vegetable matrix, allowed to identify 4-MMP extracted from Box tree and Broom (Tominaga & Dubourdieu, 1997).

pHMB was used to identify and determine volatile thiols also in red wines (Bouchilloux *et al*., 1998). Once formed thio-adducts can be dried dryness, redissolved in water, where the employment of excess in cysteine and organic solvent allows to de-combine thiols and extract them. Since it is a very specific method that avoid phenols extraction, it allowed to identify 3-MH and 3-MHA in red wines, especially Cabernet Sauvignon and Merlot (Bouchilloux *et al*., 1998). Despite this, the employment of three liters of the same wine to be extracted made this procedure not easy.

The application of this method, with slightly modification, allowed to identify 4-MMP, 3-MH, 4-mercapto-4-methylpentan-2-ol and 3-mercapto-3-methylbutan-1-ol in Sauvignon blanc wines by gaschromatography-mass spectrometry technique (Tominaga *et al*.,1998a). Even if such sensitive detection technique was used, one liter of wine was necessary to reach satisfactory sensitivity.

The employment of a new purification step applied to this method allowed to reduce wine volume. In particular, a strong basic anion exchange column was used to retain pHMB-thiol complexes, once formed (Tominaga *et al*., 1998b). In this method, thiols were extracted from

dichloromethane using a pHMB solution in water which was loaded onto anion exchange resin. Once washed by acetate buffer solution, thiols were selectively released by a cysteine solution. The original aspect of this modified method was that the application of the resin allowed to eliminates compounds other than thiols in the aqueous phase of pHMB (Tominaga *et al*., 1998b), leading to a more clean chromatogram and higher sensitivity.

As a result of those improvements, the application of such method allowed to identify and quantify thiols in wines made from several *Vitis vinifera* grape varieties (Tominaga *et al*., 2000a) suggesting a key role of such components in the aroma of different white wines. Moreover, thiol molecules with a very low perception threshold as 2-Furanmethanethiol (Tominaga *et al*., 2000b) and Benzenemethanethiol (Tominaga *et al*., 2003a) were identified and quantified in wines. The identification of these two latter molecules, together with the identification of ethyl 3-mercaptopropionate, allowed to establish the role of certain volatile thiols in the bouquet of aged champagne wines (Tominaga *et al*., 2003b).

Although very efficient, this procedure is time consuming. An effort in reduce required time in sample preparation was the employment of covalent chromatography for the enrichment of the thiols from the wine extract. In particular, Affigel 501, a cross-linked agarose gel containing phenylmercurium chloride was used (Full *et al*., 1994). Such method extracted volatile thiols from wine using dichloromethane; then, the organic solvent was loaded on covalent chromatography gel. Finally, a dithiotreitol solution in dichloromethane was use elute thiols which were concentrated to a final concentration factor of 5000 employing 0.5 liters of wine (Schneider *et al*., 2003).

The ability of some common solid phase extraction sorbents to retain organomercuric salts for selective concentration of thiols in wines was also investigated (Mateo-Vivaracho *et al*., 2009). In particular styrene-divinylbenzene copolymer sorbents were used. Since these kind of sorbents can work both in aqueous and organic-hydrophobic media retaining their chromatographic and retention properties, this work allowed an improvement in thiol extraction system (Mateo-Vivaracho *et al*., 2009). The organomercury salt (considered as hazardous poison) still remain the key point of this method.

Although described methods are powerful in obtaining purified extracts of thiol aroma compounds, their major drawback was the internal standard. In particular, a tertiary aliphatic thiol and ether was used: 4-methoxy-2-methyl-2mercaptobutane (Tominaga *et al*., 1998b). This compound was only partially functionally similar to the target compounds. As a result a non-

accurate quantification can be achieved, especially in the case of reactive compounds as in the case of thiols (Kotseridis *et al*., 2000, Hoffmann *et al*., 1996).

Indeed, 3-MH and 3-MHA are secondary thiols and, in addition, primary alcohol and acetate, respectively, while 4-MMP is also a ketone; as a result, their physicochemical properties can be different from those of the internal standard used. Development and Application of Stable Dilution Assay (SIDA) allowed an efficient quantification of voletile thiols in mass spectrometry (Schneider *et al*., 2003).

A faster method in volatile thiol determination was reached by derivatizing thiols directly in wine and purifying the adducts by anion exchange resins (Tominaga & Dubourdieu, 2006). In particular, 50 mL is enough to reach satisfactory sensitivity to quantify 2-methyl-3-furanthiol and 2-furanmethanethiol in wines (Tominaga & Dubourdieu, 2006).

3.3. Pentafluorobenzyl bromideas derivatizing agent in volatile thiol analysis

The key feature of pentafluorobenzyl bromide (PFBBr) employment in volatile thiols analysis is the transformation of mercaptans into their corresponding pentafluorobenzyl derivatives (Figure 2.1).

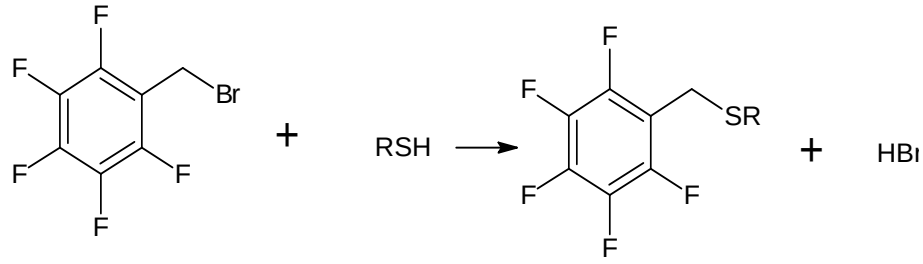


Figure 2.1. Reaction scheme between thiols and pentafluorobenzyl bromide (PFBBr).

The reaction is a nucleophilic substitution, which proceeds via the corresponding thiolate of the sulfydryl group. Similar reaction are used for the derivatization of fatty acids or phenols (Jia *et al*., 2003, Lerch & Zinn, 2003).

As derivatized products, thiol-conjugate extraction would become easier. As thiols are quite stable, considering the short period of the derivatization, required reactions are usually carried out in aqueous media using quite energetic conditions (high concentration of alkali, high temperature). Such condition cannot be used directly in wines, since in these conditions phenols would react with mercaptans leading to their oxidation and/or degradation processes. A selective purification of thiols, prior the derivatization step, is then required. Despite this, the most important features in reaching high sensitivity are the analytical properties of pentafluoro-compounds. In fact, by using pentafluorobenzyl bromide as derivatisation reagent, the derivatives formed show excellent electron-capturing properties. These properties can be then used by means of negative ions chemical ionization mass spectrometry (NCI-MS) (Mateo-Vivaracho *et al*., 2006). NCI shares with the electron-capture detection a selective and sensitive response to electrophilic atoms (halogens), as fluorine is.

The first application of such derivatising agent was carried out by on-fibre derivatisation of volatile thiols (Mateo-vivaracho *et al*., 2006). The main purpose of this work was to develop a simultaneous extraction and derivatisation procedure. Among pentafluorobenzyl derivatizing

agent, in fact, the employment of o-2,3,4,5,6-(pentafluorobenzyl)hydroxylamine hydrochloride allowed to determine low-molecular mass aldehyde by in-fibre derivatization (Wang *et al*., 2005).

The proposed method allowed to determine 3-MH, 3-MHA and 4-MMP in wines. Despite this, the method failed at determining 4-MMP and 3-MH at low levels (Mateo-Vivaracho *et al*., 2006). The amount of fragmentation in mass spectrometry was identified as the major reason of this result.

Stating that higer amount of analytes were necessary to reach enough sensitivity, a different kind of analytes purification was employed. In particular styrene- divinylbenzene copolymer sorbents (Lichrolut EN) were tested in their property to retain thiols of interest, which were derivatized in the solvent used as eluent (Mateo-Vivaracho *et al*., 2007). As solvent used as eluent could affect both the derivatsation reaction and the recovery of analytes, hexane, ether, ethyl acetate toluene, benzene, methylisobutylketone, tetrahydrofurane were tested. In the end benzene was chosen as solvent. Moreover, the presence of alkali was required to achieve good yields (Mateo-Vivaracho *et al*., 2007). The use of a carcinogenic solvent, which is forbidden in numerous laboratory, was the main limit of such method, despite it resolved some of the limitations of previous procedure.

The possibility to carry out the derivatization reaction in a solid-phase extraction sorbent was then evaluated (Mateo-Vivaracho *et al*., 2008). The formation of derivatives in a solid phase extraction cartridge was influenced by the kind of sorbent used. Despite this, such approach allowed to determine volatile thiols in wine (Mateo-Vivaracho *et al*., 2007). Moreover, a lower pentafluorobenzyl alkylation of 4-MMP was noticed and solved by carrying out an oximation of such compound prior to derivatizing step (Mateo-Vivaracho *et al*., 2008).

Although the extraction pentafluorobenzyl bromide derivatives by head space solid phase extraction was evaluated too (Ròdriguez-Bencomo *et al*., 2009), on cartridge derivatization of thiols and liquid injection is the most widespread method to determine 3-MH, 3-MHA and 4-MMP by means of pentafluorobenzyl bromide (Mateo-Vivaracho *et al*., 2010).

Since negative chemical ionization is not widespread in analytical laboratories, studies concerning electron ionization mass spectrometry were carried out. The developed method allowed to determine 3-MH in wines with a limit of quantification of 40 ng L^{-1} (Capone *et al*., 2011). In particular, a solvent extraction was followed by a second extraction in aqueous basic media where derivatisation step was carried out. Derivatised thiol are then extracted by

headspace solidphase microextraction. The presence of an aromatic ring allows both to modify thiols physical properties in purification steps and higher sensitivity in mass spectrometry.

Aims

The analytical methods that employ PFBBr to derivatize thiols, are usually coupled to negative chemical ionization mass spectrometry. Despite this, electron impact mass spectrometry has been reported to be a suitable tool to determine derivatized product. Thus, the aim of this experimental session was to evaluate if electron impact mass spectrometry can be looked as a suitable tool for derivatized thiol product using PFBBr as derivatizing agent in both organic solvent and aqueous media. In this stage thiols (3-MH), (3-MHA), (4-MMP), 1-heptanthiol (1-HEPT), 6-mercaptohexan-1-ol (6-MH) and 3-mercapto-2-butanol (3M2B)) leading or not to wine are used. These latter molecules are used because of their polarity, which could be useful during extraction studies. In fact, 1-HEPT is lower in polarity, while 3M2B is higher in comparison to wine occurring thiol molecules. Two different concentration in thiols were tested in order to identify, together with mass spectra, peaks of interest.

Materials and methods

3M2B (> 97%, sum of isomers) and 6-MH were purchased from SAFC, 4-MMP (98%), was purchased from AK Scientific, 3-MH was purchased from AlfaAesar (96%), 3-MHA from Endeavour (98%) and 1-HEPT from Sigma-Aldrich.

Mercaptoglycerol (> 98%), 1,8-diazabicyclo[5,4,0]undec-7-ene (DBU), and 2,3,4,5,6-pentafluorobenzyl bromide (PFBBr) were obtained from Sigma-Aldrich (St. Louis, MO). Dichloromethane, hexane and methanol were obtained from Panreac.

Derivatisation procedure was applied as reported by Mateo-Vivaracho et al., 2007 with minor modifications. Nine hundred microliter of thiol standard in organic solvent was transferred to a clean and dry 3 mL screw-capped vial. Then, 50 μL of the reagent solution (2 g L^{-1} PFBBr in methanol) and 50 μL of the alkali solution (20% DBU in methanol) were added. The reaction was left for 40 min at room temperature. Excess of reagent was then removed by adding 1 mL of a 2 g L^{-1} solution of mercaptoglycerol in 6.7% DBU acqueous solution, and letting the reaction 20 min at room temperature. After this time, the organic phase is washed twice with 1 mL of brine. The organic phase is finally transferred to a 2 mL autosampler vial and spiked with a small amount of anhydrous sodium sulfate. 1 μL of this sample is then injected into the

GC-EI-MS system. Trial were carried out in duplicate. Two organic solvent were used: hexane and dichlorometane.

GC-EI-MS analysis

Agilent 6890N gas chromatograph (Santa Clara, CA) equipped with 5975 mass selective detector was used. The inlet temperature was held at 240°C, 1 µL was manually injected in splitless mode, and delivered onto a HP-Innowax capillary column (30 m x 0.250 mm ID, 0.25 µm film thickness) using helium as carrier gas at a constant rate of 1 mL min^{-1}. The initial temperature of the column was 48 °C for 5 min. the column was then heated to 240 °C at 6 °C min^{-1}; remaining at that temperature for 10 min. The temperature of the interface line is set to 250 °C. The ion source, operating in electron impact mode at 70 eV, was held at 250 °C. The quadrupole temperature is set at 150 °C. SCAN mode (40 ÷ 400 m/z) is used.

Results and discussion

In derivatizing reaction at least 40 equivalents in PFBBr were used. In particular, 384 µmol PFBBr were used during this reaction. As reported in table 2.1, derivatising agent exceeds the thiol groups.

thiol	amount (g) I	mol I	eq of PFBBr I	amount (g) II	mol II	eq of PFBBr II
1HEPT	$0.892\ 10^{-3}$	$6.75\ 10^{-6}$	56.89	$0.445\ 10^{-3}$	$3.38\ 10^{-6}$	113.78
3M2B	$0.960\ 10^{-3}$	$9.06\ 10^{-6}$	42.38	$0.480\ 10^{-3}$	$4.53\ 10^{-6}$	84.76
4MMP	$1.120\ 10^{-3}$	$8.48\ 10^{-6}$	45.28	$0.560\ 10^{-3}$	$4.24\ 10^{-6}$	90.56
3MHA	$0.963\ 10^{-3}$	$5.47\ 10^{-6}$	70.20	$0.4815\ 10^{-3}$	$2.74\ 10^{-6}$	140.40
3MH	$1.008\ 10^{-3}$	$7.52\ 10^{-6}$	51.06	$0.504\ 10^{-3}$	$3.76\ 10^{-6}$	102.12
6MH	$1.010\ 10^{-3}$	$7.53\ 10^{-6}$	50.99	$0.505\ 10^{-3}$	$3.76\ 10^{-7}$	101.98

Table 2.1 . Amount, moles and equivalent in derivatising agent of volatile thiols used.

Under these conditions, the derivatized products were identified by GC EI mass spectrometry. Moreover, no remaining thiol was present after the derivatizing reaction.

The amount of fragmentation did not allow to identify the molecular ion. Only in the case of 3M2B it was possible to identify such ion. Despite this, the existence of fragmentation allowed to identify analytes and choose ions working in single ion monitoring mode (SIM). At least two

ions are required: one quantifying ion (the most abundant and selective) and one qualifying ion (the next most abundant).

In the case of 1-heptanethiol alkyl derivatives, molecular ion (m/z 312) could not be identified. Most abundant and characterizing ions are m/z 181, corresponding to the thioalkyl loss, and 131, which correspond to the pentafluorobenzyl loss. Ion m/z 71 (-241) and m/z 97 (-215) were identified, as well (figure 2.2).

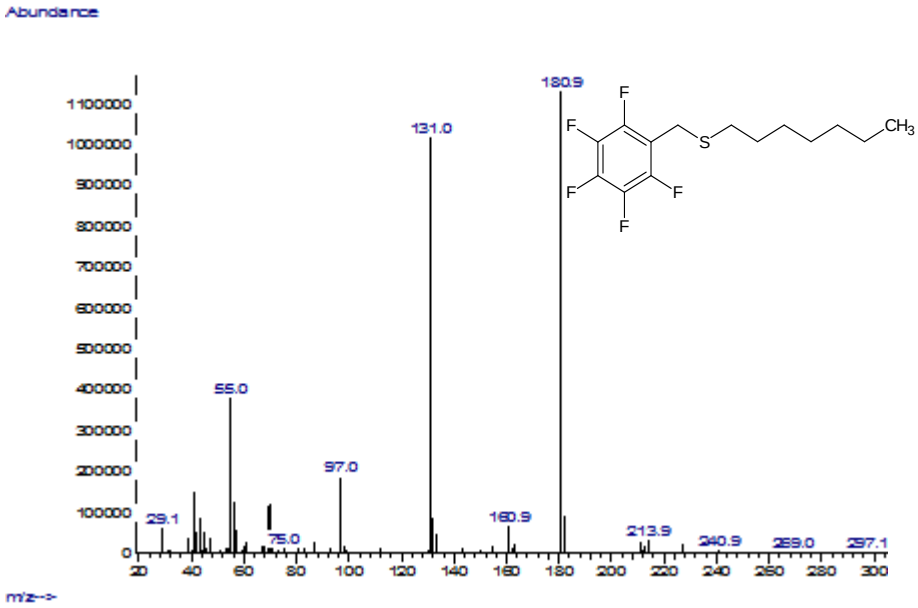


Figure 2.2 . EI positive mode MS spectra of PFB-1HEPT, obtained from derivatization with PFBBr of standard solution of 1-HEPT in hexane.

The most hydrophilic molecule used in this section, 3M2B, was identified as sum of isomers. As a result, two peaks were identified even if a non-chiral column was used during chromatographic separation (figure 2.3). Both recognized peaks showed characteristic fragmentation pattern as shown in figure 2.4 and 2.5.

The most abundant ion was ion m/z 181, corresponding to the thioalkyl loss. Among characteristic ions, ion 286 was chosen as identifier, since it is the molecular ion. Ions m/z 105 (-181) and m/z 61 (-225) were identified as identifier ions.

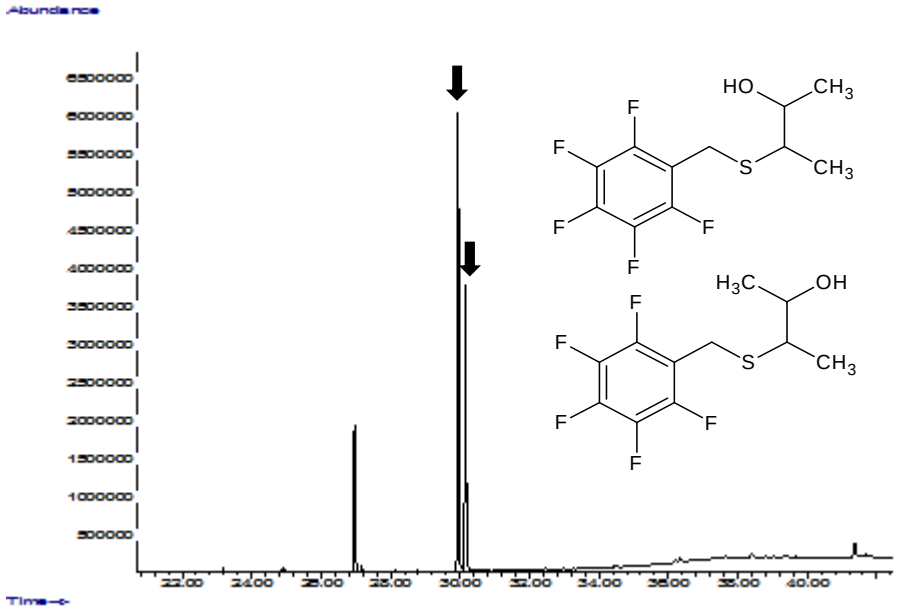


Figure 2.3. GC separation of PFB-3M2B, obtained from derivatization with PFBBr of standard solution of 3M2B in hexane.

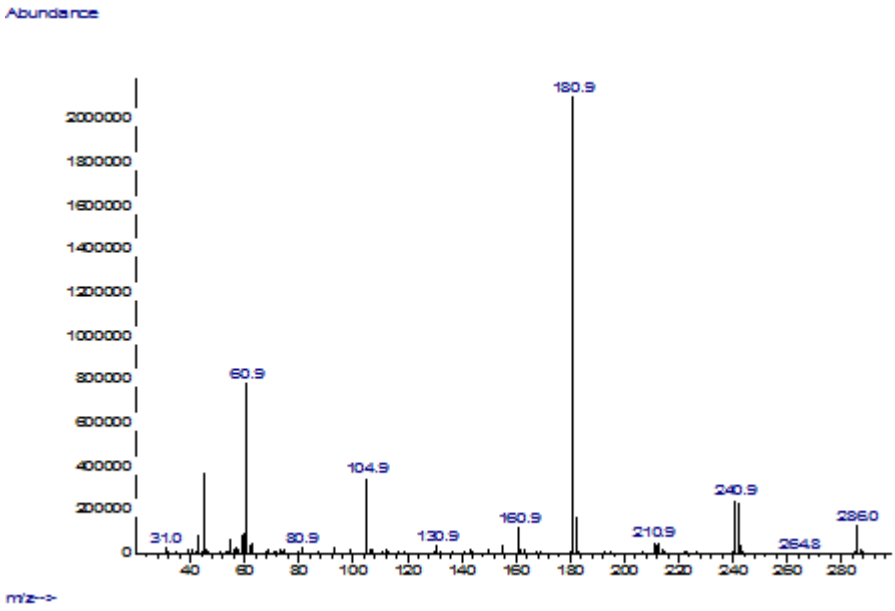


Figure 2.4 . EI positive mode MS spectra of PFB-3M2B, isomer 1.

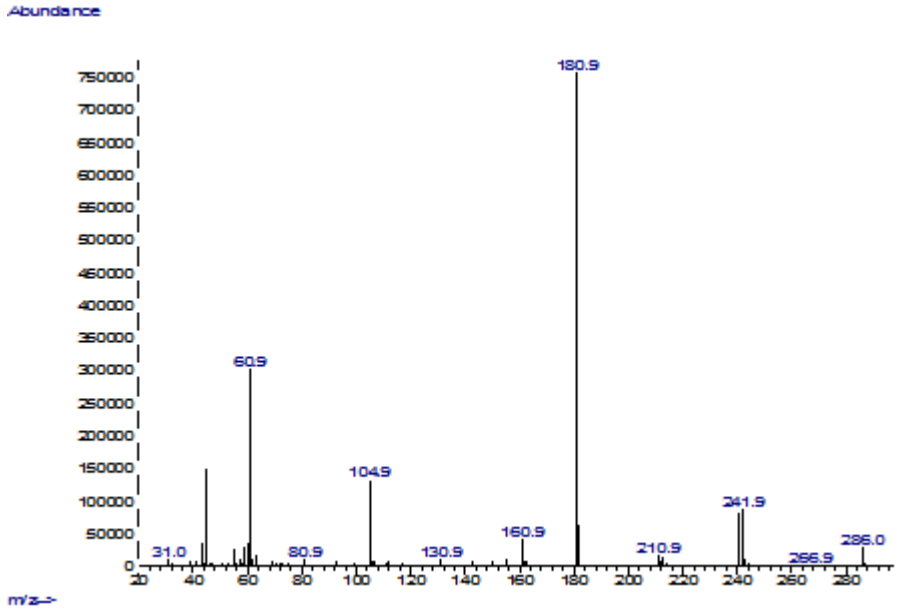
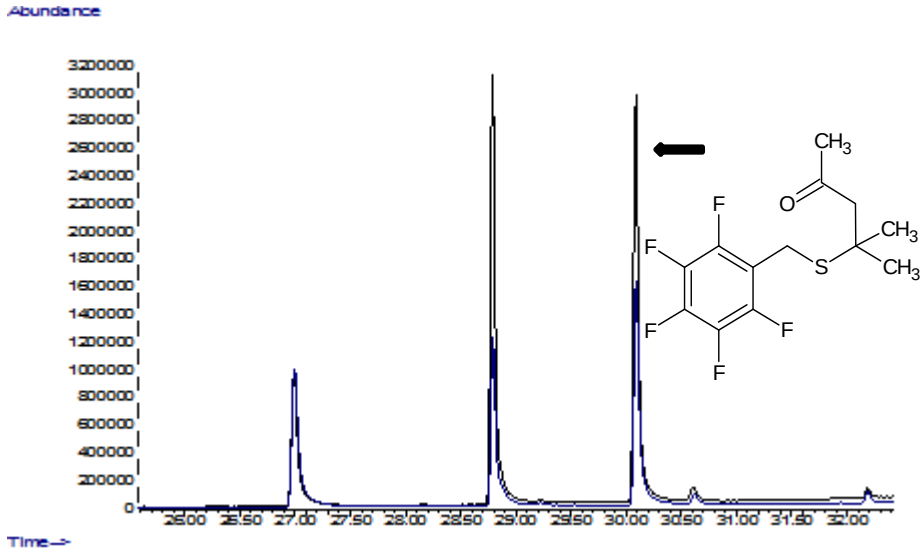


Figure 2.5. EI positive mode MS spectra of PFB-3M2B, isomer 2.

Derivatisation of 4-MMP by using PFBBr showed surprisingly two different peaks related to derivatization procedure (fig 2.6). The area of those peaks was related to thiol concentration. Despite this, only peak at 30 min showed a fragmentation pattern which could be related to 4MMP derivative product (figure 2.7). Also in this case, although the amount of fragmentation did not allow to identify molecular ions (m/z 312), m/z 255 (-57), 181 (-131), 131 (-181), 99 (-213) allows to identify the derivative product as reported in figure 2.7. It was assumed that the other peak was related to byproducts of derivatization reaction caused by impurities in the used standard.



Figue 2.6. GC separation of PFB-4MMP, obtained from derivatization with PFBBr of standard solution of 4MMP in hexane.

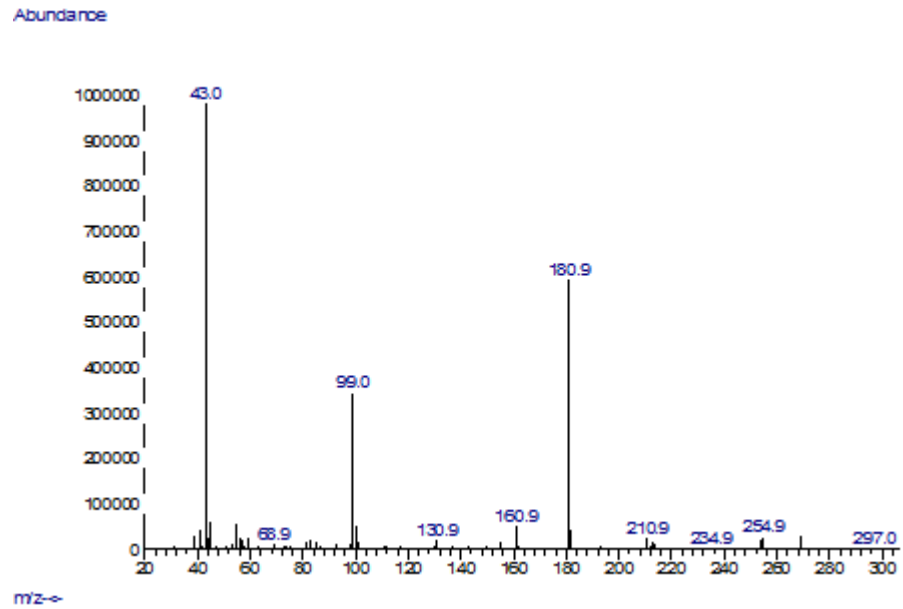


Figure 2.7. EI positive mode MS spectra of PFB-4MMP.

Although molecular ion of 3-mercaptohexyl acetate derivative product was not identified under experimental conditions, fragmentation pattern allowed peak identification. In particular,

fragments m/z 253, 181, 161, 115 and 83 are recognized as characteristics (figure 2.8). The most abuntant ion was ion m/z 115 which is also identified; ion m/z 181 is also useful for identification in single ion monitoring acquisitions.

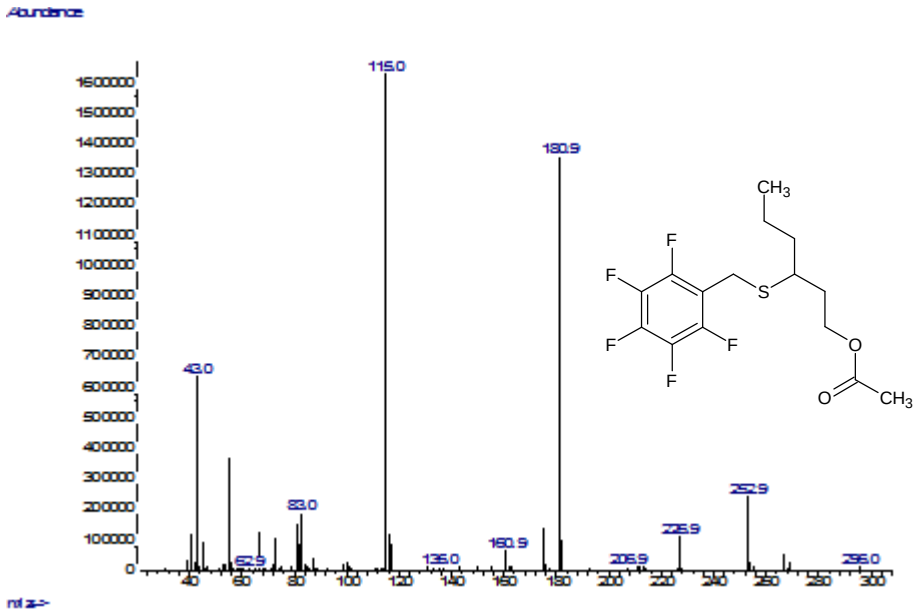


Figure 2.8. EI positive mode MS spectra of PFB-3MHA obtained from derivatization with PFBBr of standard solution of 3MHA in hexane.

Also in the case of 3-mercaptohexanol alkyl derivatives, molecular ion cannot be identified (m/z 314). Despite this, fragmentation patterns identify this molecules (figure 2.9). In particular, the most abundant ion was m/z 181, corresponding to the thioalkyl loss and m/z 133, which corresponded to the loss of the pentafluorobenzyl ring loss. Fragments m/z 100, 82 and 55 are related to 3-MH fragmentation.

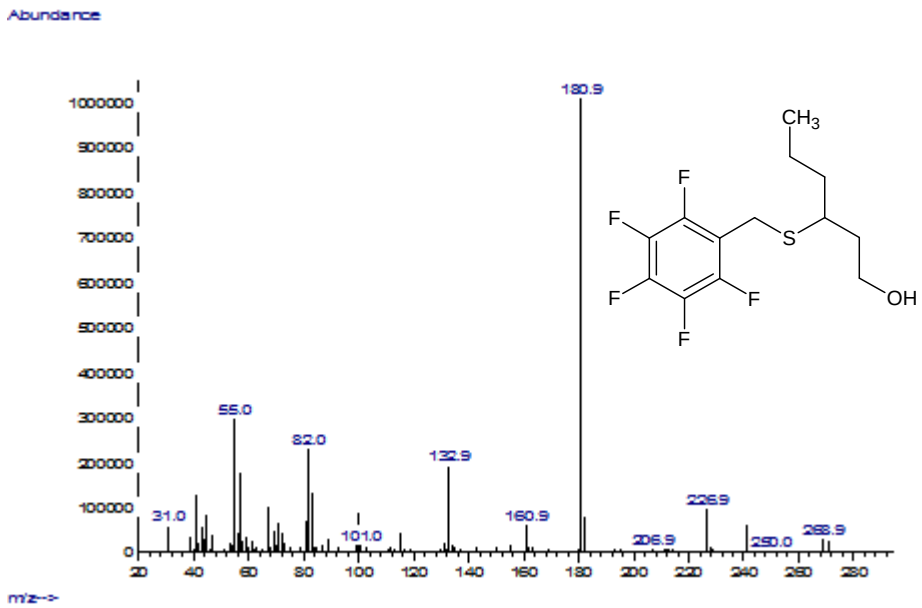


Figure 2.9 . EI positive mode MS spectra of PFB-3MH, obtained from derivatization with PFBBr of standard solution of 3-MH in hexane.

Derivative product of the last thiol employed, 6-MH, was characterized by a molar mass of 314. Amount of fragmentation did not allow to identify molecular ion. Despite this, fragmentation pattern allowed to identify the derivative product between PFBBr and 6MH (figure 2.10). In particular m/z 181 correspond to the loss of thiol alky chain, whereas m/z 133 corresponds to pentafluorobenzyl loss. Ions m/z 115 and 81 were recognized to be part of fragmentation pattern of such molecules.

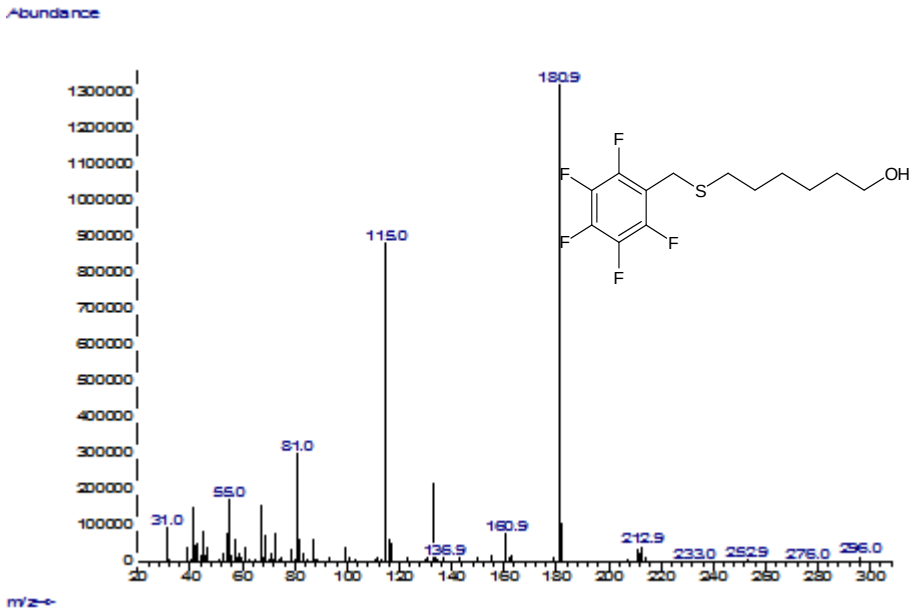


Figure 2.10. EI positive mode MS spectra of PFB-6MH, obtained from derivatization with PFBBr of standard solution of 6MH in hexane.

Due to the interpretation of the obtained mass spectra data, the quantifier and identifier ions for single ion monitoring acquisition were chosen as reported in table 2.2. In almost every derivatized thiols, ion m/z 181 was the most abundant in fragmentation pattern.

thiols	retention time (min)	Quantitation ion	Qualifier ions
PFB-1HEPT	26.62	181	131; 97
PFB-3M2B	29.93; 30.13	181	286; 105; 61
PFB-4MMP	30.08	181	131; 99
PFB-3MHA	32.21	115	181; 83
PFB-3MH	36.64	181	133; 100
PFB-6MH	37.38	181	133; 115

Table 2.2. Retention times and ions of quantification and qualification of the derivatized mercaptans analyzed by Positive EI mass spectrometry.

Since different organic solvents are reported in literature in order to purify thiols during this trial two solvents, characterized by different polarity, were tested. Evaluation of the influence of the relative yield of the derivatization, expressed as the percentage of the maxima, suggested that derivatization yield is very similar in both the tested organic solvents (figure 2.11).

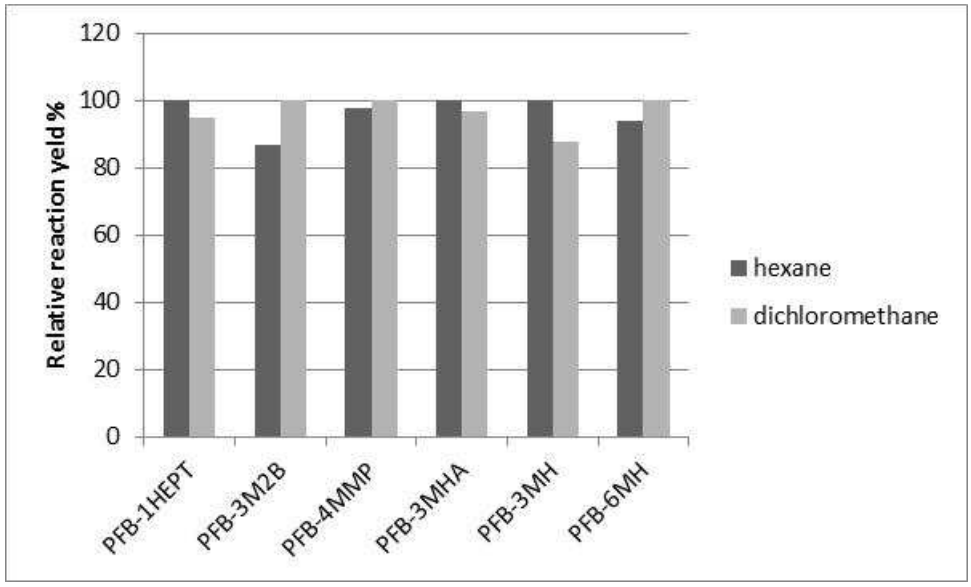


Figure 2.11. Influence of the solvent on the relative yield of the derivatization (expressed as percentage of the maxima).

Even if Pentafluorobenzyl bromide has been studied as derivatizing agent in organic solvent, it has been reported to be reactive in water in the presence of alkali; once the derivatives are formed theycan be extracted from aqueous media by HS-SPME (Capone et al., 2011). Moreover, the employment of both organic solvents and aqueous media is a useful tool to purify and determine 3-MH in wines (Capone et al., 2011). Thus, derivatization process was also tested in acqueous media. In this purpose the derivatives were extracted by using solid phase extraction. In particular, C_{18} (500 mg, 3 mL volume, Waters) and polymeric sorbents (500 mg, 3 mL volume StrataX polymeric reversed phase, Phenomenex) were used.

Derivatization procedure in aqueous media

Derivatisation procedures was carried out as follows: thiol standards (9.06 μmol 3M2B, 7.53 μmol 6-MH, 8.48 μmol 4-MMP, 5.47 μmol 3-MHA and 7.52 μmol 3-MH) were prepared in 10 mL NaOH 1 N. Then, 100 μL of the reagent solution (PFBBr 1 g L^{-1} in methanol, 384 μmol) were added. The reaction was left for 40 min at room temperature. The exceeding PFBBr was removed by adding 2 mL of a 2 g L^{-1} water solution of mercaptoglycerol, then leaving the mixture 20 min at room temperature. The latter was acidified to pH 3.0 by using a tartaric acid solution 0.75 M. Aqueous solution was then loaded onto a solid phase extraction, C_{18} and

polymeric (SDVB) sorbents were used previously conditioned using 5 mL dichloromethane, followed by 5 mL of methanol and 5 mL of milliQ water. Derivatised thiols were then eluted by using 10mL dichloromethane. The organic phase was then dried using anhydrous sodium sulfate. One microliter of this sample was then injected into the GC-EI-MS system. Peak areas were compared with derivatised product prepared as follows: 9.06 μmol 3M2B and 7.53 μmol 6-MH were dissolved in 8.9 mL hexane. Then, 100 μL of the reagent solution (PFBBr 1 g L^{-1} in methanol, 384 μmol) and 1 mL of the alkali solution (20% DBU in methanol) were added (10 mL final volume). The reaction was left for 40 min at room temperature. Excess of reagent was removed by adding 2 mL of a 2 g L^{-1} solution of mercaptoglycerol in 6.7% DBU acqueous solution, and letting the reaction 20 min at room temperature. After this time the organic phase was washed twice with an equal volume of brine. The organic phase was then dried using anhydrous sodium sulfate. One microliter of this sample was then injected into the GC-EI-MS system Trial were carried out in duplicate.

Results and discussion

Results suggest that derivatisation in hexane and in water solution are comparable in yield. In fact, peak areas were similar. Moreover both C_{18} and polymeric sorbents are able to retain pentafluorobenzyl derivative products of 3M2B and 6-MH, which can be eluted by using dichloromethane as shown in figure 2.12. Derivatization in aqueous media and purification by solid phase extraction is a suitable tool for these compounds.

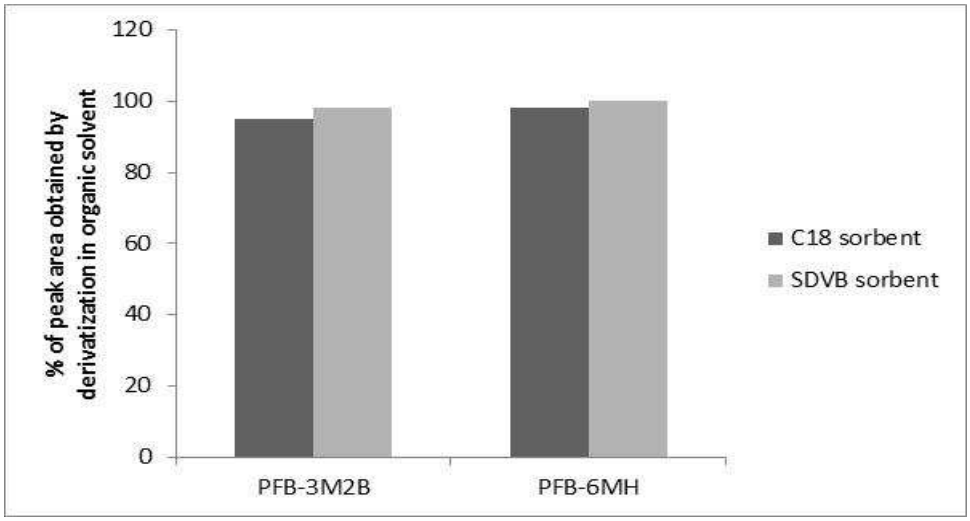


Figure 2.12. Recovery of pentafluorobenzyl derivatives of thiols on different resins, expressed as percent of peak area obtained by derivatization of equal amount in organic solvent.

Although thiol derivatisation can be carried out in aqueous media under basic condition, required high pH were responsible for 3-MHA hydrolysis. As shown in figure 2.13, coeluting peaks were obtained in both 3-MH and 3-MHA derivatives if reaction was carried out in sodium hydroxide. Retention times were characteristics for derivatized product between 3-MH and pentafluorobenzylbromide. Moreover, no peak was obtained for 3-MHA derivative product. Fragmentation pattern (Figures 2.14 and 2.15) confirm that in both cases the product obtained was the derivatized 3-MH. This result confirmed that high pH determined the total hydrolysis of 3-MHA into 3-MH during drivatization step.

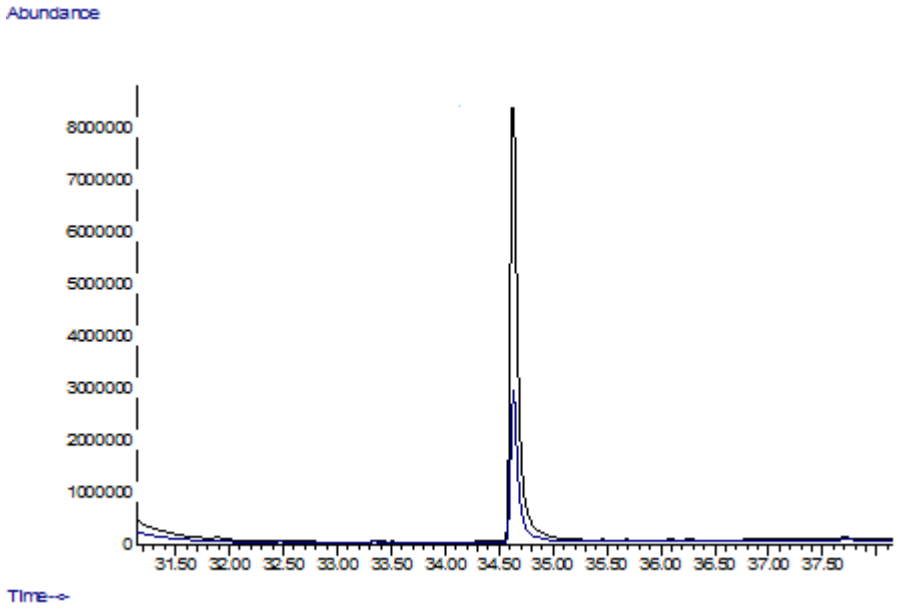


Figure 2.13. GC separation of PFB-3MH and PFB-3MHA, obtained from derivatization with PFBBr of standard solution of 3-MH and 3-MHA in NaOH 1 N

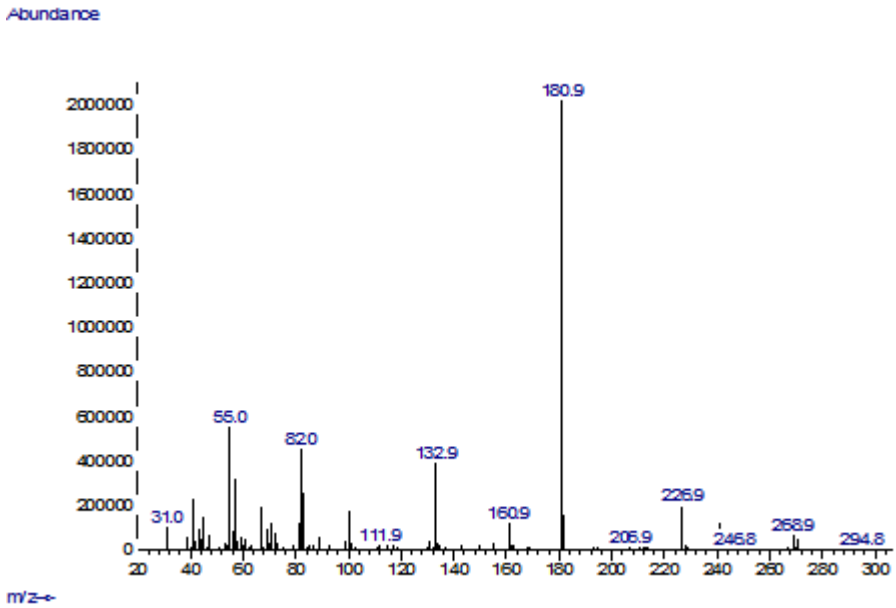


Figure 2.14. EI positive mode MS spectra of PFB-3MH obtained from derivatization with PFBBr of standard solution of 3-MH in NaOH 1 N.

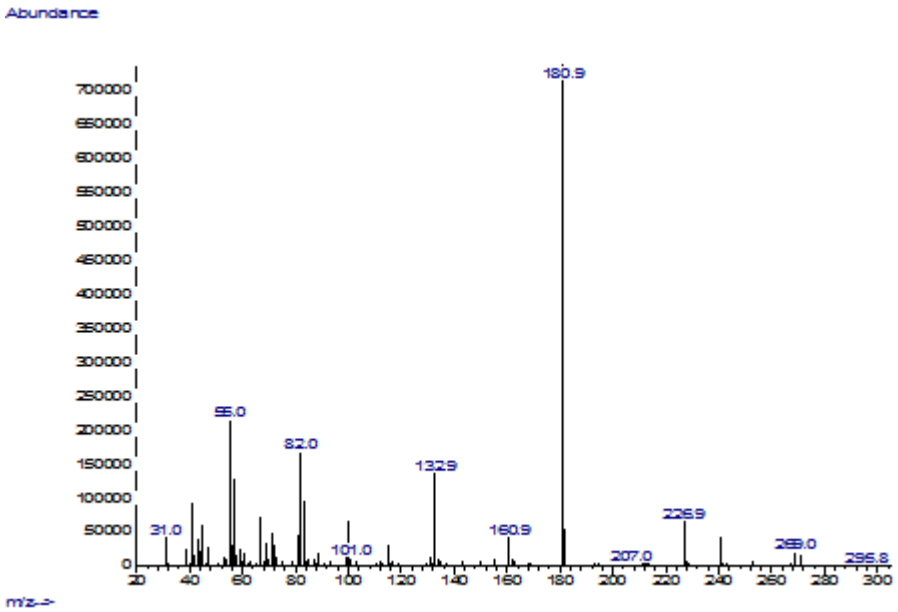


Figure 2.15. EI positive mode MS spectra of PFB-3MHA obtained from derivatization with PFBBr of standard solution of 3-MHA in NaOH 1 N.

In conclusion it was noted that identification of derivative products between volatile thiols and PFBBr using electron impact mass spectrometry as detector was possible. Although the amount in fragmentation did not allow to identify molecular ion for derivative products, they were identified by their fragmentation pattern. Moreover ions for single ion monitoring in mass spectrometry were chosen.

Among organic solvent used, reaction was possible in similar yield in both hexane and dichloromethane.

Derivatization can be carried out in aqueous media too. Moreover, derivative products can be purified by solid phase extraction by using both C_{18} and styrene divinylbenzene sorbents. Despite this, high concentration in alkali is required during derivative procedure. In such conditions hydrolysis of 3-MHA was observed, thus suggesting that this derivative conditions are not optimal for derivatization of thiols aroma in wines, where both 3-MHA and its hydrolysis product, 3-MH, are present.

As a consequence, derivatization in organic solvent was preferred. Extraction and purification of analytes can be carried out using various techniques. Among them, extraction of thiol aromas on solid phases has been reported (Mateo-Vivaracho et al., 2008). Thus, different polymeric sorbents will be evaluated in their ability to retain thiols from wine-like model system. Dichloromethane will be used as solvent since it was evaluated to be a suitable organic solvent where derivatization can be carried out.

Materials and methods

Polymeric reversed phase (strataX, 500 mg, 3 mL volume, Phenomenex) and styrene-divinylbenzene sorbents (Lichrolut EN, 40-120 μm, 500 mg, 3 mL volume, Merk) were used.

Ten milliliter of wine-like model solution (tartaric buffer 33 mM, pH 3.20, 12% v/v ethanol) containing 100 μg L^{-1} 1-HEPT, 100 μg L^{-1} 3M2B and 100 μg L^{-1} 6-MH were loaded onto spe cartridges previously conditioned using 10 mL of dichloromethane, followed by 10 mL of methanol and 10 mL water. Thiol compounds were eluted by passing 10 mL of dichloromethane. The organic phase was then dehydrated using anhydrous sodium sulfate and 1μL microliter of this sample was injected into the GC-EI-MS system. Recovery of such extraction method was calculated in comparison to a solution 100 μg L^{-1} 1-HEPT, 100 μg L^{-1} 3M2B and 100 μg L^{-1} 6-MH prepred in 10 mL dichloromethane.

Results and discussion

1-heptanthiol (1-HEPT), 6-mercapto-1-hexanol (6-MH) and 3-mercapto-2-butanol (3M2B) were chosen to assess the recovery of thiols on polymeric sorbents because of their different polarity properties being 3M2B highly polar, whereas 1-HEPT shows low polarity. Together with 6-MH these three thiol compounds represent a quite large range of polarity of thiols in wine.

As shown in figure 2.16 styrene-divinylbenzene copolymer sorbents (Lichrolut EN) and polymeric sorbents Strata X were suitable tool to retain thiols with different polarity. In fact, recovery of such compounds were high and close to 100% for both used cartridges using dichloromethane as eluent.

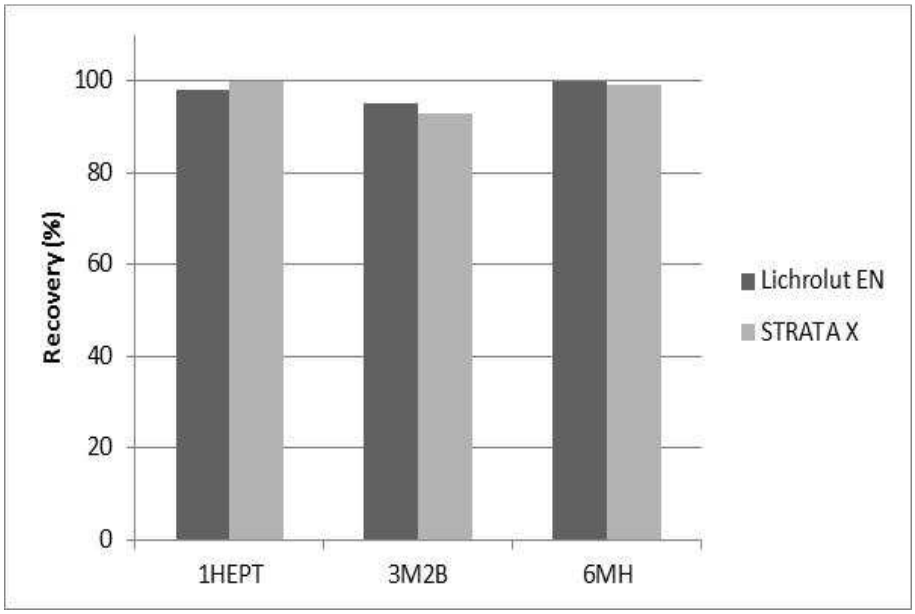


Figure 2.16. Recovery of volatile thiols on different polymeric sorbents.

Once aromatic thiols can be extracted and purified from wine-like model system by solid phase extraction and eluted using dichloromethane as organic solvent, they can be derivatized both on SPE cartridge (Mateo-Vivaracho *et al*., 2008) or in the organic solvent (Mateo-Vivaracho *et al*., 2007), when eluted. Moreover, dichloromethane has been demonstrated to be a suitable organic solvent for both elution of volatile thiols and derivatiation procedure.

Because of this, both methods (on cartridge and derivatization of thiols in organic solvent used as eluent) will be evaluated to determine thiols.

Solid-phase extraction and in-sorbent pentafluorobenzyl alkylation of volatile thiols

Experimental procedure

Ten milliliters of wine-like model solution (tartaric buffer 33 mM, pH 3.20, 12% v/v ethanol) containing 89 μg L^{-1} 1-heptane thiol (1-HEPT), 112 μg L^{-1} 3-mercapto-2-butanol (3M2B) and 97 μg L^{-1} 6-mercaptohexan-1-ol (6-MH) were loaded onto SPE cartridges previously conditioned using 10 mL of dichloromethane, followed by 10 mL of methanol and 10 mL of water. Retained thiols were then derivatized by using PFBBr as described by Mateo-Vivaracho *et al*. (2008) with minor modifications. 3 mL of an aqueous solution of DBU (6.7%) are loaded onto the cartdridge, then 2 mL of 2 g L^{-1} solution of PFBBr in hexane was loaded onto the column and letting the cartdridge imbibited with the reagent for 30 min at room temperature. Excess of reagent was removed by adding 2 mL of 2 g L^{-1} solution of mercaptoglycerol in 6.7% DBU solution, and letting the cartridge again for 30 min at room temperature. Derivatized analytes were finally eluted with 10 mL of dichloromethane. The organic phase was then dehydrated using anhydrous sodium sulfate. One microliter of this sample is then injected into the GC-EI-MS system.

Peak areas were compared with derivatised product prepared as follows: 9.06 μmol 3M2B and 7.53 μmol 6-MH were prepared in 8.9 mL dichloromethane. Then, 100 μL of the reagent solution (PFBBr 1 g L^{-1} in methanol, 384 μmol) and 1mL of the alkali solution (20% DBU in methanol) were added to 8.9mL dichloromethane containing thiols. The final volume of the reaction mixture was 10 mL containing 89 μg L^{-1} 1-HEPT, 112 μg L^{-1} 3M2B and 97 μg L^{-1} 6-MH. The reaction was left for 40 min at room temperature. Excess of reagent was eliminated by adding 2 mL of a 2 g L^{-1} solution of mercaptoglycerol in 6.7% DBU acqueous solution, and letting the reaction 20 min at room temperature. After this time the organic phase is washed twice with an equal volume of brine. The organic phase was then dehydrated using anhydrous sodium sulfate. One microliter of the organic phase was then injected into the GC-EI-MS system. Results were expressed as percentage of the maxima obtained in organic solvent.

Results and discussion

As reported by Mateo-Vivaracho *et al.* (2008), the extraction and formation of derivatives in solid phase extraction cartridge by using a microporous structure (Lichrolut EN) was a failure. In fact, peak area were lower than 10% if compared to the derivatization carried out in organic solvent.

The employment of polymeric sorbents with a different structure (Strata X) allowed to obtain derivatives in higher yield. Despite this, high differences were present among replicates.

Moreover even if polymeric sorbent (Strata X) allowed higher derivatization yield, relative area were lower than 80%.

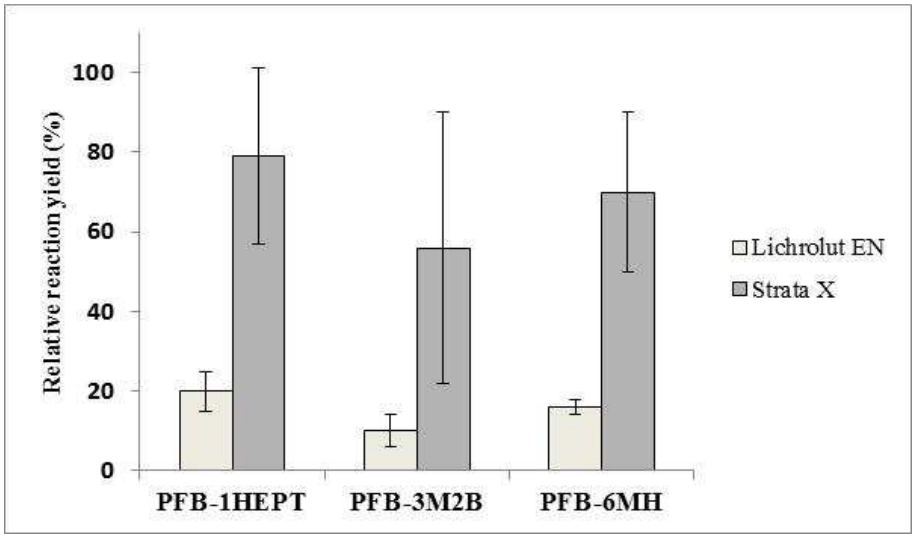


Figure 2.21. Relative yield of derivatization carried out on thiols (1-heptane thiol PFB-1HEPT, 3-mercapto-2-butanol 3M2B, 6-mercaptohexan-1-ol 6MH) extracted by polymeric sorbents. Results expressed as percentage of the maxima obtained in organic solvent.

Solid-phase extraction of volatile thiols and pentafluorobenzyl alkylation in elution organic solvent

Materials and methods

60mL of Sauvignon blanc wine, spiked using 12.6 μ gL^{-1} 3-MH, 15.2 μg L^{-1} 3-MHA, 9.5 μg L^{-1} 4-MMP and 11.4 μg L^{-1} 6-MH were loaded onto 1 g, 3 mL Lichrolut EN resin previously conditioned using 10 mL of dichloromethane, followed by 10 mL of methanol and 10 mL water. The cartridge was then rinsed by passing 10 mL phosphate buffer 20 mM, pH 7.0. Retained thiols were eluted by passing 10 mL of dichloromethane. Then, 100 μL of the reagent solution (PFBBr 1 g L^{-1} in methanol, 384 μmol) and 1 mL of the alkali solution (20% DBU in methanol) were added in order to derivatize thiols. The mixture was left 40 min at room temperature. Excess of reagent was removed by adding 2 mL of a 2 g L^{-1} solution of mercaptoglycerol in 6.7% DBU acqueous solution, and letting the reaction 20 min at room temperature. After this time the organic phase was washed twice with an equal volume of brine. The organic phase was then dehydrated using anhydrous sodium sulfate. Dichloromethane was then concentrated under gentle N_2 gas flow to ~ 100 μL. One microliter was then injected into the GC-EI-MS system. Spiked wine was evaluated in comparison to non-spiked wine.

Results and discussion

The amount of thiol aromas (above 10 μg L^{-1}) used to spike analysed wine is extremely high in comparison to the concentration naturally occurring in wines, which is lower than 100 ng L^{-1}. Despite this, the method employed did not allow to identify peaks related to derivatized thiol compounds in spiked wine (fig 2.22), even if single ion monitoring acquisition was used. The two overlapped chromatograms are acquired by following the only fragment ion m/z 181, which represent the most abundant ion in derivatized thiols fragmentation pattern in electron impact mass spectrometry.

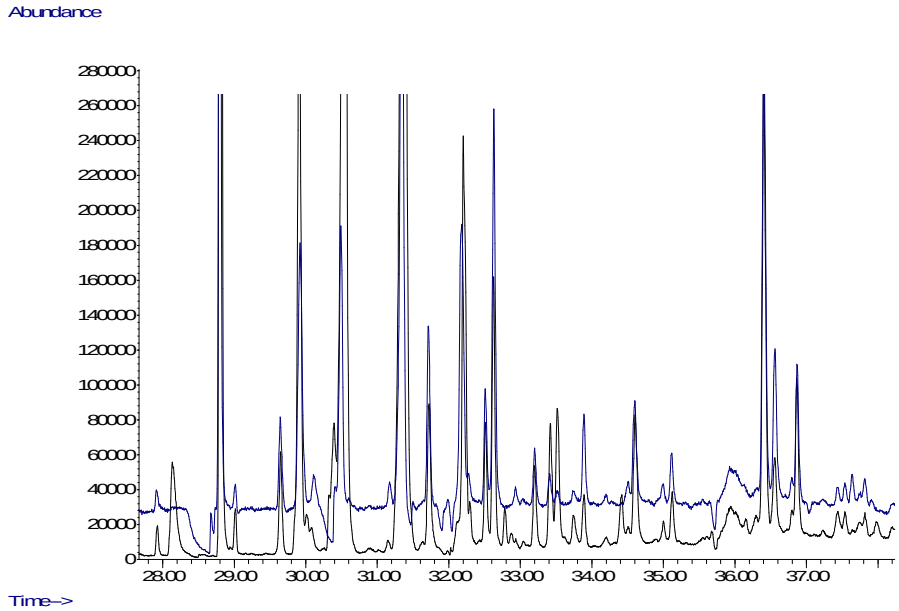


Figure 2.22. GC separation of wine (black line) and wine spiked with thiols (10 μg L^{-1} level) (blue line). Thiol extraction on polymeric sorbents and derivatization in organic solvent. SIM acquisition m/z 181.

Solid phase extraction can be used to extract thiols from wine-like model system, in particular copolymer sorbents were identified to be characterized by good recovery. Although in-sorbent derivatization by using PFBBr as derivatizing agent has been reported in literature, poor recovery and reproducibility was noted in experimental conditions applied.

Thiols extraction from wines using polymeric sorbents in solid phase extraction, and derivatization in organic solvent, did not allow to reach enough sensitivity, even if SIM mode was applied in GC-MS and final concentration factor was 500.

As a result, PFBBr is evaluated to a useful tool to derivatize thiol compounds in organic solvents. Despite this, the application of proposed method did not allow to identify volatile thiols in white wines.

Conclusions

Electron impact mass spectrometry is a suitable tool to determine derivative products between thiols and pentafluorobenzyl bromide. Derivatization process can be carried out in both organic solvent and aqueus media; despite this, presence of alkali is required. As a consequence, hydrolysis of 3MHA was noted in aqueous media, thus leading derivatization procedure in organic solvent the only suitable method.

Polymeric sorbents allowed good recovery of volatile thiol from wine-like model system. Despite this, in-sorbent derivatization procedure showed low derivatization yield and/ or poor recovery in derivatives. On the other hand, in organic solvent derivatization of thiol extracted with solid phase sorbents did not allow to determine volatile thiols in real matrices with enough sensitivity.

3.4. Ethyl propiolate as derivatizing agent in volatile thiol determination

Ethyl propiolate is a compound largely used in organic chemistry. Its reaction towards thiol function is due to a Michael-type addition and the reaction can be carried out also in water solution in good yields (Randive *et al*., 2010). This addition of thiols or amines to ynones and ynonates structures has been known for long time in organic chemistry (Xiao & Alper, 1997, Xu *et al*., 2008).

Among ynonates, ethyl propiolate has been studied for its reactivity in water with nucleophilic structures as thiols (Arcadi *et al*., 2009).

The reaction mechanism is based on the nucleophilic attack of the thiolate ion to the α-carbon atom to the triple bond generating a stable alkylthioacrylate compound (figure 2.23). The reaction rate depends on the dissociation of the thiol group which reacts as nucleophilic reagent. Since thiol group should be firstly deprotonized to act as nucleophile, generally ETP reacts under basic pH.

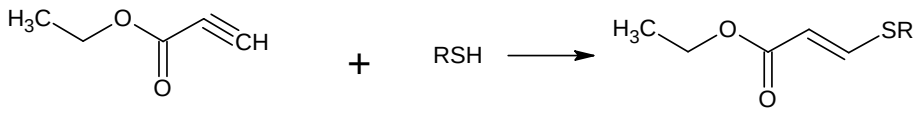


Figure 2.23. Reaction between ethyl propiolate and thiols.

The main advantages connected with this reaction are its rapidity, effectiveness and the generation of UV active species (Owen, 2008). In analytical chemistry, ethyl propiolate has been evaluated as derivatizing agent for thiol group for development of liquid chromatofgraphic method for the determination of glutathione (Zacharis *et al*., 2009, Tzanavaras *et al*., 2010, Zacharis *et al*., 2011).

Even though derivatisation of thiol species followed by detection via LC-UV, the detection limit reached without pre-concentration was not satisfactory and when a SPE step was preliminarily introduced, the presence of other species absorbing in the UV region made the identification of derivatised thiols impossible.

Chemicals and reagents

Ethyl propiolate was purchased from Sigma-Aldrich (St. Louis, MO), The sulfur compounds studied *i.e.* 3-mercaptohexan-1-ol (3-MH) was from Acros Organics, 3-mercaptohexyl acetate (3-MHA) was from Oxford Chemicals and 4-mercapto-4-methyl pentan-2- one was purchased from Aldrich. Inorganic compounds, methanol, dichloromethane and solid phase extraction resins were purchased from Sigma-Aldrich (Castle Hill, NSW, Australia)

Synthesis and identification of reference compounds

Synthesis of ethyl 3-((1'-hydroxyhexan-3'-yl)thio)acrylate. (3MH-ETP). To 0.1 g of ethyl propiolate (1mmol) methanol (0.5 mL) was added, followed by 50 mM phosphate buffer pH 9.0 (9.5 mL). 3-Mercaptohexan-1-ol (9.6 mg, 0.072 mmol) dissolved in 0.5 mL methanol was then added. The reaction mixture was stirred for 5 min at room temperature. Purification of the product was carried out by extracting the reaction crude twice with an equal volume of dichloromethane (10 mL). Organic layer was then dried with Na_2SO_4, and evaporated under reduced pressure (750 mbar, 45 °C) to obtain the product.

^{1}H NMR ($CDCl_3$): 0.93 (3H, t, *J* =7.2 Hz, 6'-CH_3); 1.29 (3H, t, *J* =7.0 Hz, C$\underline{H}_3CH_2O$); 1.40 – 1.67 (4H, m, 4'-CH_2 and 5'-CH_2); 1.78 – 2.05 (3H, m, 2'-CH_2 and OH); 3.00 – 3.07 (1H, m, 3'-H); 3.78 – 3.81 (2H, m, 1'-CH_2); 4.19 (2H, q, *J* = 7.0 Hz, OC$\underline{H}_2CH_3$); 5.84 (1H, d, *J* = 10.2 Hz, 2-H); 7.19 (1H, d, *J* = 10.2 Hz, 3 –H); 7.71 (0.1H, d, *J* = 15.4 Hz, (E)-3H). **^{13}C NMR ($CDCl_3$):** 13.7 ($\underline{C}H_3CH_2O$); 14.2 (C-6'); 19.8 (C-5'); 37.8, 38.1 (C-3', C-4'); 46.9 (C-2'); 59.5, 59.9 (C-1' and O$\underline{C}H_2CH_3$); 112.6 (C-2); 148.8 (C-3); 166.6 (C-1). **HRMS** (ESI +) found (MNa^+) 255.1034 $C_{11}H_{20}NaO_3S$, required 255.1025

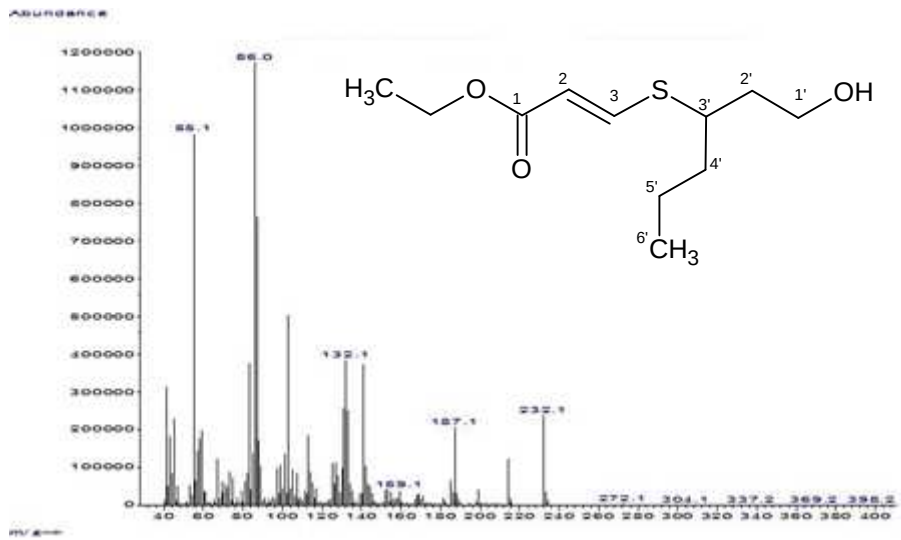


Figure 2.24. 3-((1'-hydroxyhexan-3'-yl)thio)acrylate (3MH-ETP) structure and characteristic spectrum (MS EI+).

Synthesis of ethyl 3-((1'-acetoxyhexan-3'-yl)thio)acrylate (3MHA-ETP) To 0.1 g of ethyl propiolate (1mmol), methanol (0.5 mL) was added, followed by 9.5 mL of 50 mM phosphate buffer pH 9.0. 3-Mercaptohexyl acetate (9.8 mg, 0.055 mmol) dissolved in 0.5 mL of methanol was then added. The reaction mixture was stirred for 5 min at room temperature. Purification of the product was carried out by extracting the reaction crude twice with an equal volume of dichloromethane (10 mL). Organic layer was then dried over Na_2SO_4, and evaporated under reduced pressure (750 mbar, 45°C) to obtain the product.

^{1}H NMR ($CDCl_3$): 0.93 (3H, t, *J* =7.12 Hz, 6'-CH_3); 1.29 (3H, t, *J* =7.2 Hz, $OCH_2C\underline{H}_3$); 1.41 – 1.70 (4H, m, 4'-CH_2 and 5'-CH_2); 1.86 – 2.05 (2H, m, 2'-CH_2); 2.06 (3H, s, CH_3CO); 2.89 – 2.91 (1H, m, 3'-H); 4.15 – 4.31 (4H, m, $OC\underline{H}_2CH_3$ and 1'-CH_2); 5.85 (1H, d, *J* = 10.1 Hz, 2-H); 7.10 (1H, d, *J* = 10.1 Hz, 3–H); 7.66 (0.1H, d, *J* = 15.3 Hz, (E)-3H). **^{13}C NMR ($CDCl_3$):** 13.7 ($OCH_2\underline{C}H_3$); 14.3 (C-6'); 19.9 (C-5'); 20.9 ($\underline{C}H_3CO$); 34.4 (C-3'); 37.6 (C-4'); 47.1 (C-2'); 60.2 (C-1'); 62.0 ($O\underline{C}H_2CH_3$); 113.2 (C-2); 148.0 (C-3); 166.6 (C-1); 120.8 ($\underline{C}OCH_3$). **HRMS** (ESI +) found (MNa^+) 297.1136, $C_{13}H_{22}NaO_4S$, required 297.1131.

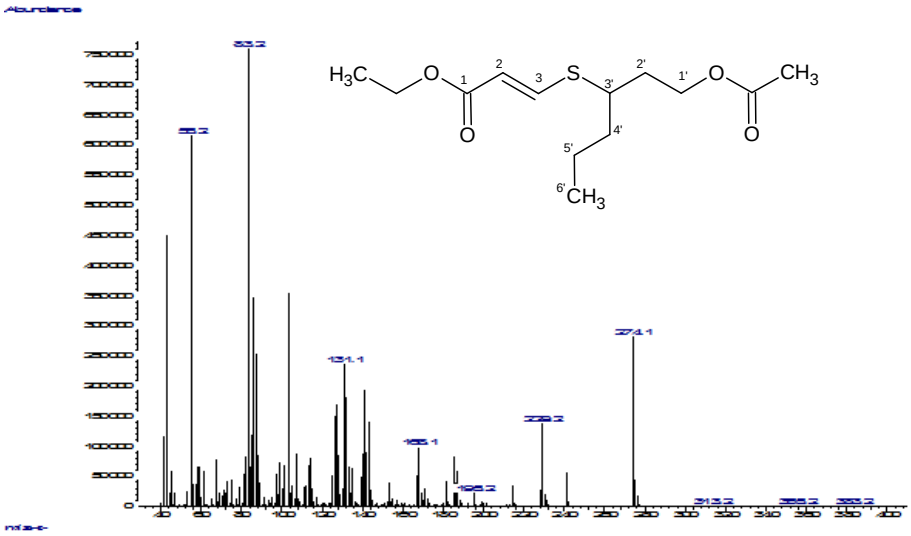


Figure 2.25. 3-((1'-acetoxyhexan-3'-yl)thio)acrylate (3MHA-ETP) structure and characteristic spectrum (MS EI+).

Synthesis of ethyl 3-((2'-methyl-4'-oxopentan-2'-yl)thio)acrylate (4MMP-ETP). 0.1 g of ethyl propiolate (1 mmol) were added with 0.5 mL methanol, and 9.5 mL of 50 mM phosphate buffer pH 9.0. 4-Mercapto-4-methyl pentan-2-one (11.2 mg, 0.085 mmol) dissolved in 0.5 mL methanol was then added. The reaction mixture was stirred for 5 min at room temperature. The mixture was loaded onto a 1200 HPLC (Agilent, Australia) equipped with a C_{18} semi-preparative column (Synergi Fusionn RP column, 15 cm x 1 cm, 4µm, Phenomenex, Australia) and coupled to a Diode Array Detector. Eluent A was 0.9% water solution of formic acid and eluent B was 100% acetonitrile. A 5 mL min^{-1} constant flow rate was applied. The eluting gradient was he compound was 5% to 80% in 16 min and 80% for 7 minutes as eluent B. Elution of target compound was monitored spectrophotometrically at λ 285 nm. Compound of interest was collected at 21.5 min elution. The eluted fraction was diluted with an equal volume of water and twice extracted with equal volumes of dichloromethane. The organic phase was then dehydrated with Na_2SO_4, and evaporated under reduced pressure (750 mbar, 45°C) to obtain the product.

^{1}H NMR ($CDCl_3$): 1.28 (3H, t, *J* =7.3 Hz, $OCH_2C\underline{H}_3$); 1.51 (6H, s, 2 x 1'-CH_3); 1.95-2.07 (2H, m, 3'-CH_2); 2.18 (3H, s, 5'-CH_3); 4.10 (2H, d, *J* =7.3 Hz, $OC\underline{H}_2CH_3$); 5.88 (1H, d, *J* = 10.4 Hz, 2–H) 7.32 (1H, d, *J* = 10.4 Hz, 3–H). **^{13}C NMR ($CDCl_3$):** 13.7 ($OCH_2\underline{C}H_3$); 27.5 (2 x

1'-CH_3); 29.1 (C-2'); 31.4 (C-5'); 44.8 (C-3'); 59.5 ($O\underline{C}H_2CH_3$);112.6 (C-2); 144.2 (C-3); 166.0 (C-1); 205.6.

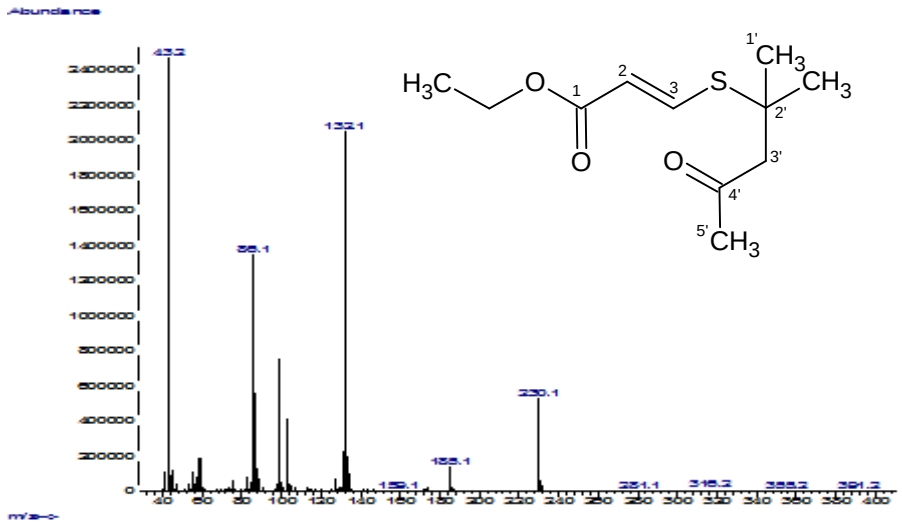


Figure 2.26. 3-((2'-methyl-4'-oxopentan-2'-yl)-thio)acrylate (4MMP-ETP) structure and characteristic spectrum (MS EI+).

Although formation of Z and E isomers were found during synthesis of reference compounds, Z to E ratio was at least 90:10.

Factors affecting thiols derivatization procedure with ethyl-propiolate

Although the reaction between ETP and volatile thiol is fast and selective, pH play a key role in derivatization reaction and product stability. On the one hand, the yield of this reaction is strongly affected by the presence of free ETP in the media and by the presence of the thiolate group acting as nucleophile. On the other hand, derivatization proceeds at mild basic pH, where hydrolysis of ester groups can occour (Makhotkina & Kilmartin, 2012).

Relative area of 3MH-ETP, 3MHA-ETP were then considered at different pH between 6.0 and 9.0. Neutral or mild basic conditions are required to carry out the derivatization procedure. In fact, no derivative product could be identified under mild acidic conditions (pH 6.0) (figure 2.27). Moreover, the higher was the pH, no decrease in 3MHA-ETP area was noticed, thus suggesting that no hydrolysis took place under the experimental conditions. On the other hand, 3MH-ETP and 3MHA-ETP showed a practically linear increase with the pH.

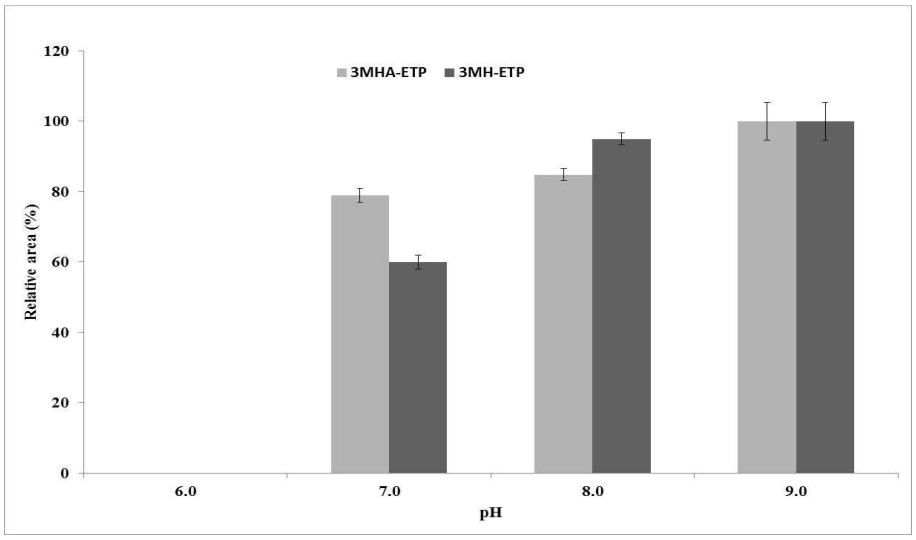


Figure 2.27. pH effect on derivatization yield between ethyl propiolate and 3-MH, 3-MHA.

As a result, the application of this novel derivatising agent and of a pre-concentration step allowed to identify 3MH and 3MHA in both wine and spiked wine at μgL^{-1} level (figure 2.28).

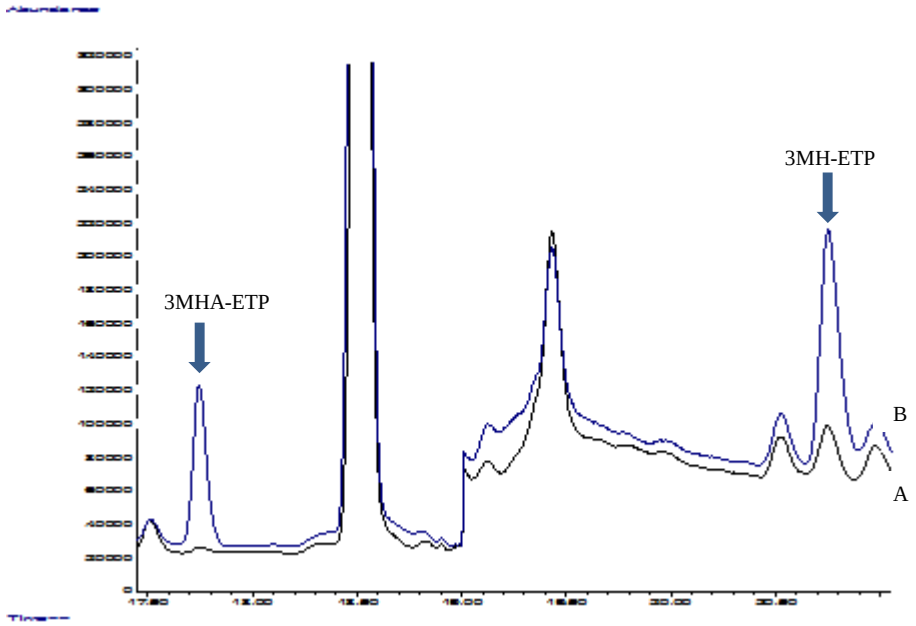


Figure 2.28. Identification of reference compound in wine (A) and wine containing 10 μg L^{-1} 3-MHA and 3-MH (B).

Conclusions

Michael type reaction between thiols and ethyl propiolate in water gives stable products. These products were identified by their NMR and mass spectra. Moreover, the fragmentation pattern allows to identify characteristic fragments thus increasing sensitivity. Neutral or mild basic pH is required to obtain derivatization. Despite this, hydrolysis of 3MHA does not occour. Reference compounds can be retained on solid phase cartridges. Derivatization and extraction procedure can be applied to real matrices too. As a consequence, volatile thiols can be identified at concentration close to their perception threshold in wines.

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4. Liquid chromatographic approaches in volatile thiols determination in wines

Aromatic thiol compounds involved in Sauvignon blanc aroma have boiling point higher than 90°C (Mestres *et al*., 2000). Therefore, the extraction of such compounds from wine matrix involving their volatility needs harsh conditions to reach enough sensitivity (Fedrizzi *et al*., 2008). This suggest that, although gaschromatography has been identified as excellent tool for analysis of wine aroma, in certain condition liquid chromatography could be more reliable to analyse thiol related aroma compounds.

Various analytical methods have been reported in literature to assess thiol containing molecules in grape juices and wines employing high-performance liquid chromatography. In particular, several methods have been carried out to determine glutathione in its reduced form. Among derivatizing agents, glutathione has been reported to react with p-benzoquinone, to introduce chromophores making detection of this thiol molecule by UV possible (Tirelli *et al*., 2010).

If fluorescence detector is employed, *o*-phthalaldehyde (Park *et al*., 2000, Janet *et al*., 2010), as well as 2,3-naphtalenedialdehyde (Marchand de Revel, 2010) can be used as derivatizing agent of glutathione. Capillary electrophoresis was used to separate reaction fluorescent adducts of glutathione with monobromobimane (Lavigne *et al*., 2007).

Since thiol aroma compounds are water soluble, the analytical approaches described above could be suitable tools to their evaluation. Despite this, thiol occurrence at trace concentration is the main obstacle to direct application of such derivatizing agents to wines.

4.1. *p*-benzoquinone as derivatizing agent in volatile thiol determination

Reaction between thiol compounds and *p*-benzoquinone (pBQ) has been reported. The mechanism of this reaction is a nucleophilic addition carried out by the thiol to an unsaturated carbonyl (figure 4.1) (Jocelyn, 1972).

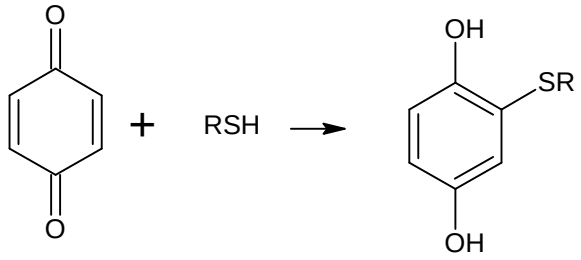

Figure 4.1 reaction of thiols with pBQ.

As extremely reactive compounds, thiols rapidly react with pBQ. The formation of thiol-substituted hydroquinones is fast and stechiometric. As a conseguence, sensitive and reliable quantification of glutathione and cysteine of yeast cell-wall fractions (Tirelli *et al*., 2010) as well as reduced glutathione in grape juice and white wine (Fracassetti *et al*., 2011) can be carried out. Derivative products are detectet at 303 nm.

The main advantages connected with this reaction are its rapidity, effectiveness and the generation of UV active species. Moreover, pBQ is a symmetric molecule and thiol addition makes molecules which are equal in their structure. As a consequence, a single product is obtained during derivatization reaction which means a single peak in chromatography.

Thanks to this, the employement of pBQ was evaluated to be a suitable tool to test in order to determine volatile thiols.

Materials and methods

3-mercaptopropionic acid (3MPA) and *p*-benzoquinone (pBQ) were purchased from Fluka (Swizerland). Trifluoroacetic acid (TFA) was purchased from Sigma-Aldrich (St. Louis, MO).

The derivatisation was conducted as described by Tirelli *et al*. (2010): 2mL of thiol standard in citate buffer 75 mM pH 5.0 were added with 100 μL of 43.2 mg L^{-1} pBQ and mixed for 1 min. One milliliter 53 mg L^{-1} MPA was added, in order to remove the the exceeding amount of pBQ. The reaction mixture was mixed again and then microfiltered (0.22 μm, PVDF, Millipore). The reversed phase (RP)-HPLC of thiol substituted hydroquinones and *p*-hydroquinone (pHQ) was

performed with a Water Alliance 2695 (Milliford MA) equipped with a photodiode array detector Waters 2996. The separation column was a hexyl-phenyl column, 250 mm x 4.6 mm, 5 µm, 110 Å (Phenomenex, Torrence, CA). Eluting solvents were water/trifluoroacetic acid (0.05% v/v) and methanol; the concentration of the latter increased from 30% to 80% in 15 minutes, to carry out the separation at 1.0 mL min^{-1} flow.

Results and discussion

As reported in figure 4.2 derivatisation of 3M2B, 3MH, 6MH and 3MHA using the proposed procedure was possible at mg L^{-1} level. Moreover, separation of derivatised products was possible.

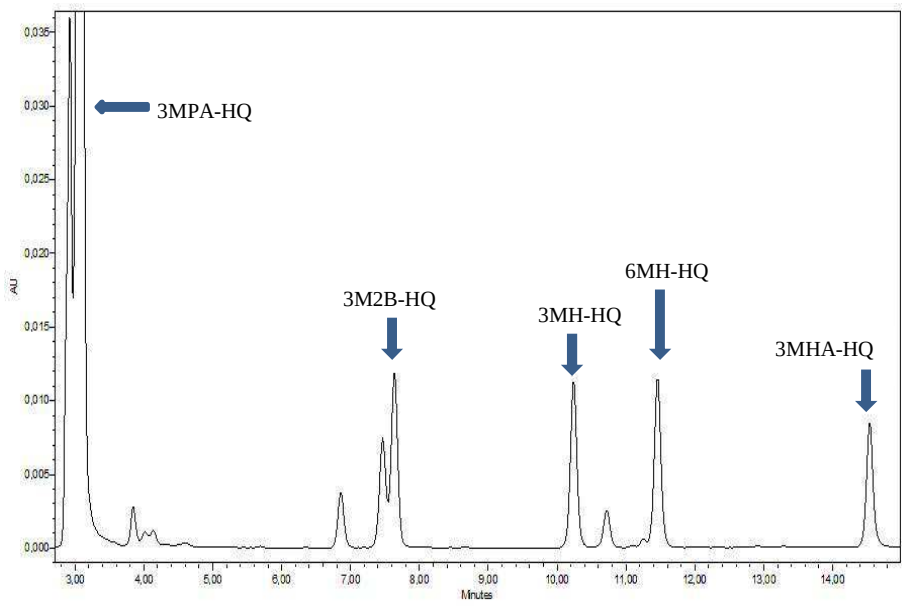

Figure 4.2 HPLC separation of 3MPA-HQ, 3M2B-HQ, 3MH-HQ, 6MH-HQ and 3MHA-HQ.

Despite this, volatile thiol concentration in wines is 4 to 5 orders of magnitude lower than the concentration reported in figure 4.2. As a result, this technique is not sensitive enough for a direct use in wines and it needs the analytes to be concentrated. Because of thiol reactivity and volatility under certain condition, it is not possible to concentrate wine, thus a specific extraction of volatile thiols should be carried out.

Among various technique to extract volatiles from wine, the employment of liquid-liquid extraction with various organic solvent has been widely used for many compounds. Depending on their repartition constant, volatiles (and other compounds) are distributed between sample

matrix and the organic solvent used as extractor. As a result, a specific extraction of analytes can be carried out.

Although different solvents can be used to extract volatiles from wines, usually dichloromethane, pentane, or a mixture of them are used. In particular, dichloromethane (DCM) has been proposed as solvent to extract volatile thiols from wines (Tominaga *et al*., 1998). Thus, in this work DCM will be used to extract volatile thiols.

Depending on the nature of the sample matrix, the solvent used, the characteristic hydrophobicity of the analyte and the presence of modifiers (i.e. salts), volatile compounds can be extracted from wine by using DCM. Stating that thiols (as other molecules in wine) are at least partially soluble in aqueous system, part of the analytes will remain in aqueous system during the liquid-liquid extraction. A quantitative measure of the distribution between aqueous system and organic phases is the distribution coefficient K, which is constant for a single analyte and the solvents used. The constant K is the ratio of the concentration of the solute in the two solvents once the system reaches the equilibrium. It can be used to calculate the effectiveness of multiple small volume extraction in extracting the total amount of analytes from the aqueous system.

In the case of volatile thiols (RSH), if the mercapto group is in its uncharged form (RSH form), the hydrophobicity depends on the alkyl chain R. Different volatile thiols are present in wines: although 3-mercaptohexan-1-ol, its corresponding acetate and 4-mercapto-4methyl-pentan-2-one are considered the most important and studied, many other less hydrophobic thiol molecules can contribute to aroma complexity. In order to develop a method able to determine such less hydrophobic molecules too, two different thiols will be used in this trial: 6-mercaptohexan-1-ol, which is as hydrophobic as wine thiols and 3-mercapto-2-butanol, which is more hydrophilic than the previous one.

Experimental procedure

10 mg L^{-1} solution of 6-mercaptohexan-1-ol (6-MH) and of 3-mercapto-2-butanol in 50mL wine-like model system (tartaric buffer 5 g L^{-1}, pH 3.20, 12% v/v ethanol) was extracted by using an equal volume (50 mL) of dichloromethane. The presence of NaCl (50 g L^{-1}) was evaluated. the emulsion was stirred for 1 hour to reach the equilibrium, then the two phases were separated. Concentration of volatile thiol in aqueous media before and after the extraction was determined by pBQ derivatization, as reported below. Trial was carried out in duplicate

As expected the more hydrophobic thiol (6-MH) was more easily extracted using dichloromethane, while 3M2B showed a lower distribution constant. The presence of NaCl (50 g L^{-1}) clearly improve the extraction yield for both thiols used in this section (Table 4.1).

Analytes	$K_{DCM/Wine;\ (NaCl\ 0g/L)}$	$K_{DCM/Wine;\ (NaCl\ 50g/L)}$
3-mercapto-2-butanol **3M2B**	4.57	8.95
6-mercaptohexan-1-ol **6MH**	5.83	16.82

Table 4.1 . Repartition constant (K) of thiols between dichloromethane and wine-like model system.

The yeld of extraction following to n extraction steps can be described for each analyte as:

$$x^n = x_0 \left(\frac{KV_s}{KVs + Va}\right)^n$$

Where:

x^n is the amount of analyte exctracted during the n^{th} extraction

x_0 is the amount of analyte at the beginning of the extraction procedure

K is the distribution constant specific for an analyte between two specific solvents

Vs is the volume of solvent used during the extraction

Va is the volume of liquid to be extracted

The ratio between the amount of analyte extracted during each extraction and the total amount of analyte represent the yield of the extraction (Y)

$$\frac{x^n}{x_0} = \left(\frac{KVs}{KVs + Va}\right)^n$$

$$\frac{x^n}{x_0} = Y$$

As a consequence, if the initial volume of liquid to be extracted as well as the number of multiple extraction is established, it is possible to calculate the volume of organic solvent to be used during the extraction to gain certain yield

$$Y^n = \left(\frac{KVs}{KVs + Va}\right)^n$$

$$V_s = KV_a \left(\frac{1 - Y^{1/n}}{Y^{1/n}}\right)$$

If the lower distribution constant in the presence of NaCl is used ($K_{DCM/W}$ 3M2B) to calculate its total extraction starting from 1 L solution by multiple extraction, three volumes of 129 mL dichloromethane allow to extract 99.9% of total 3M2B. Since the other thiol showed an higher distribution constant, this volume of organic solvent is clearly sufficient to completely extract total 6MH from wine-like model system.

Once extracted in organic solvent, volatiles can be concentrated thus allowing to increase the sensitivity of the analytical method. Despite this, dichloromethane cannot be injected in HPLC system. Moreover, derivatisation procedure can be used for aqueous system under acid pH (Fracassetti *et al*., 2011). Thus, thiols should be back-extracted in aqueous media.

Hydrophobicity of alkyl mercaptans is clearly influenced by the status of the thiol group. In fact, if this group is dissociated the hydrophobicity is lower and solubility of thiols in aqueous media higher. Stating that the pKa of such molecules is in the range pH 9÷11, the employment of alkaline solution seemed to be the best choice for this back-extraction step. Moreover, the extraction of mercaptans from organic solvents and gasoline by using alkaline solution have been reported in literature (Yabroff, 1940).

From a theoretical point of view, the distribution of a mercaptan between an oil phase and an aqueous solution depends on the solubility of the un-dissociated mercaptan in the organic solvent. Despite this, if alkaline solution is used, the mercaptan will be present in the aqueous phase in both dissociated and undissociated forms. If the former molar fraction is great, more undissociated mercaptan will be solubilized in the aqueous media, thus determining a stronger extraction from the organic layer.

On this idea, distribution constant between DCM and sodium hydroxide 10 mM (pH 12) were calculated in order to back-extract mercaptans from the organic layer.

Experimental procedure

Solution of mercaptans (3M2B 4.9 μM, 6-MH 4.2 μM, 3-MH 3.5 μM and 3-MHA 2.7 μM) were prepared in sodium hydroxide (Merk chemicals) 10 mM, 50 mL. An equal volume of dichloromethane (50 mL) was added. The emulsion was stirred for 1hour to reach the equilibrium, then the two phases were separated. Sample pH was then adjusted to 5.0 with tartaric acid 1 M before the derivatizing was carried out. Concentration of mercaptans in aqueous media was then determined and distribution constant were then calculated.

Results and discussion

As reported in table 4.2 distribution constant under experimental condition showed that NaOH 10 mM was not a suitable tool for back-extraction of mercaptans from dichloromethane. In fact, solubility of thiols was still higher in dichloromethane, even if pH is higher than dissociation constant for thiols thus too high volume in alkaline solution has to be used to extract thiol, even if multiple extraction are used.

Analytes	$K_{NaOH/DCM}$
3-mercapto-2-butanol **3M2B**	8.91
6-mercaptohexan-1-ol **6MH**	0.91
3-mercaptohexan-1-ol **3MH**	0.61
3-mercaptoheyl acetate **3MHA**	n.d.

Table 4.2. Repartition constant (K) of thiols between NaOH 10 mM and dichloromethane.

Despite higher alkaline concentration has been suggested to improve back extraction of mercaptans from oil phases (Yabroff, 1940), this condition has not been tested because of two main reasons. On the one hand, NaOH concentration used allowed to have higher pH than thiol dissociation constant. Moreover, hydrolysis of 3MHA has been noticed under higher pH also during gaschromatographic approaches.

This trial showed that back-extraction of mercaptans from dichloromethane with alkaline solution did not allowed to back extract thiols from dichloromethane in an efficient way.

Solubility of mercaptans in dichloromethane has been demonstrated to be higher than in water system. Despite this, from a physical point of view, DCM density is higher than water density and this two solvents are unsoluble, thus implying that if both water and solvent are present, the bottom layer will be dichloromethane. Moreover, thanks to its low boiling point, dichloromethane is a suitable solvent in which mercaptans can be concentrated.

Since thiols showed they cannot be back-extracted in water by dissociating thiol group using alkaline solution, trial to “force” thiol to pass from dichloromethane to water by removing the organic solvent under reduced pressure was carried out.

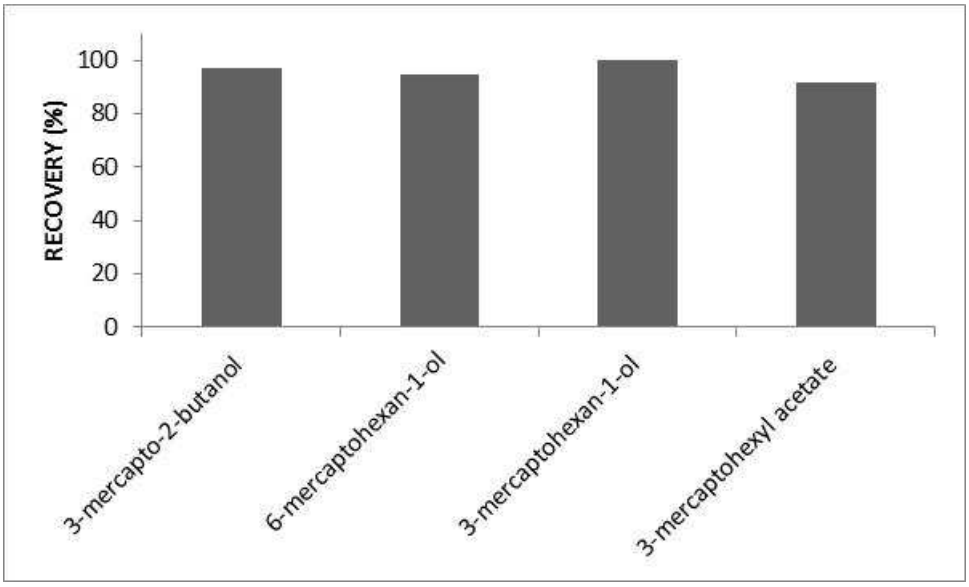

Figure 4.3. Recovery of thiols during back-extraction from dichloromethane using water under reduced pressure.

Although the solubility of mercaptans in dichloromethane is higher than in water, good recovery could be achieved if such molecules were "forced" to solubilize in water by eliminating the organic solvent (fig 4.3). Moreover, in this case, distribution constant did not influence the extraction, since the end of the process only water is present.

Water and dichloromethane were identified as suitable tool to purify thiol from wine-like model system and carry out their derivatization by using p-benzoquinone as derivatizing agent.

Despite this, the method, as described, needed to use 10 mL of water to back-extract thiols from dichloromethane to reach good recovery. Considering that 50 µL were injected, further concentration of the sample would be necessary (up to 1 mL) in order to reach a final concentration factor of 1000, which would allow to gain enough sensitivity.

In this purpose, extraction of derivatized thiols by solid phase extraction was carried out. Since derivatized thiol hydrophobicity is clearly different if compared to thiols, different resins were used. In particular C_{18}, polymeric sorbents, and PVPP were used. Because of their different hydrophobicity, two thiols not present in wines were chosen for this trial: 6-MH and 3M2B. The former being very similar to thiol present in wines, the latter being lower in hydrophobicity.

Experimental procedure

10 mL citrate buffer 50 mM pH 5.0 containing 3M2B 56 µM and 6-MH 45 µM were derivatized using pBQ. Derivatization was carried out on 10 mL sample by adjusting amount of pBQ and MPA in order to have the same final concentration (pBQ 258 µM, MPA 630 µM).

C_{18}, polymeric and polyvinyl polypyrrolidone (PVPP) sorbents were tested during this trial.

Derivatized thiols were loaded onto solid phase extraction resins previuosly conditioned by passing 5 mL methanol followed by 5 mL of water. Elution step was carried out by passing 2 mL methanol 100%.

Determination of derivatized thiols by HPLC-UV detection was carried out as previously described.

Results

As hydro quinone derivatives (RS-HQ), thiols are very hydrophobic molecules and they were retained on various sorbents. In fact, no derivatized thiol could be measured in citrate buffer coming from the sample loading step of the resin. Moreover, elution of such compounds using methanol allowed to gain satisfactory recovery in every sorbents used (figure 4.3).

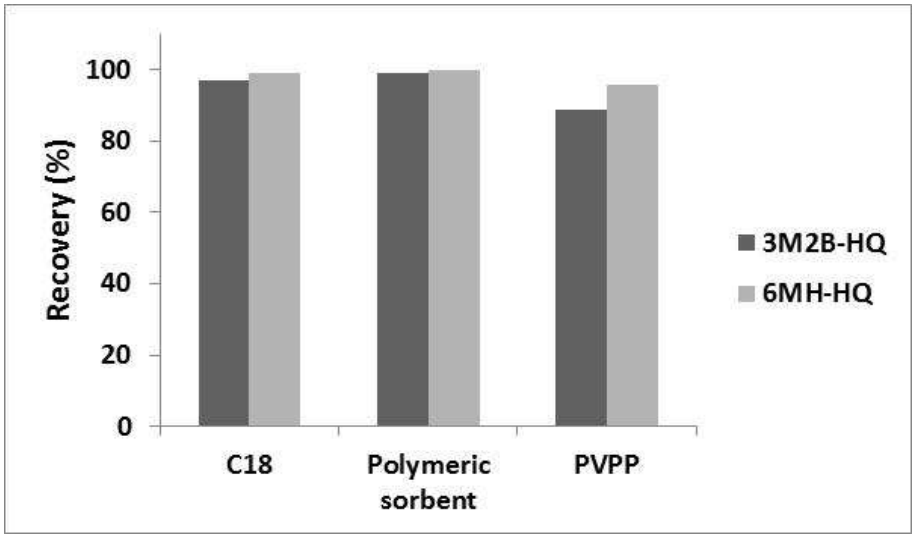

Figure 4.3 Recovery of 3M2B-HQ and 6MH-HQ using different resins as sorbents.

During recovery trial, at least 2 mL of methanol were to be used during the elution step of analytes thus affecting final factor concentration. Despite this, analytes in methanol could be concentrated under reduced pressure with good recovery and the absence of interfering species absorbing at specific wavelength was noted.

Thus, extraction and final concentration factor of 1000 was reached. Considering the detection limit showed by pBQ method in determining glutathione (Tirelli *et al*., 2010), this concentration factor could be enough to reach the needed sensitivity to determine volatile thiols in wines. As a consequence, extraction and derivatization was applied to wine like model system containing volatile thiols.

Experimental procedure

3M2B 7.2 nM, 6-MH 5.7 nM were prepared in 1 L wine-like model system (tartaric buffer 5 g L^{-1}, pH 3.20, 12% v/v ethanol). Wine was added with 50 g L^{-1} NaCl and extracted three times using 150 mL DCM. The organic phases were then combined and concentrated up to 10 mL under reduced pressure. Ten milliliters of water were then added and the organic layer was eliminated. Water pH was then adjusted to 5.0 and derivatization step was carried out with pBQ as previously reported. Derivatized thiols were then retained onto a polymeric sorbent (200 mg, 3 mL volume, Strata X Phenomenex) and eluted by passing 2 mL methanol 100%. Methanol was then concentrated to 1 mL under reduced pressure.

50 µL were injected in the HPLC-UV system to determine derivatized thiols as described below.

Results

Experimental trial showed that the presence of other species absorbing at the same wavelength (303 nm), made the identification of derivatised thiols impossible (figure 4.3). Even if trial was carried out in wine-like model system, where no interference should be present, impurities contained in the chemicals were concentrated 1000 times and were responsible for the impossibility to identify derivatised products.

As a consequence, pBQ was no longer considered a suitable tool to determine derivatised thiols using diode array (λ= 303 nm) as detector.

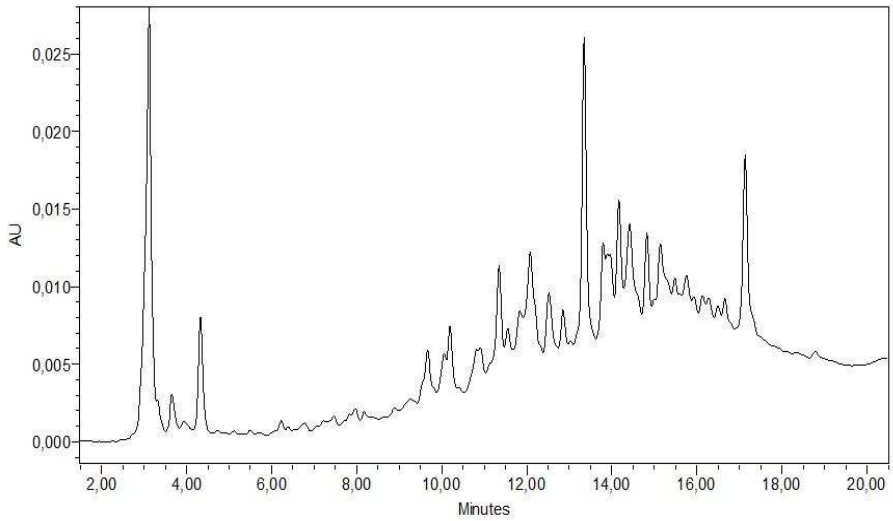

Figure 4.3 HPLC-DAD chromatogram of wine-like model system extracted three time using dichloromethane, back-extracted with water, derivatized with pBQ and concentrated to 1 mL. (λ: 303 nm).

4.2. *o*-phthalaldehyde as derivatizing agent in volatile thiol determination

Among derivatizing agent employed for thiol groups, *o*-phthalaldehyde (OPA) has been received interest because of its sensitivity. The reaction of OPA with a primary amino group and a thiol group leads to the formation of an indole (figure 4.3) (Simons & Johnson, 1978).

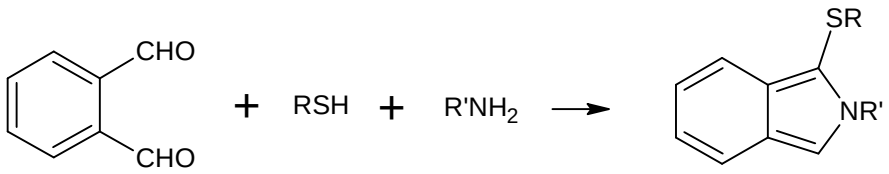

Figure 4.4. Reaction of thiols with OPA.

Derivatized products are fluorescent, thus leading to an improvement in sensitivity of the method itself. Among thiol molecules, such method has been proposed for glutathione and cysteine determination in grape juices and wines (Park *et al*., 2000).

This derivatizing agent was proposed to determine amino acids and amines (Kutlàn & Molnàr-Perl, 2003, Molnàr-Perl & Bozor, 1998, Vasanits *et al*., 2000).

Behavior and stability of amino acid derivatives have been largely studied (Molnar-Perl, 2001, Kutlàn *et al*., 2002).

Since the presence of interference UV adsorbing molecules made impossible to determine volatile thiols extracted from wine-like model system, the employment of high sensitive derivatizing agent was assumed a suitable tool to use.

Materials and methods

Amino ethanol (AE) and *o*-phthaldialdehyde (OPA) were purchased from Fluka (Swizerland).

Thiol standards were prepared in water. Pre-column derivatization was carried out as follows: 50 µL of sample were withdrawn, 5 µL of OPA (5 mg mL^{-1} in methanol) (final concentration 3 mM) and 5 µL AE (10 mg mL^{-1} in borate buffer 80 mM, pH 7.30), final concentration 9 mM were then withdrawn. The derivatization mixture (total volume 60 µL) was then let react for 1 min and then injected for analysis. The reversed phase (RP)-HPLC of derivatized thiol was performed with a Water Alliance 2695 (Milliford MA) equipped with a fluorescence detector Jasco FP-920 (λ_{ex}: 330 nm, λ_{em}: 440 nm). The separation column was a hexyl-phenyl column,

250 mm x 4.6 mm, 5 µm, 110 Å (Phenomenex, Torrence, CA). Eluting solvents were water and methanol, eluting gradient and flow is indicated in table 4.3.

time (min)	flow ($mLmin^{-1}$)	A %	B %
0.0	0.1	95.0	5
5.0	0.1	95.0	5
5.1	0.1	40.0	60
7.0	1.0	40.0	60
20.0	1.0	20.0	80
20.2	1.0	0.0	100

Table 4.3. HPLC separation gradient for thiol derivatized with OPA. Eluent A: water, eluent B: methanol.

Results and discussion

Volatile thiols could be derivatized and separated under the experimental condition used (figure 4.5). Only 4-MMP did not allow to be derivatized. Despite this, used concentration are above 1 mg L^{-1}. This concentration represents at least 10000 times real concentration of volatile thiols in wine. Thus, extraction and concentration procedure have to be applied to reach enough sensitivity.

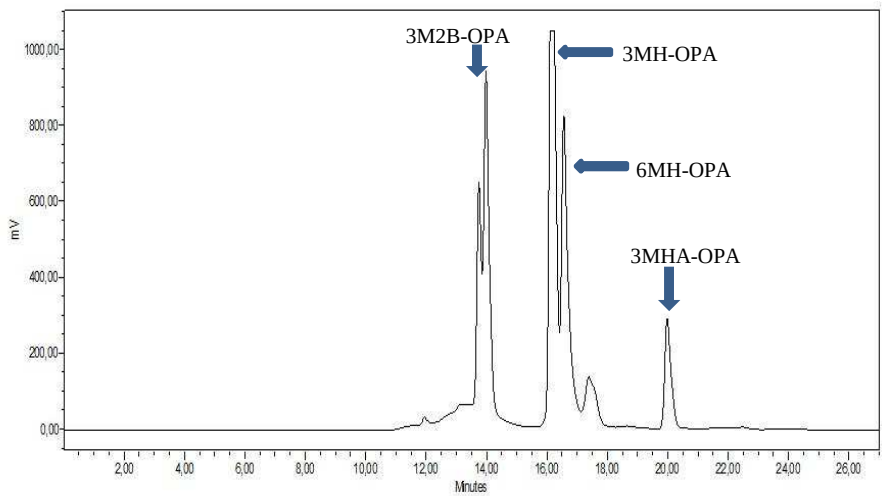

Figure 4.5 HPLC separation of 3M2B-OPA, 3MH-OPA, 6MH-OPA and 3MHA-OPA.

Optimization of derivatization procedure with OPA

Derivatization by OPA and AE of thiols back-extracted with water was then tried. From previuos trials, derivatized thiols with pBQ could be concentrated by solid phase extraction resins and, once eluted by in methanol, concentrated under reduced pressure.

As indoles are more hydrophobic than volatile thiols, extraction of indoles derivatives of analytes was tried on polymeric sorbents. Derivative products between OPA, primary amines and thiols have been reported to be high reactive species (Kutlàn *et al*., 2002). Extraction and concentration steps increased by-products formation, characterized by the same fluorescent properties, thus letting impossible to identify presence of derivatized thiols in the analyzed samples. As a result, pre-column derivatization was chosen as the best one. Despite this, many factors can affect derivatization yield. Among others, pH can influence derivatization yield (Nakamura & Tamura, 1982).

Derivative procedure was then carried out at different pH to evaluate which was the best range for volatile thiols.

Standard solutions were then prepared in citrate buffer 10 mM pH 5.0, phosphate buffer 10mM pH 6.5, borate buffer 10 mM pH 8.0, borate buffer 10 mM pH 9.0, carbonate buffer 10 mM pH 10, carbonate buffer 10 mM pH 11.0; pH 12.0 was prepared using NaOH.

Pre-column derivatization procedure and separation of derivatized product was carried out as reported previously.

Optimal pH for derivatization process was different with various volatile thiols studied (figure 4.5). pH conditions lower than 6 did not formed indole. This suggested that acidic medium was not optimal for volatile thiol derivatization with OPA-AE.

In neutral and mild basic pH, 3M2B derivatization yield was higher as pH increased. A similar behavior was noticed for 3-MH whereas 3-MHA derivatization yield dropped at high pH, while 3-MH increased thus suggesting an hydrolysis of this ester into the corresponding alcohol. 6-MH derivatization yield was constant within the pH range 6.5-12.0. As a consequence, the optimal pH range to determine volatile thiols was noticed to be the pH range between 6.5 and 9.0.

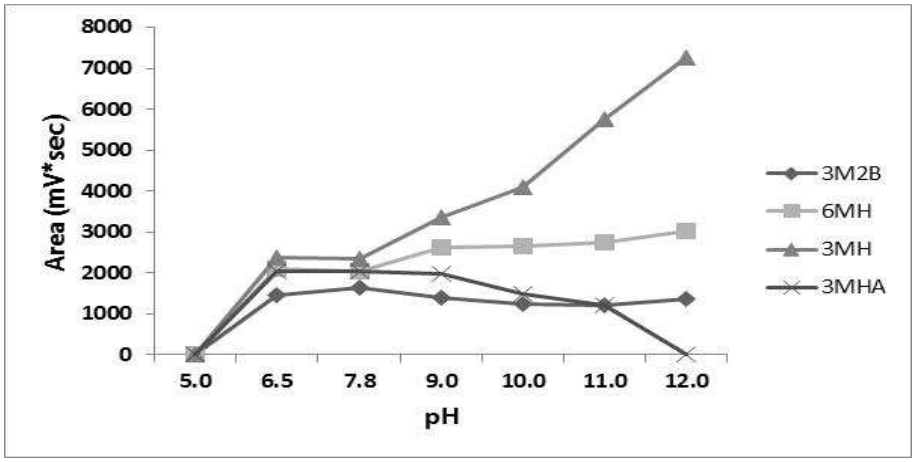

Figure 4.5 pH effect on derivatization yield using OPA as derivatizing agent.

During pre-column derivatization sample containing thiols, derivatizing agent and co-derivatizing agent (amino ethanol) can be withdrawn in various sequences, thus affecting derivatization yield. In order to maximize derivatization procedure, different combination of sample (S), amino ethanol (AE) and o-phthaldialdehyde (OPA) withdrawing sequences were tested. 6-mercaptohexan-1-ol was used as reference thiol in this optimization trial. Chosen pH was 7.0, since this pH was noticed to be optimum for every studied volatile thiol. As reported in figure 4.6 relative yield was maximum for the withdrawing sequence S-OPA-AE.

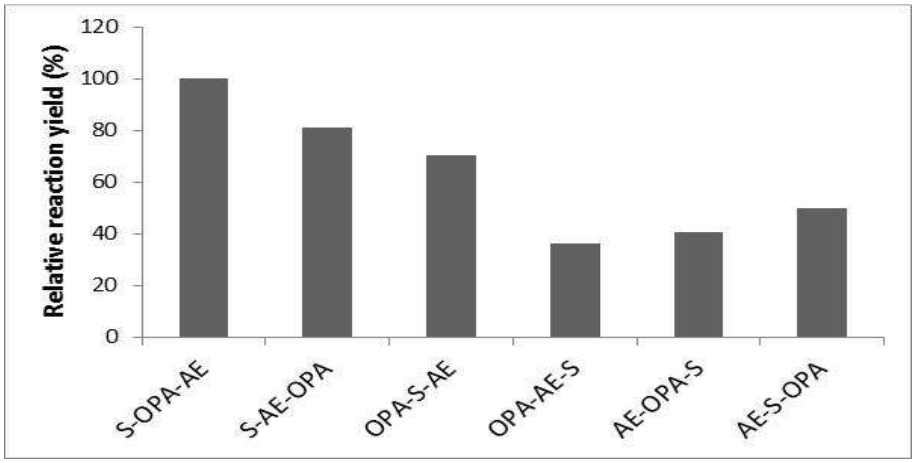

Figure 4.6. Effect of sample (S), amino ethanol (AE) and o-phthaldialdehyde (OPA) on derivatization yield.

As derivatizing process was optimized, extraction of volatile thiol from wine-like model system (tartaric buffer 5 g L^{-1}, pH 3.20, 12% v/v ethanol) was applied as follows: wine was added with 50 g L^{-1} NaCl and extracted three times using 150 mL dichloromethane. The organic phases were then mixed together and concentrated up to 5 mL under reduced pressure. One milliliter of water was then added and the organic layer was eliminated. The aqueous phase containing volatile thiol was then submitted to HPLC-Fluor analysis with pre-column derivatization as described previously.

The optimized derivatization and the employment of specific fluorescent detection, allowed to identify volatile thiols in wine like model system at μg L^{-1} levels.

Stating this result, the same extraction and derivatization procedure was applied for white wines but the identification of thiols in real matrix was not possible. The complexity of wine matrix was responsible for the lack of derivatization or thiol reactivity towards various chemical species. In order to identify the major responsible of this result and let the extraction and derivatization method work, different aspects were evaluated.

Interferents extraction from wine affecting volatile thiol determination

Although dichloromethane has been reported to be an ideal solvent to extract volatile thiols from wines (Tominaga *et al*., 1998) and other vegetable matrices (Tominaga *et al*., 1997), this organic solvent has been widely used to extract and analyze many other compounds from wines (Ortega-Heras *et al*., 2002). In particular, dichloromethane was identified to be able to extract acids, alcohols, carbonyl compounds, esters, volatile phenols, lactones and terpenes (Hernanz *et al*., 2008). Many of the cited compounds are able to interact with volatile thiols both in water and in organic solvent, or they may influence derivatization reaction yield.

Hexanoic acid, octanoic acid and decanoic acid extraction as well as hydroxybenzoic and hydroxycinnamic acids extraction could be responsible for pH modification of back-extraction water. On the other hand, extracted phenolics and corresponding quinones could react with thiols in aqueous media (Nikolantonaki & Waterhouse, 2012), as well as in organic solvent under certain conditions. Moreover, as strong nucleophilic compounds, thiols can be involved in several reactions which are based on Michael-type mechanism. Since these variables can interact in a complex way, one variable at time was investigated.

Evaluation of acids extraction: effect on back-extraction water pH

Since derivatization yield has been noticed to be strongly affected by pH, influence of wine matrix on the pH in back-extraction was firstly evaluated. In this purpose, 1 L of white wine containing 50 gL^{-1} NaCl was extracted three times using 150 mL dichloromethane. organic solvent was then concentrated to 10 mL and an equal amount of water was added. Once the organic phase was eliminated, back-extraction water pH was measured. Back-extraction water pH was 4.56 (as average of two replicates). Moreover, water color was brown thus suggesting that both acids and phenolics were dissolved in dichloromethane and in back-extraction water.

To avoid organic acids extraction from aqueous media using dichloromethane as organic solvent, dissociation of carboxylic function or the formation of the corresponding salt can be evaluated as the most interesting way.

Total dissociation of organic acids present in wines can be reached at high pH. despite this, under these condition both quinones and thiolates formation is favoured (Danilewicz *et al.*, 2008; Jocelyn, 1972), thus improving nucleophile additions of thiol to quinones, leading to the loss of aroma compounds.

The addition of $CaCO_3$ to transform carboxylic acids into the corresponding Calcium salts was then evaluated. As deacidification tool of musts and wines, calcium carbonate is well-known (Steele & Kunkee, 1978). In fact, it reacts rapidly with tartaric and malic acids (the major acids in grapes and white wines) to form calcium tartrate and calcium malate unless the wine being treated is maintained at pH 4.5 or above. When the pH is higher, calcium malate-tartrate (double salt) can be formed. This treatment can be carried out in order to increase must and wine pH. Despite this, it can be used to develop salt precipitation processes and avoid solubilization in dichloromethane of (Ca) salified organic acids.

In this purpose, white wines were submitted to deacidification. In particular, $CaCO_3$ was added adjusting pH to 5.0.

Back-extraction water pH of treated wines was then evaluated as follows: 1 L of white wine was added of 50 g NaCl. pH was the adjusted to 5.0 using $CaCO_3$. Treated wine was extracted three times with 150 mL volume of dichloromethane. The combined organic phases were then concentrated to above 10 mL under reduced pressure. 10 mL of water were then added and the organic solvent was eliminated under reduced pressure. Back-extraction water pH was then measured.

$CaCO_3$ treatment allowed to obtain optimal pH for OPA derivatization process in back-extraction water. Measured pH was 6.75 as average. On the opposite, not treated wine lowered back-extraction water pH: measured pH was lower than 5.0.

Evaluation of phenolics extraction

The reaction between thiol compounds and quinones has been reported to be a major responsible of thiol loss in wines. The reactivity between these two species depends on the characteristics of both thiol compounds (Nikolantonaki & Waterhouse, 2012) and phenolics (Nikolantonaki *et al*., 2010, Kilmartin *et al*., 2001).

Conjugate addition of thiol compounds to quinones has reported to proceed both in wine (Nikolantonaki *et al*., 2010), in water (Yadav *et al*., 2007).

In general, this type of addition reaction of nucleophiles to unsaturated carbonyl compounds requires basic condition or the presence of a catalyst is required (Yadav *et al*., 2007).

Depending on their repartition constant, phenols and quinones could be extracted in dichloromethane, thus being responsible for thiol loss during extraction procedures.

Ferric Chloride-Phenol reaction has been used to identify phenolic compounds (Wesp & Brode, 1934). Many studies have been carried out to improve such specific test for phenols in various solvents. The production of a blue, violet, or red coloration by the addition of ferric chloride to solutions of phenols has been used as a qualitative test for this aromatic hydroxyl group (Soloway & Wilen, 1952).

Qualitative evaluation of the presence of phenolics in back-extraction water from wine extraction was then carried out by ferric chloride test. When treated with this salt, back-extraction water turned brown, suggesting the extraction of phenolics from wine with dichloromethane.

Effect of phenols extraction on volatile thiol determination

Effect of phenols in back extraction water was the evaluated as follows: (+)-Catechin 5 mM and caffeic acid 5 mM were prepared in dichloromethane (50 mL). 6-mercaptohexan-1-ol was then added to the organic solvent to give a final concentration 100 μM. The organic solvent was then evaporated under reduced pressure and back-extracted using 1 mL of water as described previously.

In the presence of such reactive phenolic species in dichloromethane, identification of 6-mercaptohexanol was not possible. On the other hand, even if the thiol standard was added to

the back-extraction water, no peak was measured. Thus suggesting that thiol loss could proceed in organic solvent or in aqueous media if phenols were extacted during liquid-liquid extraction.
To confirm the presence of reactive species in back-extraction water from wine able to determine thiol loss during extraction process or interfering with derivatization reaction, addition of 6-mercaptohexan-1-ol to back extraction water was carried out.

Experimental procedure

One liter of wine containing 7.6 nM of 6-MH, 50 g L^{-1} NaCl, pH adjusted to 5.0 with CaCO3, was extracted three times using 150 mL of DCM. The organic phases were combined, reduced under reduced pressure and back-extracted with 1 mL water. Increasing amounts in 6-MH (0.76 µM, 1.60 µM, 2.52 µM, 3.55 µM) were then added to back-extraction water and determination of thiol with OPA as derivatizing agent was carried out as previously reported.
As reported in figure 4.7, 6-MH identification was not possible in extracted wine. Moreover, identification of 6-MH was possible only at high concentration (3.55 µM), thus suggesting that interferents extracted from wine matrix can interact with thiols causing their loss and/or not allowing derivatization procedure.

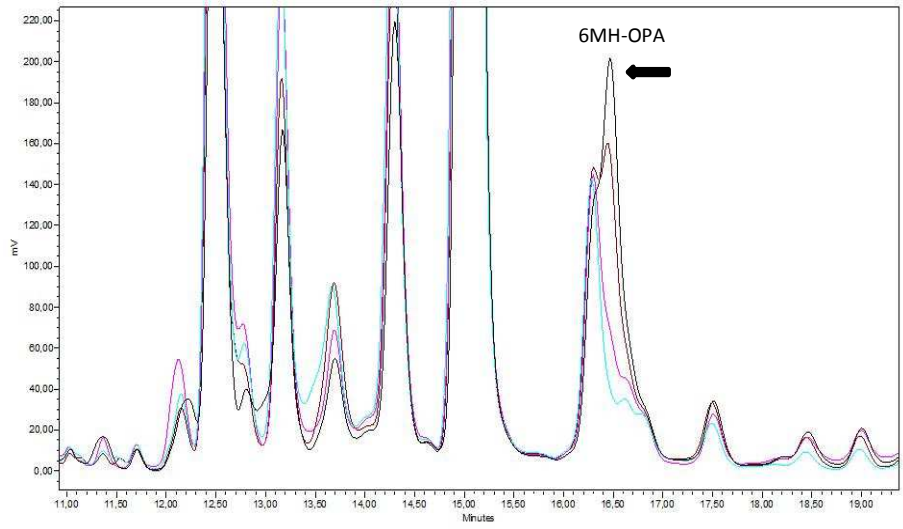

Figure 4.7. HPLC separation wine extracted sample spiked in back-water extraction with 6-MH.

Since phenolics affect the presence of volatile thiol in back-extraction water and/or interfering with derivatization reaction, and their putative presence has been noticed during extraction from white wines, fining agents and washing step of the organic solvent have been evaluated.

Phenolic compounds can be largely eliminated from wine by adsorbing them on fining agents. Among those latter, water-insoluble polyvinyl polypyrrolidone (PVPP) is a synthetic polymer which is used to adsorb phenols from beverages. It is though that the adsorbtion of polyphenols by PVPP is through hydrogen bonding between the proton donor from the polyphenol and the carbonyl group from PVPP, together with π-bond overlap and hydrophobic interaction between the aromatic ring of the polyphenol and the PVPP ring (Laborde *et al*., 2006). The affinity of PVPP increases as the number of phenolic hydroxyl groups increase, as more groups are available for hydrogen bonding (Doner *et al*., 1993). The use of PVPP revealed to be a very efficient for adsorption of phenolic compounds such as catechin, epicatechin and quercetin (Magalhaes *et al*., 2010), as well as proanthocyanidins (McMurrough *et al*., 1995).

Although PVPP is able to adsorb polyphenolics in a selective way, entire elimination of such molecules is difficult. In fact, number and kind of substituents on the aromatic ring of phenolics can affect the interaction between phenols and the fining agent (Laborde *et al*., 2006). As a consequence, phenolic compounds which are not eliminated from wine can be extracted during the process thus leading to thiol loss.

Washing step of organic solvents during liquid-liquid extraction is a usual procedure to eliminate interferents. Phenolics have been reported to be extracted from organic solvents and oil by using aqueous media. In particular, good recovery can be achieved by using alkaline solutions (Murray, 1949). Despite such media showed good recovery in extracting phenols from gasoline, strong alkaline solution cannot be used in the case of thiol extraction. As discussed above, sodium hydroxide could extract thiols from DCM (Capone *et al*., 2011) thus meaning their loss. On the other hand, alkaline solution would lead to the formation of quinones which would react quickly with thiols and also in this case the result would be thiol loss (Danilewicz *et al*., 2008). Thus, washing step by using water seems to be the most suitable choice. With the aim to minimize phenols content in dichloromethane during the extraction process and in the back-extraction water, PVPP treatment on wine samples and washing dichloromethane with water were tested. Presence of phenolic compounds in both water used for washing step and back extraction water was evaluated by ferric chloride test.

Experimental procedure

3.0 g $Na_2S_2O_5$ and 5 g of PVPP were added to 1 L of white wine and stirred for 15 min. The sample was then centrifuged at 4500 rpm for 10 min to remove unsoluble PVPP. 50 g NaCl were then added to the sample and the pH was adjusted to 5.0 with $CaCO_3$. Wine was then extracted three times with 150 mL dichloromethane. The emulsion was broken by centrifugation (4500 rpm 10 min). Collected organic phases were mixed (450 mL) and washed three times with 50 mL water. The organic solvent was then concentrated under reduced pressure to above 2 mL. then an equal amount of water was added and dichloromethane was eliminated under reduced pressure.

Presence of phenolic species in water used during washing step was carried out by concentrating water (150 mL) to above 2 mL under reduced pressure and treating it with ferric chloride.

Back-extraction water was submitted to thiol analysis using OPA as derivatizing agent as reported above. Once analyzed, back-extraction water was treated with ferric chloride to evaluate the presence of phenolics.

Results

Brown color obtained in ferric chloride test applied to water used during the washing step suggested that PVPP treatment was not able, if used alone, to eliminate extractable phenolics from wine. On the other hand, the capability of the washing step to partially remove extracted phenolics was tested. Back-extraction water, when treated with ferric chloride, showed light-yellow color. This result suggested that PVPP treatment and washing step carried out on organic layer were able to drastically reduce phenolics content in back-extraction water. Despite this, volatile thiols were not detectable in spiked wine.

To evaluate interactions between thiol compounds and applied treatments, 3.0 g $Na_2S_2O_5$ and 5 g of PVPP were added to 1 L of wine like model system containing thiols and stirred for 15 min. The sample was then centrifuged at 4500 rpm for 10 min to remove unsoluble PVPP. 50 g NaCl was then added to the sample and the pH was adjusted to 5.0 with $CaCO_3$ and extracted once with 150 mL dichloromethane. The organic solvent was washed with water 50 mL, then concentrated under reduced pressure and back-extracted with water 1mL as described above. Peaks area was compared to non-treated wine-like model system. $CaCO_3$ and PVPP treatment, together with the washing step of dichloromethane by water did not affect thiol concentration in comparison to non-treated wine like model system (figure 4.8). As a consequence, those

treatments can be considered as selective in elimination of interfernts which can be regarded as responsible of thiol loss during extraction process.

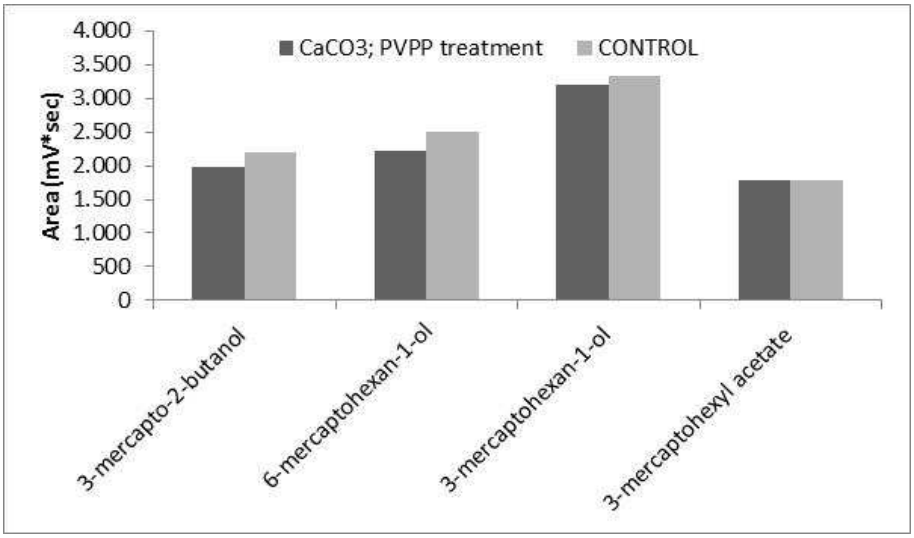

Figure 4.8. Effect of $CaCO_3$ and PVPP treatments on volatile thiols in wine-like model system.

Evaluation of various Michael addition type reactions responsible for thiol loss

Reactivity of thiols as strong nucleophilic group is the reason of their conjugation reactions. The latter are the main responsible for thiol loss during wine aging as well as during their extraction from wines. In particular, Michael type addition explains the reaction mechanism. Quinones, and in particular *p*-quinones can be considered as the most reactive species for the conjugate addition of thiols (Yadav *et al*., 2007). Despite this, many other structures can be considerate as putative strong electrophile in both aqueous media and organic solvent. In fact, the Michael addition accepted mechanism involves a direct addition of a nucleophile to a remote carbon of the conjugated C=O, or related system. Among various putative molecules, αβ-unsaturated acids and esters are able to give Michael type additions in the presence of thiol group. Among thiols, the addition between thiol acetic acid and molecules containing "enal" group have been studied (Ilyashenko *et al*., 2010, Rossiter & Swingle, 1992).

In particular, during the proposed extraction method, some phenolics can be still present in organic solvent, although fining treatments and washing step are carried out during the

extraction procedure of thiols. As a result, on the one hand many putative structures for nucleophilic addition of thiols are present in the organic solvent. On the other hand, concentration step of the organic solvent and back-extraction can force such addition. As a consequence, conjugate additions of volatile thiols can occour.

As reported in previous session, thiol compounds could not be identified in spiked wine although phenols concentration was minimized and optimal derivatizing conditions were present.

Among reducing agents, sodium borohydride is responsible in the reduction of different functional groups in organic synthesis. In particular, reactivity towards conjugated carbonyl compounds was observed while chemo- and regioselectivity demonstrated (de Souza & Vasconcelos, 2006). On the other hand, borohydride hydrolysis reaction in water generates hydrogen (Liu & Li, 2009), thus suggesting that sodium borohydride interaction with interferents compound in wine matrix, organic solvents or water is complex. Despite this, such reducing agent is not soluble in dichloromethane, while the presence of sodium borohydride during derivatization step may be responsible for reducing carbonyl group of o-phtalaldehyde (OPA). The effect of $NaBH_4$ treatment on interferents in wine prior to the liquid extraction with dichloromethane was then evaluated.

Experimental procedure

One liter of Sauvignon blanc wine spiked with thiols was added with 3.0 g $Na_2S_2O_5$ and 5 g of PVPP and stirred for 15 min. The sample was then centrifuged at 4500 rpm for 10 min to remove unsoluble PVPP. 50 g NaCl were then added to the sample and the pH was adjusted to 5.0 with $CaCO_3$. 3.84 g $NaBH_4$ were added. Wine was then extracted three times with 150 mL dichloromethane. The emulsion was broken by centrifugation (4500 rpm 10 min). Collected organic phases were combined (450 mL) and washed three times with 50 mL water. The organic solvent was then concentrated under reduced pressure to above 5 mL. then an equal amount of water was added and dichloromethane was eliminated under reduced pressure.

Determination of thiols was then carried out using OPA as derivatizing agent as previously described.

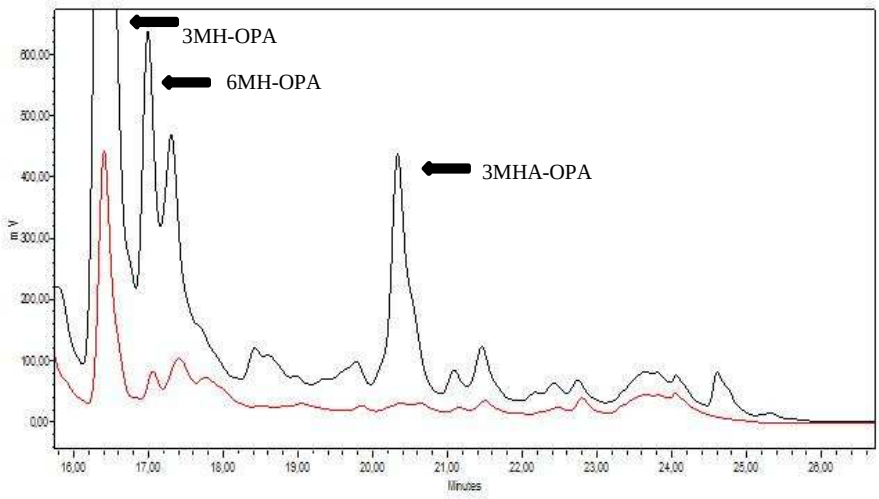

Figure 4.9. HPLC-FLUO separation of wine (red line) and wine containing 1μgL^{-1} 3MH, 6MH and 3MHA (black line) extracted with the proposed method. Derivatization of thiols with OPA.

The reduction treatment with sodium borohydride, together with PVPP fining treatment and washing step, allowed to identify volatile thiols in wine at μgL^{-1} level in spiked wine. Moreover, 3-MH was detectable also in Sauvignon blanc wine using the proposed method (figure 4.9).

Conclusions

Although volatile thiols have always been analyzed by gaschromatographic methods, their quantification with HPLC approaches can be carried out, thanks to their high boiling point and to the employment of specific derivatizing agents. Extraction procedure based on organic solvent employment and back extraction in aqueous media allows to reach required detection limit in methods development. Despite this, the key factor in achieving good results is the evaluation of interferents responsible both for lack of derivatized species detection and derivatization reaction, as well as the elimination of reactive species towards thiols. In particular, UV absorbing derivatized thiol by *p*-benzoquinone as derivatizing agent were affected by many interferents UV absorbing molecules.
The employment of fluorescent derivatizing agent allowed higher sensitivity. Despite this, only the evaluation of the effect caused by interferent species allowed to reach identification of thiol aromas in real matrices. Due to the extraction capacity of dichloromethane, variuos interferents were extracted. In particular, on the one hand derivatization process was not possible because of extraction of species causing low pH in back-extraction water. On the other hand, phenolics have been noticed to be responsible for thiol loss during extraction process. Moreover, the

minimization of Michael-type reactions by reductive agents was needed. Specific treatments and washing steps were evaluated to be successful in eliminating interferents. As a result, volatile thiols were determined both in spiked wine at μg L^{-1} level, and in Sauvignon blanc wines.

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5. Putative 3-mercapto-1-hexanol precursors: evaluation of the reaction of bisulfite with *trans*-2-hexenal

Various studies concerning 3-mercaptohexan-1-ol (3-MH) biogenic pathway have been carried out (Peña-Gallego *et al*., 2012). As a result, cysteine and glutathione conjugates were evaluated to be the major precursors. The identification of intermediates cysteinilglycine precursor (Capone *et al*., 2011), together with the action of γ-glutamyltranspeptidase and carboxypeptidase, suggested that cysteinilated precursor of 3-mercaptohexan-1-ol is alongside the metabolic pathway of the degradation of the corresponding glutathione conjugate thus confirming that this latter compound can be regarded as the major precursor of 3MH in wines following the described biogenic pathway (Roland *et al*., 2010).

Alternative pathway for the biogenesis of 3-MH in wine has been proposed focusing on *trans* 2-hexenal as starting material leading to thio-conjugates (Schneider *et al*., 2006). The proposed pathway is based on Michael addition of the sulfydryl group from different molecules which act as sulfur donor. Yeast lyase activity would be then responsible for 3-MH formation. On the other hand the addition of hydrogen sulfide would lead directly to the smelling molecules.

The occurrence of *trans*-2-hexenal and other unsaturated C6 compounds in grape juice is related to oxidative metabolism of unsaturated fatty-acids. They are present or form after grape damage (Joslin & Ough, 1978). Thus, C6 unsaturated compounds concentration in grape juice increases during skin contact (Ramney *et al*., 1986). Moreover, *trans*-2-hexenal content decreases during storage period after machine-harvesting, thus suggesting reactivity towards other compounds present in grape juice (Capone *et al*., 2012).

Among thiol compounds hydrogen sulfide and L-Cysteine were evaluated to react with hexenal (Schneider *et al*., 2006). Despite this, the former sulfur donor compounds is produced during the alcoholic fermentation, while the concentration of *trans*-2-hexenal is known to decline prior to the fermentation (Joslin & Ough, 1978, Capone *et al*., 2012). Cysteine and, most of all, glutathione can be regarded as sulfydryl group donor during prefermentative operation leading to glutathionylated thiol precursor formation (Roland *et al*., 2010).

The evaluation of the biogenic pathway of this thiol precursor based on C6 unsaturated aldehyde, explain higher concentration in both cysteine and glutathione conjugates in grapes submitted to oxidative treatments and transportation prior to crushing (Capone *et al*., 2012, Capone *et al*., 2011, Capone & Jeffery, 2011). Despite this, the highest content of thiol precurors is not related to the highest content in thiols in the corresponding wines (Allen *et al*., 2011, Pinu *et al*, 2012) thus suggesting that thiol precursors and aromatic thiol are related in a more complex way and/or the 3MH in wine may derive from alternative pathway.

Occurrence of aromatic thiols in wine can be affected by SO_2 following to various mechanism, including both enzyme inhibition and direct chemical effects. Bisulfite can act as an antioxidant and lower the build-up of reactive quinones (Makhotkina & Kilmartin, 2009). Reduced glutathione loss can be lowered thanks to the presence of free SO_2, at least until this latter is bound by fermentation products. As a result, on the one hand a higher amount in sulfydryl donor molecules is preserved. On the other hand, the lower the quinone content in the juice the lower the oxidative losses of the 3-MH formed during the early stages of alcoholic

fermentation. At the same time, SO_2 is responsible for disrupting grape membrane structures leading to higher extraction of compounds into the juice, which could include some thiol precursor and polyphenols.

Effect of SO_2 in preventing oxidative degradation of 3-MH precursors can be excluded, since thiol conjugates are not sensitive to oxidation. In fact, the sulfydryl group is involved in a carbon-sulfur bond and such products do not decrease as a result of juice oxygenation (Roland *et al.*, 2010). Likewise, no difference was noted in glutathione conjugates in the presence of SO_2 at nil or 50 mg L^{-1}. On the other hand, excessive addition of SO_2 led to lower concentration in both cysteine and glutathione conjugates (Capone & Jeffery, 2011) thus suggesting that bisulfite has some influence in thiol precursor formation during prefermentative stage.

Bisulfite is a strong nucleophile reagent which could act as a sulfur donor, leading to a sulfonate product that yeast might be able to convert to 3-MH. Moreover, interaction between unsaturated aldehyde and bisulfite are characterized by complex behavior leading to the formation of various species (Dufour *et al.*, 1999, Barker *et al.*, 1983).

In order to evaluate if this putative precursor can be generated during prefermentative stages, interaction between *trans*-2-hexenal and bisulfite will be studied. Synthesis of sulfonate products will be then carried out to obtain pure species and evaluate if they can be considered as putative precursors of 3MH in wines.

5.1 Interactions between bisulfite with trans-2-hexenal

As a strong nucleophile, bisulfite may interact with the carbonyl group of *trans*-2-hexenal (**A**) to generate trans-1-hydroxyhexen-2-ene-1-sulfonic acid (**B**). On the other hand, following to a Michal-type addition reaction (similarly to sulfydryl group of Cysteine, glutathione and hydrogen sulfide), 1-oxohexane-3-sulfonic acid (**C**) can be produced. In the end, if both Michael reaction and nucleophile addition to carbonyl group are followed, 1-hydroxyhexane-1,3-disulfonic acid (**D**) will be the major product (figure 5.1).

Mono adducts (species B and C) are characterized by the same accurate mass, even if their similarity to 3-MH is different.

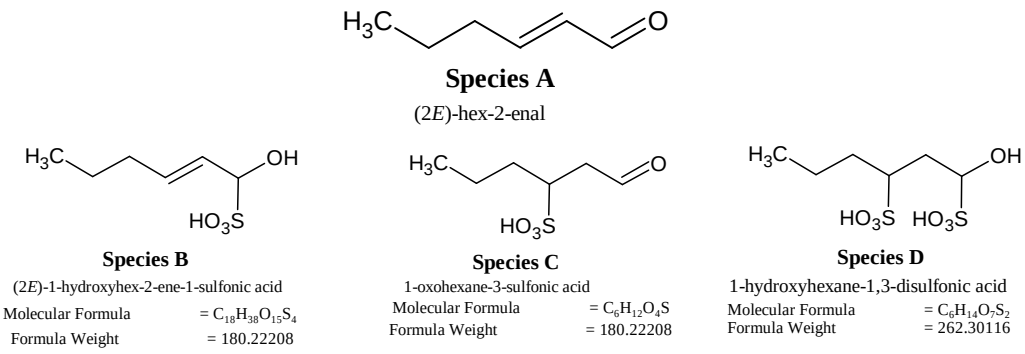

Figure 5.1. Chemical structures of trans-2-hexenal and putative products generate from bisulfite addition to species A.

Materials and methods

trans-2-hexenal (98%), Sodium bisulfite (40% solution), deuterium oxide and deuterated methanol were purchased from Sigma-Aldrich. ^{1}H and ^{13}C NMR spectra of addition products between the unsaturated aldehyde and bisulfite were obtained on a Bruker Avance 300 spectrometer operating at 400 MHz.

^{1}H and ^{13}C NMR spectroscopy could be used to characterize both starting material and species formed during the interaction between the unsaturated aldehyde and bisulfite. The most interesting protons to describe structure during bisulfite addition are proton at 9.33 ppm (doublet) which is characteristic of the carbonyl group, and protons at 6.05 and 7.25 ppm that are characteristics of the double bond between carbons in the starting material. Protons at lower ppm are linked to the alkyl chain.

^{13}C NMR data allowed assignments for species A in its most interesting carbon. ^{13}C at 200 ppm is characteristic for the carbonyl group, while carbons at 161 and 134 ppm were assigned to be the carbon linked with the double bond.

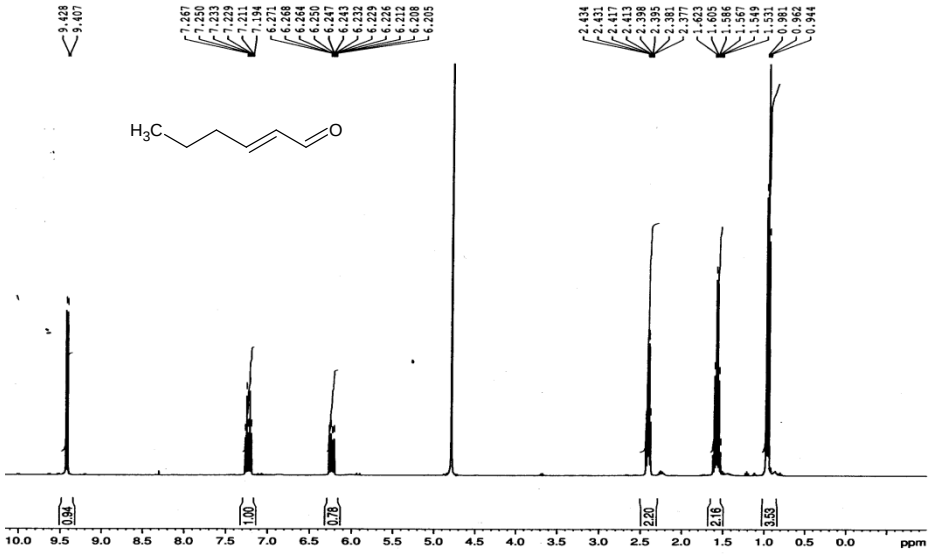

Figure 5.2. ^{1}H NMR spectra of *trans*-2-hexenal.

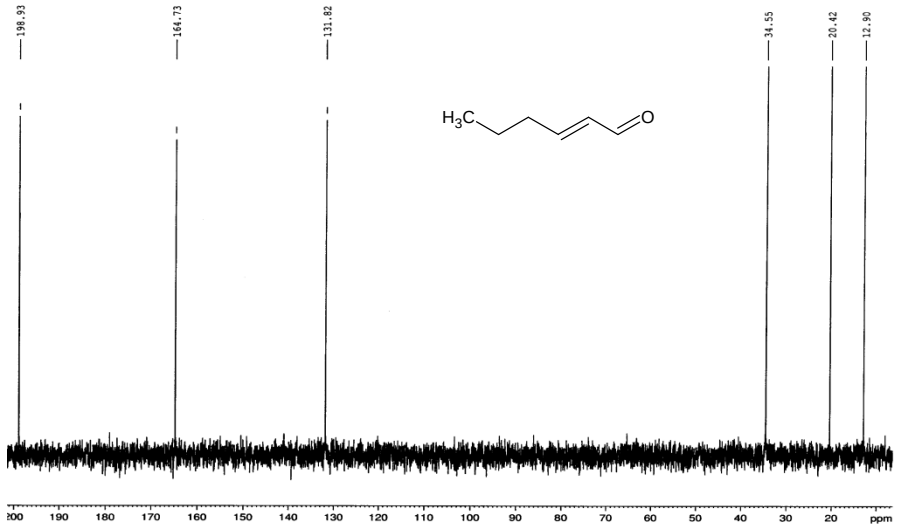

Figure 5.3. ^{13}C NMR spectra of *trans*-2-hexenal.

The level of bisulfite in grape juice is in the ppm range, whereas *trans*-2-hexenal is present at ppb levels. Nevertheless, the level of free bisulfite in juice available to react with the unsaturated aldehyde is variable and dependent on competing reactions. In fact, various species can react with bisulfite in grape juice, first of all quinones. Consequently, the bisulfite concentration relative to the unsaturated aldehyde may or not be in excess. Therefore the analysis of the addition of bisulfite to trans-2-hexenal was investigated both in excess or not of bisulfite (table 5.1).

Formation of 3MH glutathione and cysteine conjugate precursors is a dynamic process, influenced by various operations. Among others, prolonged storage time has been identified to be crucial. On the other hand hexenal concentration during this time evolves. As a consequence, time was evaluated to be an interesting variable for bisulfite and hexenal interaction too (table 5.1).

Entry	***Equiv of $NaHSO_3$***	***time (h)***
1	0.6	4
2	100	4
3	0.6	24
4	100	24

Table 5.1. Reaction condition for the sulfonylation of *trans*-2-hexenal.

The initial addition of the bisolfite to *trans*-2-hexenal was to the aldehydic function. This occourred within the first four hours and represents the only reaction product both in case of bisulfite and usaturated aldehyde excess (entry 1 and 2 table 5.1).

If enough bisulfite was present to bind every carbonyl group, no starting unsaturated aldehyde was present, while if excess in aldehyde was present only two species were detectable: species

A and species B (figure 5.4). In particular, proton at 4.85 ppm was assigned to be the proton related to the carbon bounded with the hydroxyl and sulfonate groups, while protons at 5.6 and 6.05ppm are characteristics of the remaining double bond with an upfield shift (figure 5.4).
^{13}C NMR data confirmed the formation of the species B as the major product. In particular 13C at 84.97 ppm was assigned to be the carbon bounded to the sulfonate group, while carbons at 123.11 ppm and 138.79 ppm were assigned to the remaninig carbons bounded with double bond (figure 5.5).

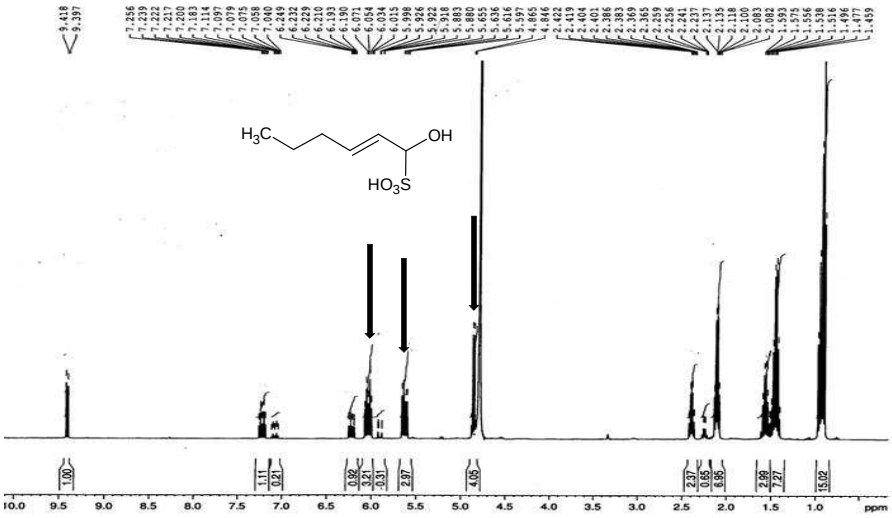

Figure 5.4. ^{1}H NMR spectra of bisulfite addition to *trans*-2-hexenal, 4h stirrig at room temperature.

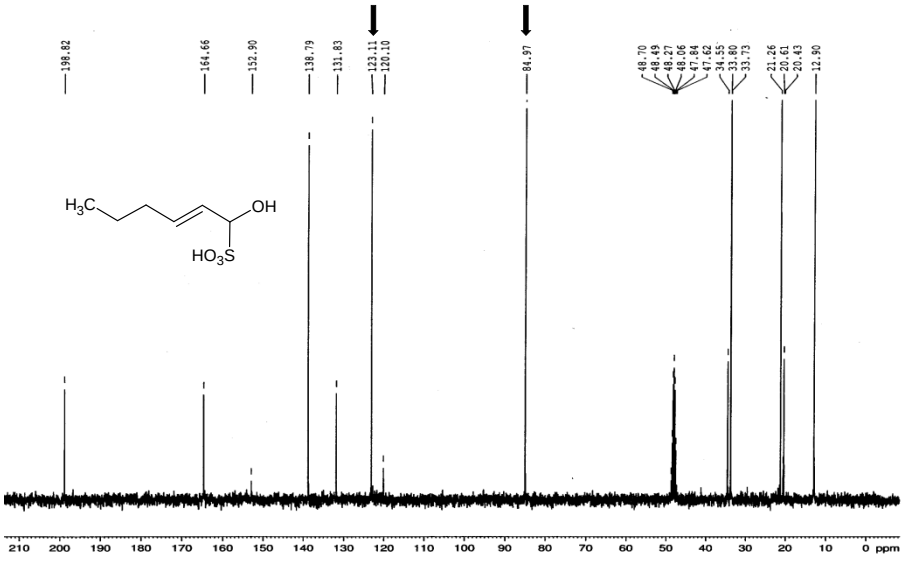

Figure 5.5. ^{13}C NMR spectra of bisulfite addition to *trans*-2-hexenal, 4h stirrig at room temperature.

Although the addition to the carbonyl function was evaluated to be the first occurring during interaction between bisulfite and hexenal, other products could be noted. In fact species D was produced as minor compound after 4 hours at room temperature even if bisulfite was present as 0.6 equivalents of the unsaturated aldehyde. Protons at 4.8and 4.5ppm were assigned to be linked to the carbons bounded to the sulfonates groups. Moreover, the absence of left protons related to the double bound (zone 6 ÷ 7 ppm) confirmed the formation of species D (figure 5.6). Moreover, species D could be purified by drying crude reaction mixture to dryness.

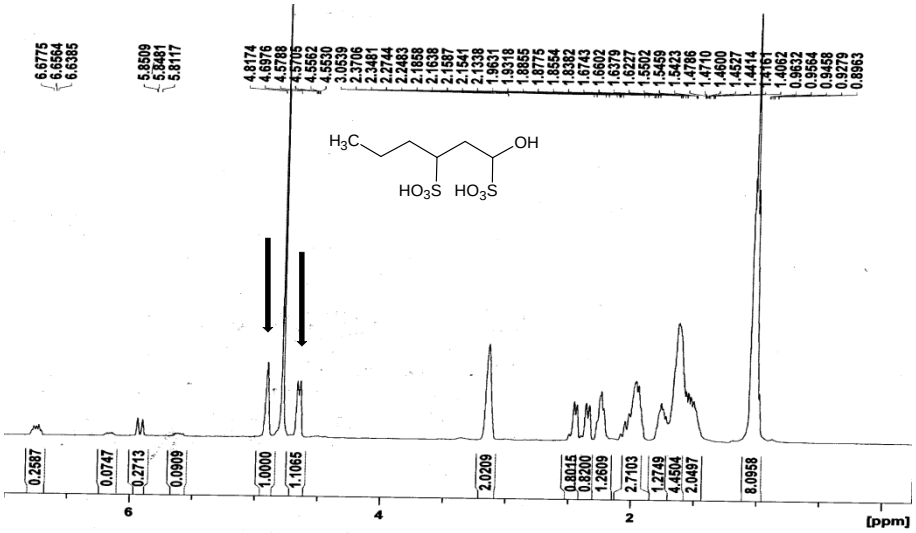

Figure 5.6. ^{1}H NMR spectra of spectra of bisulfite addition to *trans*-2-hexenal, 4h stirrig at room temperature.

Species D was the major product deriving from the interaction between *trans*-2-hexenal and bisulfite after 24 hours stirring if the bisulfite was in excess in comparison to the unsaturated aldehyde (entries 3 and 4 table 5.1).

Stating these results, the addition of bisulfite to the double bond of species B to yield the disulfonates suggested to take place. Despite this, the absence of the carbonyl group would lead to the lack of an acidic carbon which would be necessary for the nucleophilic addition of bisulfite. As a result, the fornation of disulfonates would imply that the first bisulfite addition would follow a Michael reaction type.

Since reflux conditions were necessary for Michael addition of bisulfite to αβ-unsaturated carbonyl compounds (Kellog *et al.*, 2003), similar conditions were used to increase yield in mono-sulfonate in order to investigate the pathway leading to the species D.

Synthesis was carried out in acetate buffer pH 5.0, 10% v/v methanol, since at that pH molar fraction of bisulfite form is maximum. Amount in *trans*-2-hexenal and bisulfite are reported in table 4.2. Crude reaction was stirred for 12 hours under reflux.

It is noteworthy that under these conditions only C and D species are produced suggesting that these two addition products between bisulfite and hexenal are strictly related.
Stating that more unsaturated aldehyde was present in comparison to the nucleophilic reagent, some starting material is still present. Its presence was shown by both ^{1}H and ^{13}C NMR spectra (figures 5.5 and 5.6).
Presence of both species C and D was suggested by both ^{1}H and ^{13}C NMR spectra(figures 5.7 and 5.8). In particular, protons at 9.5 ppm and ^{13}C at 205.48 ppm were assigned to species C, while protons at 4.66 ppm and carbon at 83 ppm and 53 ppm allowed to identify species D.

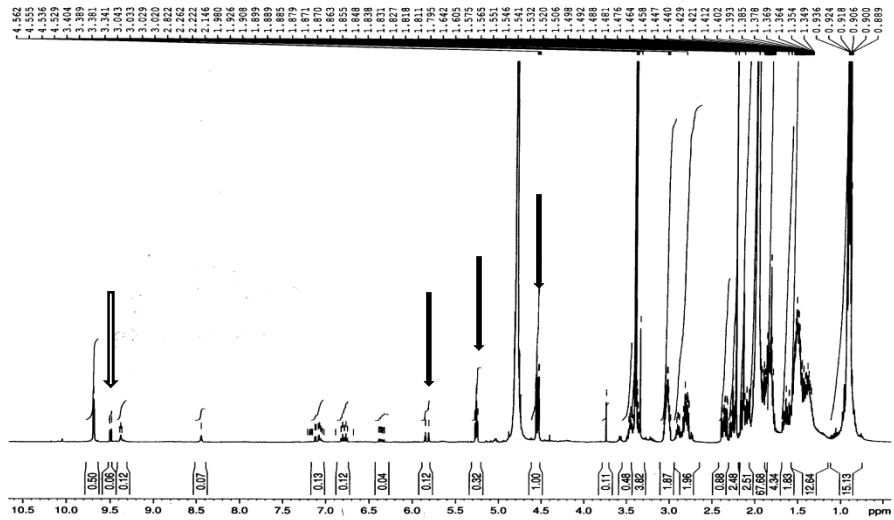

Figure 5.7. ^{1}H NMR spectrum of bisulfite addition to *trans*-2-hexenal, 12 h stirrig under reflux.

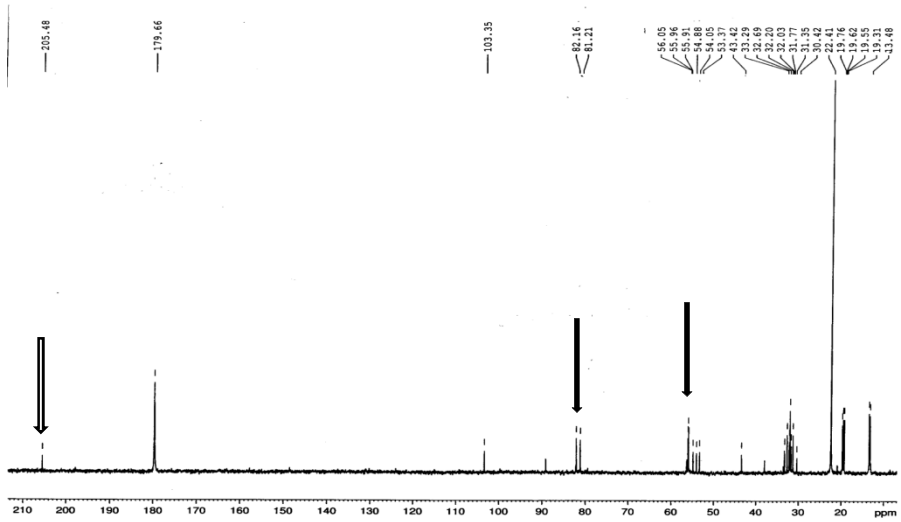

Figure 5.8. ^{13}C NMR spectrum of bisulfite addition to *trans*-2-hexenal, 12 h stirring under reflux.

Although less equivalent of bisulfite in comparison to unsaturated aldehyde were used, both monosulfonates and disulfonates were obtained. Moreover, ratio between species C and D was 1:4. It was assumed that once 1-oxohexane-3-sulfonic acid was formed, it competed with *trans* -2-hexenal to bind bisulfite to the aldehydic function.

Thanks to the obtained results potential equilibria of *trans*-2-hexenal/bisulfite was proposed as reported in figure 5.9

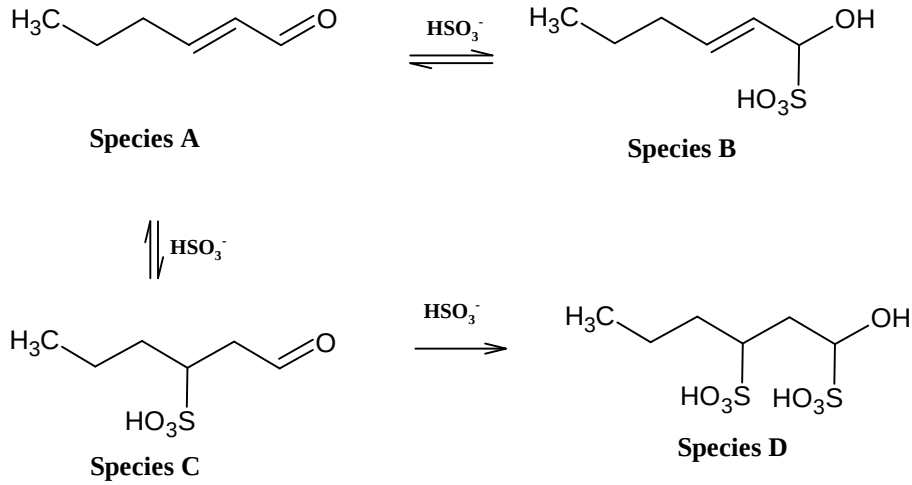

Figure 5.9. Proposed *trans*-2-hexenal bisulfite equilibria.

The initial addition of bisulfite to αβ-unsatured aldehyde is at the aldehydic function. This reaction occours rapidly and species B was seen as the kinetic product. The formation of species D (disulfonates) occours much slowly than the first. As species D evolved, a concomitant decrease in the level of species B was observed. Within 24 hours, there was a complete loss of the proton signals associated with the double bond of species B, and species D was the most abundant one. As a result, species D can be regarded as the most stable product in the interaction between *trans*-2-hexenal and bisulfite, if this latter is in excess amount.
Formation pathway of species D was shown to be related to the species C, formed following Michael-type addition reaction. Once formed, spicies C competes with *trans*-2-hexenal to bind bisulfite to the aldehydic function. In fact, 1-hydroxyhexane-1,3-disulfonic acid was formed even if less than one equivalent of bisulfite was used during synthesis.

5.2. Synthesis and evaluation of 1-hydroxyhexane-1,3-disulfonic acid as 3-mercaptohexan-1-ol putative precursor

During the evaluation of interactions between trans-2-hexenal and bisulfite, 1-hydroxyhexane-1,3-disulfonic acid was noted to be the most stable product. In fact, the reaction leding to its formation proceeded even if less than one equivalent of bisulfite was used. The evaluation of this product as putative 3-mercaptohexan-1-ol was then carried out.

Synthesis of 1-hydroxyhexane-1,3-disulfonic acid

To 0.098 g of *trans*-2-hexenal (1 mmol, 1 eq), in 25mL acetate buffer 50 mM, pH 5.0, 10% methanol, 0.78 mL $NaHSO_3$ 3.84 M (3 mmol, 3 eq) were added. The reaction mixture was then stirred for 12 hours under reflux. Crude reaction was then dried dryness leading to the pure standard.

1H NMR spectra (figure 5.10) and 13C NMR spectra (figure 5.11) allowed to identify the disulfonate product. **HRMS** (ESI +) found (MNa^+) 282.9934, $C_6H_{13}NaO_7S_2$, required 283.2829.

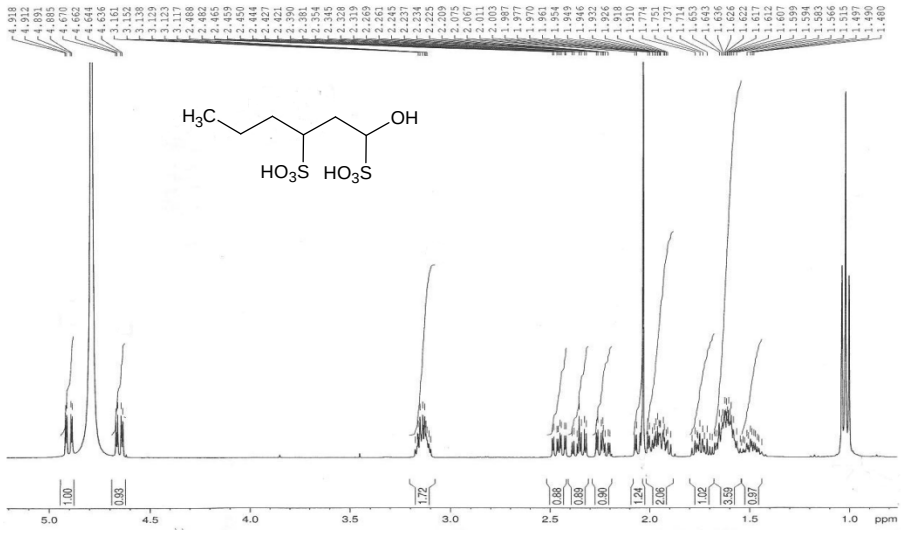

Figure 5.10. ^{1}H NMR spectrum of 1-hydroxyhexane-1,3-disulfonic acid

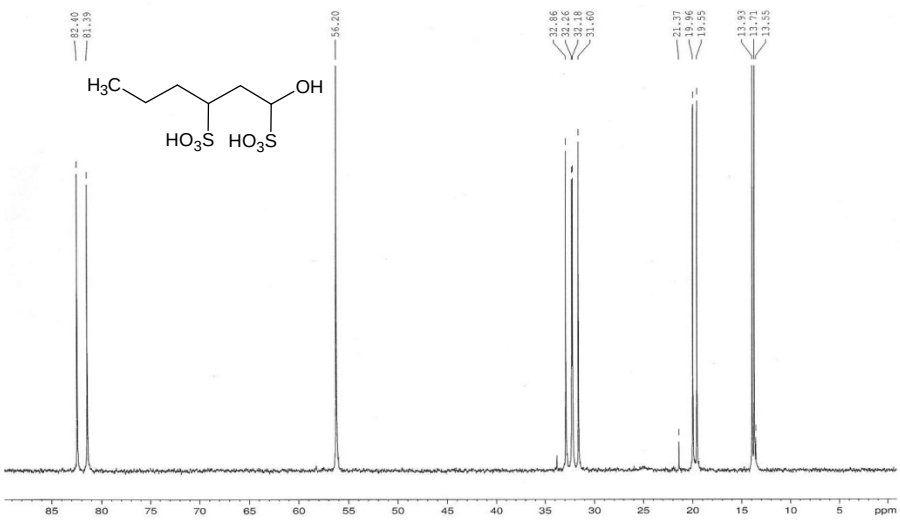

Figure 5.11. ^{13}C NMR spectrum of of 1-hydroxyhexane-1,3-disulfonic acid.

Micro-fermentation in presence of 1-hydroxyhexane-1,3-disulfonic acid

The yeast strains used in this study were purchased from School of Biological Science, University of Auckland (NZ). In particular two yeast strains tipically used for alcoholic fermentation of New Zealand Sauvignon blanc grape juices were used: ECIII8 and X5. Yeasts were cultured for 48 h at 28° C in YPD medium (1% yeast extract, 2% tryptone, 2% glucose) before inoculation into experimental microferments. Concentration of 100 μg L^{-1} and 500 μg L^{-1} of disulfonates were used. Ferments were initially conducted at 25°C with 100-rpm shaking in 250mL Erlenmeyer flasks with 210 mL synthetic grape media (SGM) or sterile Sauvignon Blanc grape juice. The grape juice was sterilized by incubation with 200 μL dimethyl dicarbonate per litre of grape juice at room temperature overnight to kill all microbes. Fermentation progress was monitored daily by weighing the flasks. At the end of alcoholic fermentation, the ferments were harvested. The contents of the flask were centrifuged at 6000 x *g* for 10 min to pellet the solids. The supernatant was then submitted to further analysis. Fermentation were carried out in duplicate.

3-mercaptohexan-1-ol and 3-mercaptohexyl acetate analysis

Thiols were extracted and quantified as follows: five mL of 1 mM Na-4-hydroxymercuribenzoate (pHMB) and 0.5 mL of 2 mM butylated hydroxyanisole (BHA) were added to 50mL of wine and then 50 μL of a mixture of the deuterated compounds for 3-MH and 3-MHA were added (Hebditch et al., 2007) to standardize the quantification. The pH was adjusted to 7 and the samples were loaded onto a washed Dowex resin column and passed through at a flow rate of one drop every 5 seconds. The column was then washed with 50 mL of 0.1 M Na-acetate, pH 6, 0.02 mM BHA at a flow rate of one drop every 4 seconds. Any bound thios were eluted with 50 mL cysteine elution buffer (0.1 M Na-acetate, 0.02 mM BHA, 400 mg cysteine-HCl, adjusted to pH 6) at a flow rate of one drop every 7 seconds. The thiols were extracted with 4 and then 2 mL of dichloromethane. Each time, the lower organic phase was recovered and then dried with anhydrous Na_2SO_4, filtered and concentrated under N_2 gas flow to above 25 μL. The thiols were detected and quantified by injection of 1 μL in splitless mode into an Agilent 6890N gas chromatograph (Santa Clara, CA) equipped with a 7683B automatic

liquid sampler, a G2614A autosampler, and a 5973 mass selective detector. The inlet temperature was held at 240°C, the column used was an Agilent HP-INNOWax capillary column (60 m x 0.250 mm ID, 0.25µm film) using helium (BOC) as carrier gas (112kPa) at an initial flow rate of 1mL/min (for 43.60 minutes). The initial oven temperature (50°C for 5 min) was ramped to 162°C at a rate of 3°C/min, then raised to 250°C at 70°C/min (held for 10min) before dropping down to 50°C. 3-MH and 3-MHA were detected in SIM mode.

Results

The addition of putative precursor (1-hydroxyhexane-1,3-disulfonic acid) to Sauvignon blanc grape juice did not increase the amount in 3-mercaptohexan-1-ol (figure 5.12) and its acetate (figure 5.13) in corresponding wines. Same results were obtained for both used yeast strains. Moreover, no 3MH or 3MHA was produced during alcoholic fermentation of syntethic grape media in the presence of disulfonate as putative precursor. Interaction studies suggested that the disulfonates product is the most stable in the addition of bisulfite to *trans*-2-hexenal, if enough bisulfite is present. Despite this, this product cannot be considered as a putative precursor of 3-MH and 3-MHA in wine.

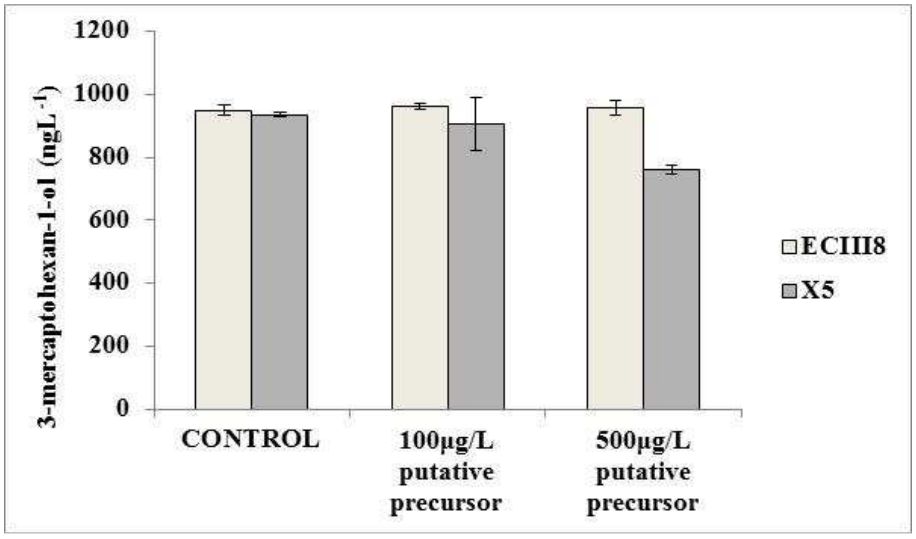

Figure 5.12. 3-mercapto-1hexanol content in wines after alcoholic fermentation of Sauvignon blanc grape juice containing 0 $\mu g L^{-1}$ (CONTROL), 100 $\mu g\ L^{-1}$ and 500 $\mu g\ L^{-1}$ of putative precursor (1-hydroxyhexane-1,3-disulfonic acid).

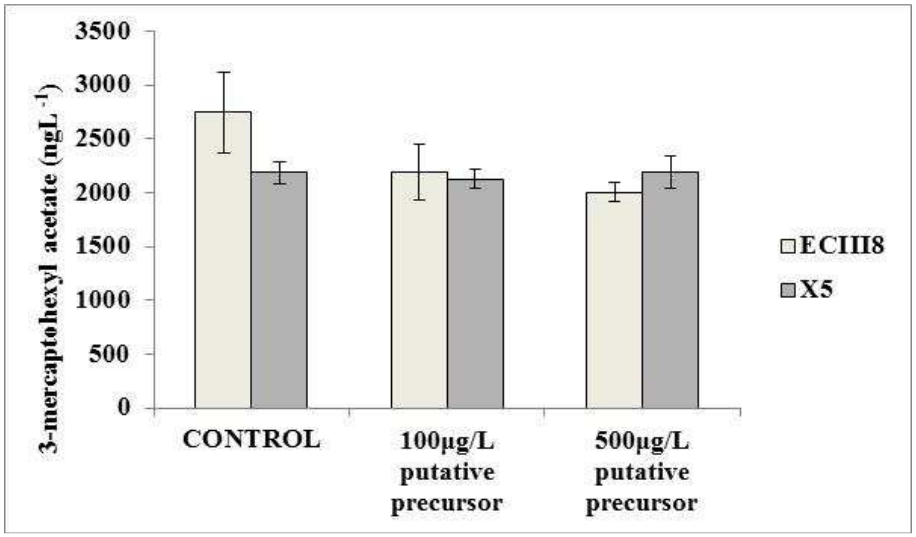

Figure 5.13. 3-mercaptohexyl acetate content in wines after alcoholic fermentation of Sauvignon blanc grape juice containing 0 µg L^{-1} (CONTROL), 100 µg L^{-1} and 500 µg L^{-1} of putative precursor (1-hydroxyhexane-1,3-disulfonic acid).

5.3. Temptative synthesis of 1-oxohexane-3-sulfonic acid

Among addition products between bisulfite and *trans*-2-hexenal, 1-hydroxyhexane-1,3-disulfonic acid cannot be considered as a 3-MH precursor in wines. On the other hand, *trans*-1-hydroxyhexen-2-ene is the most similar compound to 3-MH. Reduction of this sulfonate compound would lead directly to the aromatic compound in wines. Moreover its formation is a Michael type addition which is similar to the formation of other thiol precursors and its presence has been noted during interaction studies between bisulfite and the unsatuarated aldehyde.
Sulfonylation of unsaturated carbonyl compounds at room temperature have been studied (Fini *et al*., 2010). The presence of a Brønsted bases (i.e. amine) was evaluated to increase the reaction yield. In particular, the employment of triethyl amine as base in large excess in comparison to other reactants, together with water/methanol as solvents, was demonstrated to maximize the reaction yield (Fini *et al*., 2010).
Synthesis of 3-MH conjugates to cysteine and glutathione uses water:acetonitrile 50:50 as solvent, while pyridine is used as base. Maximum reaction yield is reached in 48 hours reaction without heating (Fedrizzi *et al*., 2012, Grant-Preece *et al*., 2010).
Interaction studies between bisulfite and *trans*-2-hexenal, suggested that if more than 2 equivalents of bisulfite are present, double addition product (disulfonates) is the only obtained species. On the other hand, in the case of less then two equivalents of bisulfite, Michael addition product may be obtained as species.
With the aim to abtain Michael addition sulfonates species as major product, the following synthesis were carried out. To 1 mmol *trans*-2-hexenal, 1 equivalent of bisulfite was added. Solvent used were water 50% acetonitrile or water 10% methanol. In the case of the former solvent used, pyridine was used as Brønsted base (2.37 eq). When the latter solvent was used, triethylamine (1.2 eq) was used as base. Synthesis conditions are summarized in table 5.2. Crude reaction mixture was stirred for 48 hours at room temperature, then dried dryness under reduced pressure. Obtained product was redissolved in deuterium oxide and submitted to ^{1}H NMR analysis.

Solvent	***Base***	***Equiv of $NaHSO_3$***
water 50% Acetonitrile	Triethylamine 1.2 eq	1
water 10% Methanol	Pyridine 2.37 eq	1

Table 5.2. Reaction conditions for the sulfonylation of *trans*-2-hexenal.

In both cases, as observed during interaction studies, both *trans*-1-hydroxyhexen-2-ene and 1-hydroxyhexane-1,3-disulfonic acid were obtained as products. Moreover, double addition product was the major obtained species. Temptative purification by SiO_2 chromatography or by anion exchange resins did not allow to gain single species.

Since direct addition of bisulfite to trans-2-hexenal in experimental conditions did not allow to obtain pure trans-1-hydroxyhexen-2-ene, the addition of thiolacetic acid was then evaluated (Ilyashenko *et al*., 2010).

Synthesis of S-(1-oxohexan-3-yl) ethanethioate

To 5.1 mmol *trans*-2-hexenal in water 50% acetonitrile, 7.55 mmol thiolacetic acid (1.48 eq) and 12.11 mmol pyridine (2.375 eq) were added. The reaction was stirred for 48 hours at room temperature. Crude reaction was then vacuum dried. The residue was redissolved in 50 mL dichloromethane and washed first with an equal volume of hydrochloric acid 2 M, then using an equal volume of saturated $NaHCO_3$. The organic layer was then dried over sodium sulfate, and evaporated under reduced pressure (750 mbar, 45°C) to afford the pure product (4.885 mmol).
^{1}H and ^{13}C NMR analysis were carried out using deuterated chloroform as solvent (figures 5.14 and 5.15)

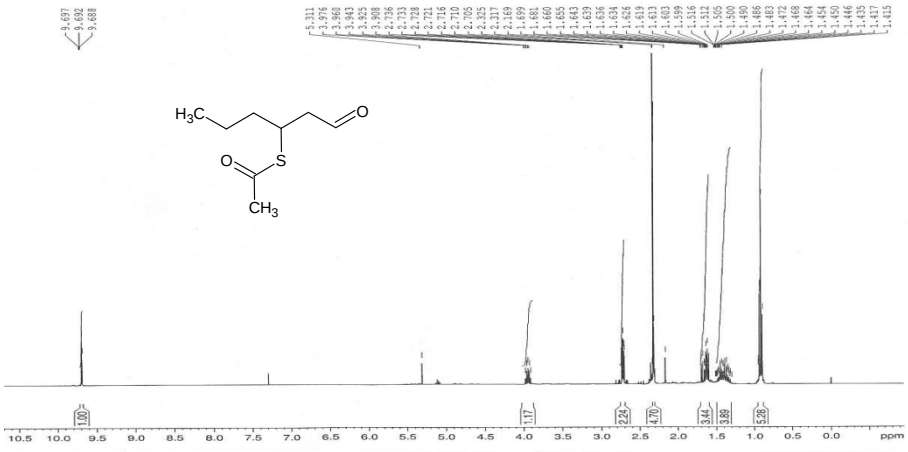

Figure 5.14. ^{1}H NMR spectrum of S-(1-oxohexan-3-yl) ethanethioate.

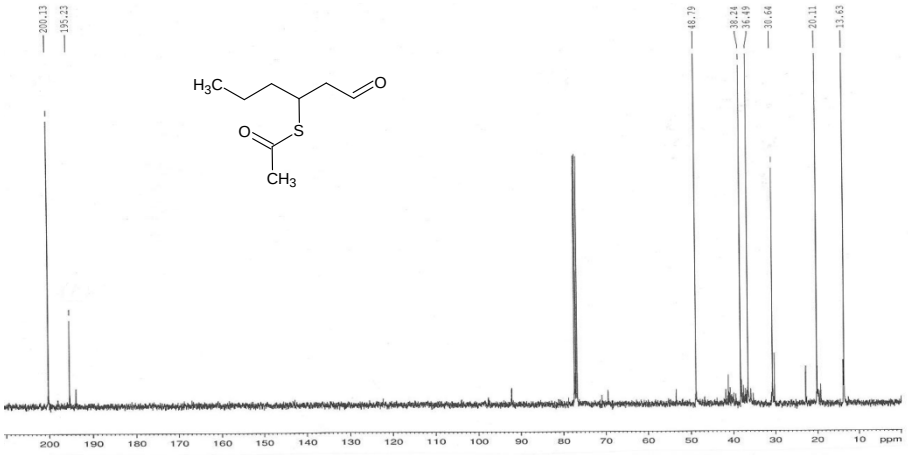

Figure 5.15. ^{13}C NMR spectrum of S-(1-oxohexan-3-yl) ethanethioate.

Oxidation of acetylthio conjugates have been reported to be successful to obtain corresponding sulfonate product (Zhao *et al*., 2010). With this purpose the following synthesis trial was carried out: to S-(1-oxohexan-3-yl) ethanethioate (0.85 g, 4.885 mmol) in acetic acid (20 mL) was added 30% H_2O_2 (6.5 mL). The mixture was stirred overnight at room temperature (25° C).
The crude reaction mixture was then vacuum dried. The product was redissolved in D_2O and submitted to ^{1}H and ^{13}C analysis.
The reaction was responsible for oxidation of the acethyltio aldehyde to the corresponding carboxylic acid. The lack of proton signal at 9.6 ppm (figure 5.16) showed the absence of the aldehyde. At the same time, ^{13}C spectrum (figure 5.17) showed very low signal at 202 ppm, correlated to the aldehyde group, while signal at 197 ppm suggested the presence of carboxylic acid. Moreover, the signal at 197 ppm was still present thus showing that the acetylthio group was not oxidized. As a result, the synthesis trial gave 3-(acetylsulfanyl)hexanoic acid as the only product.

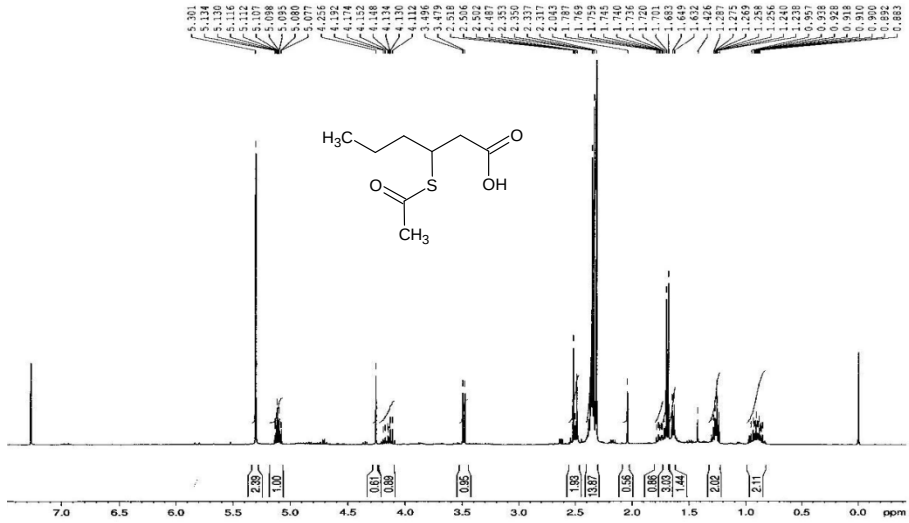

Figure 5.16. ^{1}H NMR spectrum deriving from S-(1-oxohexan-3-yl) ethanethioate oxidation.

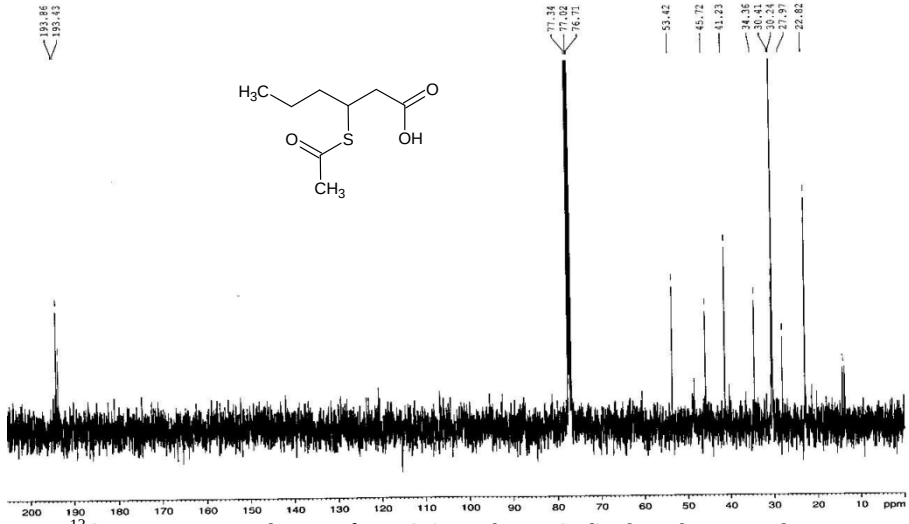

Figure 5.17. ^{13}C NMR spectrum deriving from S-(1-oxohexan-3-yl) ethanethioate oxidation.

Oxidation step applied to the acetyltio conjugate to hexanal determinded the oxidation of aldehyde to the corresponding carboxylic acid. On the other hand, addition of bisulfite to unsaturated aldehyde showed both addition to the aldehydic function and Michael type reaction leading to a mixture of sulfonate products. Purification trials both using silica gel column and anion exchange column did not allow to obtain pure products.
The addition of thioacetic acid in Michael type reactions, may be carried out on unsatured esters. Reduction of esters to corresponding alcohol has been reported by various reducing agent (Zhao et al., 2010). Among unsaturated esters, ethylhexenoate was chosen for this synthesis trial.

Ethyl hexenoate synthesis
26.76 mmol of freshly distilled butyraldehyde (1.326 g, 1.2 eq) was dissolved in 50 mL tetrahydrofuran anhydrous, then 22.3 mmol (1 eq) of triethylphosphonoacetate was added. The solution was then stirred and 22.3 mmol (1 eq) and *tert*-butyl hydroxide was added. The crude reaction mixture was then stirred for 3 hours under reflux. Reaction process was followed by TLC plates (λ: 254 nm) using hexane:ethylacetate 2:1 as eluent. Once no spot was longer visible for butyr aldehyde, an equal amount of hydrochloric acid 2 M was added and the organic solvent eliminated under reduced pressure. The aqueous layer was extracted twice with an equal volume of ethylacetate. The organic layer was then dried over sodium sulfate, and evaporated under reduced pressure to obtain a yellow oil. Ethyl hexenoate was then purified by distillation under reduced pressure at 165 °C to reach 18.5 mmol ethylhexenoate (figure 5.18).

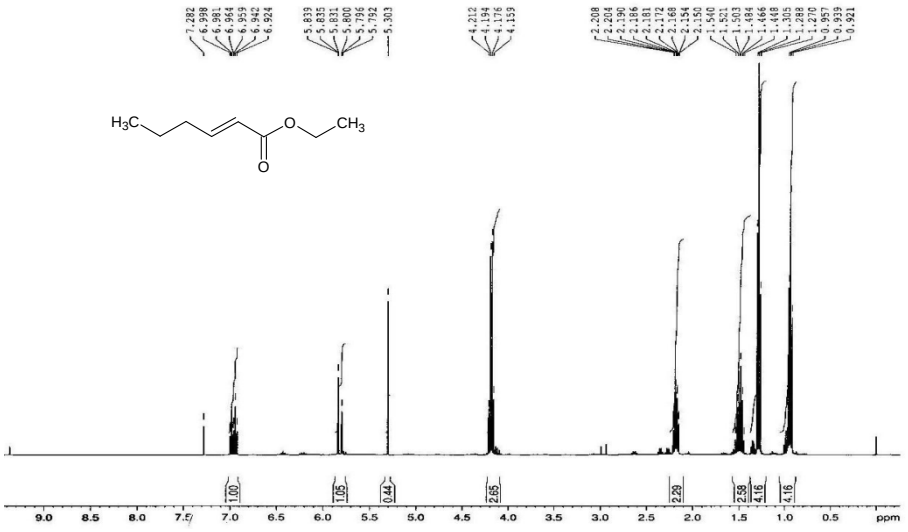

Figure 5.18. ^{1}H NMR spectrum of ethyl hexenoate.

Ethyl 3-(acetylsulfanyl)hexanoate synthesis: to ethyl hexenoate (0.3 g, 2.11 mmol) in 20 mL of THF at 0°C were added a mixture of thioacetic acid (0.222 mL, 1.48 eq) and DIPEA (diisopropylethylamine) (0.59 mL, 1.61 eq) in 20 mL THF over 20 min. After being stirred at 0°C for 1 hour, the mixture was allowed to warm at room temperature, then stirred at room temperature overnight and evaporated dryness. The residue was redissolved in dichloromethane, washed with $NaHCO_3$ (sat.), dried over $MgSO_4$, filtered, evaporated and chromatographed on

silica. The product was eluted with an ethyl acetate / hexane gradient (1:14 to 1:4) to afford the title product. ^{1}H NMR spectrum was used to identify synthetized compound (figure 5.19).

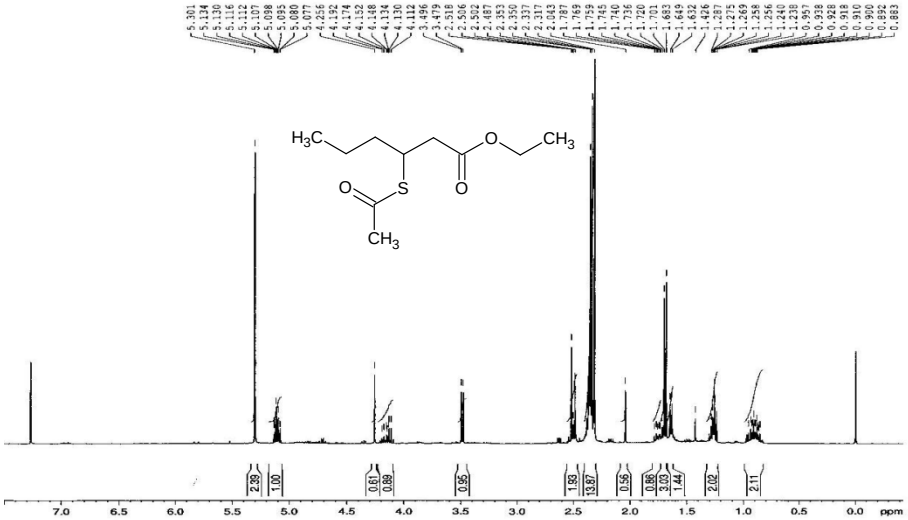

Figure 5.19. ^{1}H NMR spectrum of 3-(acetylsulfanyl)hexanoate.

1-ethoxy-1-oxohexane-3-sulfonic acid temptative synthesis by oxidation of 3-(acetylsulfanyl)hexanoate

Oxidation of acetylthio conjugate was carried out using H_2O_2 following two different synthesis trial:

- To ethyl 3-(acetylsulfanyl)hexanoate (0.414 g, 1.9 mmol) in acetic acid (7.8 mL) was added 30% H_2O_2 (3 mL). The mixture was stirred overnight, then dried dryness. (Zhao et al., 2010).
- A peroxyformic acid solution generated by adding 30% H_2O_2 (2.0mL) to 10mL formic acid (98%) and stirring 0.5 h at 0°C. Then, ethyl 3-(acetylsulfanyl)hexanoate (0.436g, 2.0 mmol) in THF (4.0 mL) and added to the peroxyformic acid solution at room temperature. The solution was then stirred overnight at room temperature (Chen & Xu, 2012).

The crude reaction mixture, in both cases, was then concentrated under reduced pressure and dried dryness. The methods used did not allow to oxidize the alkylthioacetate conjugate (figure 5.20).

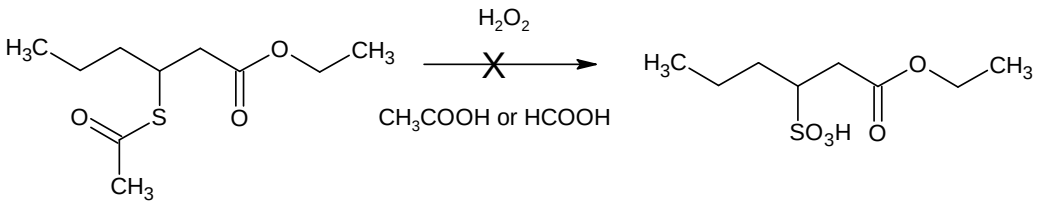

Figure 5.20. Temptative 3-(acetylsulfanyl)hexanoate oxidation to 1-ethoxy-1-oxohexane-3-sulfonic acid.

1-ethoxy-1-oxohexane-3-sulfonic acid synthesis by ethyl hexenoate sulfonylation

The sulfonylation of activated alkenes can be carry out with direct addition of bisulfite in the methanol/water solution at room temperature (Fini *et al*., 2010). Synthesis of sulfonates conjugates to ethyl hexenoate by direct addition of bisulfite to the unsaturated ester was then tried. The successful addion and purification was as follows: to ethylhexenoate (1.083 g, 7.63 mmol, 1 eq) in 50 mL water 10% methanol 2.83 mL $NaHSO_3$ 3.84 M (9.156 mmol, 1.2 eq) and 1.27 mL triethylamine (9.156 mmol, 1.2 eq) were added. The crude reaction mixture was stirred overnight under reflux. The mixture was then dried dryness and chromatographed on silica. The product was eluted with methanol/dichloromethane/acetic acid (10:90:1 to 20:80:1) to afford 0.7g of the title compound. **HRMS** (ESI +) found (M^+) 223.0645 $C_8H_{15}O_5S$, required 223.064.

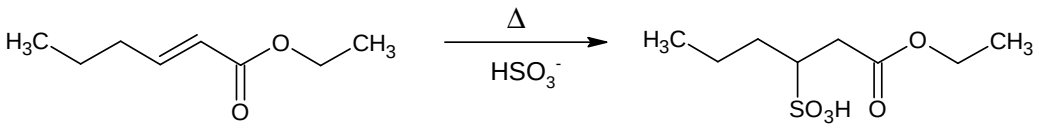

Figure 5.20. Ethyl hexenoate sulfonylation to 1-ethoxy-1-oxohexane-3-sulfonic acid.

Temptative synthesis of 1-oxohexane-3-sulfonic acidby reduction of 1-ethoxy-1-oxohexane-3-sulfonic acid

Reduction of sulfonic conjugate to saturated ester was then tried with two different reducing agents:

In the first case, Diisobutylaluminium hydride (DIBAL H) was used as reducing agent. 1-ethoxy-1-oxohexane-3-sulfonic acid (0.344 g, 1.5 mmol) was dissolved in 50 mL anhydrous THF at 0°C, then 3 mL Diisobutylaluminium hydride (DIBAL H) 1 M in cyclohexane (3 mmol, 2 eq) were added dropwise. After being stirred at 0 °C for 1 h, the mixture was allowed to warm at room temperature, then stirred at room temperature overnight. An equal volume of hydrochloric acid 2 M was added to quench DIBAL and stirred at room temperature for 0.5 h. The mixture was then dried dryness and chromatographed on silica by passing methanol/dichloromethane/acetic acid (20:80:1 to 50:50:1).

This reaction did not allow to reduce the ester to aldehyde or to alcohol. Although the reaction was carried out overnight under reflux, corresponding sulfonate conjugate to alkyl aldehyde or alcohol was not obtained.

$LiAlH_4$ was then tested as reducing agent: 2.75 g 1-ethoxy-1-oxohexane-3-sulfonic acid (12.6 mmol, 1 eq) was dissolved in 50 mL anydrous THF, then 1.43 g (37.8 mmol, 3 eq) $LiAlH_4$ and the crude reaction mixture was stirred over night under reflux. An equal volume of hydrochloric acid was added and the mixture was stirred at room temperature for 0.5 h, then dried dryness. The product was chromatographed on silica by passing methanol/dichloromethane/acetic acid (20:80:1 to 50:50:1). Also in the case of this second reducing agent, alcohol or aldehyde were not obtained. Reducing trial did not reduce the ester.

As a result, effort to obtain pure 1-oxohexane-3-sulfonic acid were not successful and the evaluation of this species as putative precursor of 3-MH in wines was not possible.

Conclusions

Biogenetic pathway of stable sulfonates formation reaction between bisulfite and *trans*-2-hexenal, is stricktly related to 1-oxohexane-3-sulfonic acid, whose structure is similar to 3-MH. The addition occours in aqueous acidic media, thus suggesting a similar biogenetic pathway in grape juice. The initial addition of bisulfite to *trans*-2-hexenal is at the aldehydic function. This reaction occours rapidly and it leads to *trans*-1-hydroxyhex-2-ene sulfonic acid, whose structure is neither similar to 3-MH nor stable. The formation of the stable 1-hydroxyhexane-1,3-disulfonic acid occours much slowly. Nevertheless, it is not a precursor of 3-MH and 3-MHA in wine.

Although *trans*-2-hexenal must exceed bisulfite to form 1-oxohexane-3-sulfonic acid, disulfonate is the most abundant reaction product. Moreover, the unsaturated aldehyde concentration in grape juice is 3 to 4 orders of magnitude lower than bisulfite concentration usually employed during harvesting. Despite this, many compounds can react with bisulfite, thus it may or not be in excess, thus suggesting that 1-oxohexane-3-sulfonic acid could be formed in grape juice as minor product of bisulfite addition to *trans*-2-hexenal.

The evaluation of this sulfonate as precursor of 3-MH was not possible since effort to synthetize 1-oxohexane-3-sulfonic acid did not allow to obtain pure species. New alternative synthesis pathway will be tested with the aim to study sulfonates as putative precursors of 3-MH in wine.

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6. Reduced and total glutathione in wine using different *Saccharomyces cerevisiae* yeast strains

Thiol nucleophilic addition to quinones is the major responsible for thiol related aroma loss in wine (Nikolantonaki *et al*., 2012). The presence of compounds able to reduce such quinone is strictly correlated to thiol stability in wine. The tripeptide glutathione(GSH), naturally occurring in grape and wine, can be responsible for this protective effect (Ugliano *et al*., 2011). Moreover, its nucleophilicity has been demonstrated to be higher than thiol related aromas (Nikolantonaki & Waterhouse, 2012) and its concentration in wine is about 1000 folds higer than volatile thiols.

GSH is constituted by L-cysteine, γ-glutammic acid and glycine and is known to reduce *o*-quinones deriving from both enzymatic oxidation carried out by polyphenoloxidase in juice and chemical oxidation in wines (Singleton et al., 1984, Li et al., 2008). During the former oxidation process in grape juice, caffeoyl-tartaric acid and coumaric-tartaric acid are the phenols mainly involved in the enzymic activity. The nucleophilic addition of GSH to oxidized caftaric acid generates the 2-glutathionylcaffeoyl-tartaric acid (Grape Reaction Product GRP) (Singleton et al., 1984) thus limiting both the condensation reaction of quinones with phenols and the brown compounds formation.

Glutathione occurs naturally in grape must up to 100 mg L^{-1} (Chenier et al., 1989), it can be present either as reduced or oxidized form. The accumulation of GSH starts at the onset of ripening (Adams & Liyanage, 1993, Okuda & Yokotsua, 1999) and its level is influenced by the nitrogen uptake in the vine (Chonè et al., 2006).

During grape crushing, GSH decreases rapidly due to its reactions with *ortho*-quinones (Singleton et al., 1985) and/or oxidation to the disulfide (Cassol & Adams, 1995). Wine making practices influence the tripetpide loss. Pressing condition, skin contact (Maggu et al., 2007), and oxygen exposure are known to influence the rate of glutathione decrease. In particular, reductive treatments (low dissolved O_2 during pressing) results in higher concentration in GSH (du Toit et al., 2007).

GSH represents an essential endogenous (Elskens et al., 1991) and exogenous sulphur source for the yeast (Grant C M et al., 1996, Kumar et al., 2003). At the beginning of the alcoholic fermentation GSH almost disappears and it increases during vinification process due to yeast activity (Fracassetti & Tirelli, 2011).

Cell lysis induces the GSH release in wine affected by the content of the nitrogen assimilable in must (Lavigne & Dubourdieu, 2004).

This tripeptide is about the 1% of the dry weight of *S. cerevisiae* (Penninckx & Elskens, 1993) and the main intracellular sulfur compounds having low molecular weight (Pennickx, 2002). Its presence is related to oxidative stresses response through glutathione peroxidase activity and detoxification process.

GSH is synthesized by the consecutive action of L-γ-glutamate-L-cysteine ligase (γ-GCS ligase) and L-γ-glutamylcysteine-glycine-γ-ligase (GSH synthetase) (figure 6.1). γ-GCS ligase appears to be a highly regulated enzyme (Lee et al 1999). Its activity is feedback-inhibited by GSH, preventing over-accumulation of the tripeptide (Penninckx & Elskens, 1993). Unlike to γ-

GCS ligase, GSH synthetase appeared to be a constituent unregulated enzyme (Inoue *et al*., 1998).

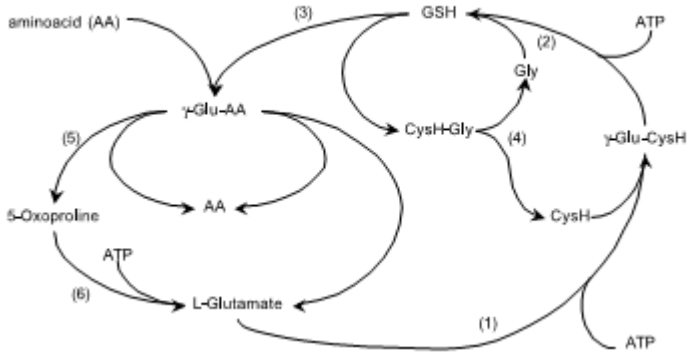

Figure 6.1. The γ-glutamyl cycle (Penninckx, 2002): (1) γ-glutamylcysteine synthetase; (2) GSH synthetase; (3) γ-glutamyltranspeptidase; (4) cysteinylglycine dipeptidase; (5) γ-glutamylcyclotransferase; (6) 5-oxoprolinase.

Glutathione is degradated by the activity of γ-glutamyltranspeptidase enzyme (γ-GT) in yeast. This enzyme catalyzes either the transfer of the γ-glutamyl residue to amino acids or the hydrolytic release of L-glutamate from GSH, different γ-glutamyl compounds, and S-substituted derivatives (Tate & Meister, 1981).
Besides methionine, homocysteine and cysteine, *S. cerevisiae* can metabolize GSH as sulfur source. It happens when *S. cerevisiae* growths with no sulfur source (Elskens et al., 1991).
In case of nitrogen starvation, more than 90% of the cellular GSH is shifted toward the central vacuole of the yeast (Mehdi & Penninckx, 1997) where γ-GT is responsible for its degradation. As a consequence, in sulfur and nitrogen starvation only small amounts of GSH can be released into the growth medium.
GSH is involved in cell response to reactive oxygen species (ROS) which may derive from yeast metabolism. These molecules are peroxides, including hydrogen peroxide (H_2O_2) and alkylhydroperoxides (ROOH) as well as lipid hydroperoxide (LOOH) which are generated in biological membranes from unsaturated fatty acids (Grant *et al*., 1996, Stephen & Jamieson, 1996), and superoxide anion.
Glutathione peroxidase is the key enzyme in the defence mechanism against hydroperoxides. Such enzyme catalyzes the reduction of hydrogen peroxide to water and the organic peroxide to the corresponding alcohol. The reduction reaction uses the reduced glutathione as equivalent source, leading to its oxidation to disulfide.
Toxic heavy metals (e.i. copper, zinc, silver, lead and cadmium) and xenobiotics can be accumulated by yeast (Penninckx, 2000). GSH plays a key role in cellular defence against reactive electrophiles such as halogenated aromatics. Many xenobiotics can react either spontaneously with the thiol moiety of glutathione to form S-conjugates, or via GSH S-transferases (GST) enzyme activity.

Even if many factors can affect glutathione concentration in wine, the yeast strain seemed have a strong effect (Lavigne *et al*., 2007). However, yeast lysis increases the concentration of amino acids, peptides and proteins and stabilize the thiol-related aromas, while yeasts absorb the oxygen (Salmon *et al*., 2000), during both wine ageing and storage.
Due to the great importance of GSH in preventing thiol aromas loss, the content of this compound was assessed (both reduced and oxidized forms) in wine which was produced after alcoholic fermentation carried by several *S. cerevisiae* strains. Moreover, the effect of prolonged wine ageing on the lees was deepened on GSH content.

Determination of reduced glutathione
Reduced glutathione was determined by HPLC-FLUO analysis using pre-column derivatization with *o*-phthalaldehyde (OPA) and 2-aminoethanol (AE) (Park *et al*., 2000). An Agilent HPLC system (1200 series) equipped with an autosampler which permits on-line derivatization was used for the analyses. This automatic HPLC system was controlled by Agilent chemstation. Using the gradient program shown in table 6.1 derivatives were separated on a Synergi 4u Hydro RP 80A column (150 mm x 4.6 mm ID 4 µm, Phenomenex, Torrence, CA) and detected by a fluorescence detector where wavelengths for excitation and emission were 340 nm and 450 nm, respectively.

time (min)	flow ($mLmin^{-1}$)	A %	B %
0.0	0.45	90.0	10
2.0	0.45	85.0	15
6.0	0.45	72.0	28
7.0	0.45	68.0	32
9.0	0.45	64.0	36
10.0	0.45	56.0	44
12.0	0.45	52.0	48
19.0	0.45	50.0	50
21.0	0.45	40.0	60
26.0	0.45	0.0	100

Table 6.1. HPLC separation gradient for thiol compounds derivatized with OPA. Eluents acetate buffer 50 mM pH 5.7 (A) and methanol (B).

N-acetylcysteine was added as internal standard (IS) (5 mg L^{-1}) to wine samples and submitted to precolumn derivatization as follows: 4 µL OPA in methanol were withdrawn (2 mg mL^{-1}), then 5 µL of sample and 4 µL AE (4 mg mL^{-1} in borate buffer 20 mM pH 8.0) were withdrawn and mixed for 2 minutes. The derivatized sample (total 13 µL) was then injected for analysis. Ratio between reduced glutathione (GSH) and IS amount were compared to ratio between corresponding peak areas to obtain calibration curve (figure 6.2). It showed linear response for GSH concentration up to 40 mg L^{-1}.

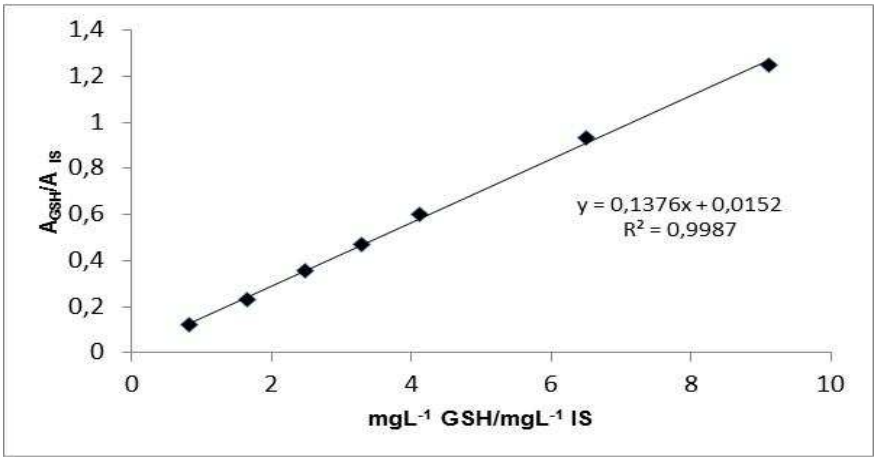

Figure 6.2. Calibration curve of reduced glutathione GSH dissolved in acetate buffer 5 mM, pH 5.40.

Determination of total glutathione

The total glutathione (tGSH) was calculated as the sum of GSH and oxidized glutathione (GSSG) which was reduced to the thiol form before the derivatization as described by Kusmierek & Bald (2008). Aliquots of standard solutions and samples (0.5 mL) were added with 0.25 mL of sodium borohydride ($NaBH_4$) 6 M dissolved in dimethyl sulfoxide and 0.12 mL HCl 3 M. The mixture was stirred for 2 minutes and then 0.12 mL HCl 3 M in order to decompose the excess of $NaBH_4$. N-acetylcysteine 500 mg L^{-1} as internal standard was added (5 µL) and pre-column derivatization was performed as previously reported.

Micro-fermentation of grape juice

S. cerevisiae yeast strains (ISE 128, ISE 77, ISE 24, ISE117, ISE81, BK1) from the collection of the CRA-Centro di Ricerca per l'Enologia (Asti, Italy) were first propagated in YPD medium (1% yeast extract, 2% tryptone, 2% glucose), then inoculated at 10^6 UFC mL^{-1} in a white grape must (20.2 Bx) (Arneis *cv*.) previously sterilized by incubation with 200 µL L^{-1} (v/v) dimethyl dicarbonate per liter of grape juice at room temperature overnight to kill all microbes, with no addition of nitrogen source. Ferments were conducted at 20 °C in 1 L Erlenmeyer flasks with 750 mL sterile grape juice. The cell growth was monitored at 600 nm and the fermentation process was monitored by HPLC analysis of the medium using a refractometric detector. At the end of alcoholic fermentation, the ferments were harvested. One milliliter of wine was centrifuged at 6000 x *g* for 10 min to pellet the solids. The supernatant was then submitted to further analysis. Fermentation were carried out in duplicate. Yeast lees were resuspended weekly for three months. The determination of reduced and total GSH was carried out in wines at the end of alcoholic fermentation and after three months of ageing on lees.

Results and discussion

The alcoholic fermentation was completed in 15 days. Similar growth curves were obtained for the yeast strains used. Only the strain ISE 128 showed a different pattern (figure 6.3)

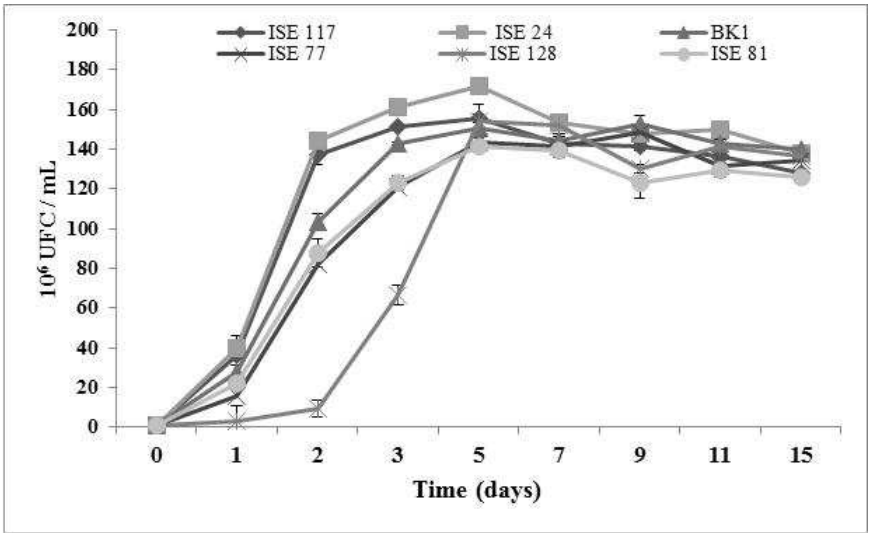

Figure 6.3. Growth curves durng alcoholic fermentation of grape juice (Arneis cv) carried out by different *S. cerevisiae* yeast strains.

GSH concentration in juice before alcoholic fermentation was lower than 1 mg L^{-1}. The formation of 2-S-glutathionyl caffeoyl tartaric acid (GRP), when glutathione reacts with certain quinones, during extraction of the must has been clearly described (Singleton *et al*., 1985). Moreover, GSH could also disappear from must by oxidation into disulfide (Cassol & Adams, 1995). Thus GSH content at the end of alcoholic fermentation, could be related to the yeast. The content of readily assimilable nitrogen could represent a limiting parameter on the *S. cerevisiae* GSH biosynthesis. The readily assimilable nitrogen content in the must used for the trial was 295 mg L^{-1}. As a result, the GSH content in wine was higher than in musts. These data are in agreement with Lavigne & Dubourdieu (2004) which suggested 200 mg L^{-1} of readily assimilable nitrogen is needed to allow the release of GSH by the yeast.

In particular, the employement of various yeast strains during laboratory-scale alcoholic fermentation led to different reduced glutathione content (figure 6.3). At the end of alcoholic fermentation, glutathione was mainly in its reduced form (GSH). In fact, the amount of GSH and tGSH (figure 6.4) was similar.

The employment of several yeast strains affected tGSH content too (figure 6.4). It is noteworthy that ISE 128 yeast strain showed the lowest GSH and tGSH release. During the three months of ageing on lees the total GSH content did not increase (figure 6.4): neither reduced nor oxidized glutathione were released during aging on lees. This suggests that production and liberation of this tripeptide is clearly related to living cells.

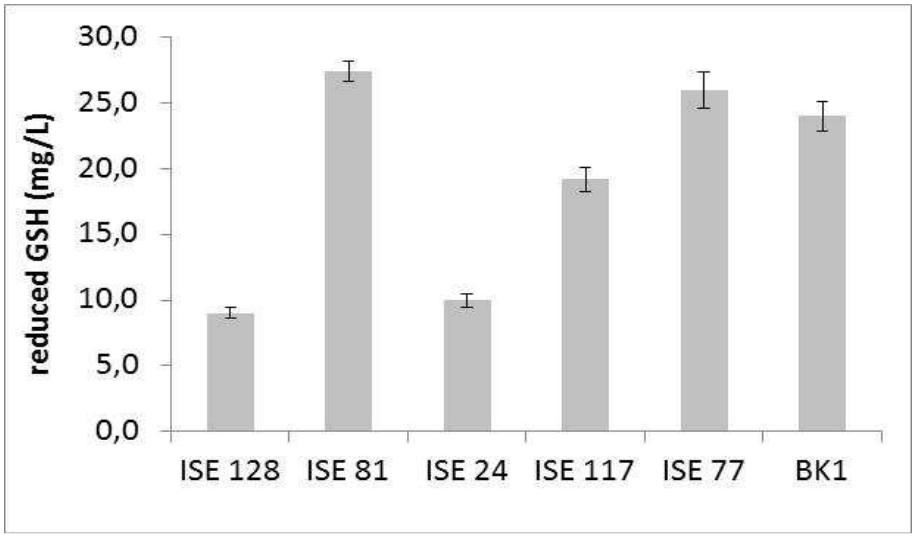

Figure 6.4. Reduced glutathione (GSH) in wines after alcoholic fermentation carried out by different *S. cerevisiae* yeast strains.

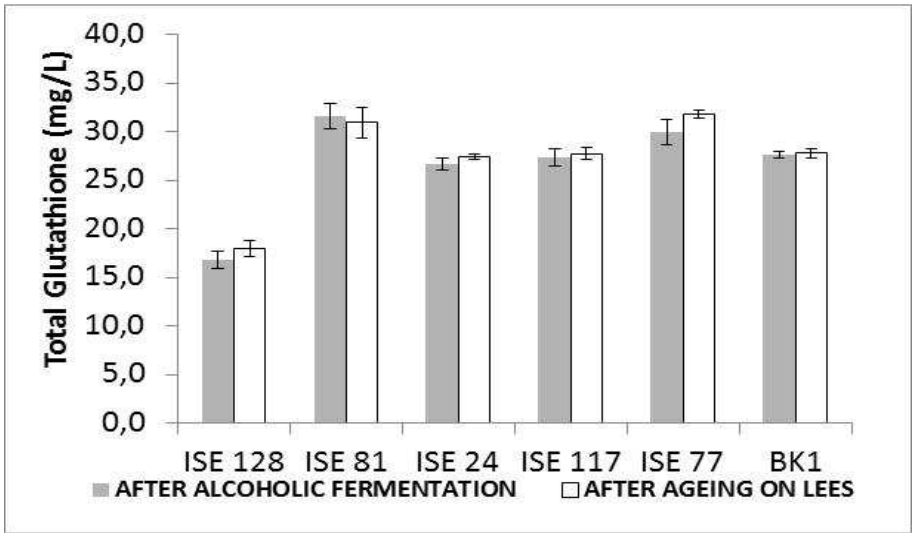

Figure 6.4. Total glutathione after alcoholic fermentation and after three months ageing on lees.

Conclusions

The fermentative activity of yeast strain influences reduced and total glutathione content in wine. During ageing on lees neither reduced nor oxidized glutathione is released, thus suggesting that production and liberation of this tripeptide in is related to living cells.

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Appendix 1 Copy of papers, oral communications and posters

JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY ARTICLE

J. Agric. Food Chem. 2010, 58, 11969–11976 11969
DOI:10.1021/jf102603r

Molecular Basis of the Interaction between Proteins of Plant Origin and Proanthocyanidins in a Model Wine System

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Plant proteins are being used as a replacement for animal proteins in wine fining. The surface hydrophobicity of plant proteins in four commercial preparations differing for their origin and processing was assessed by using a fluorescent hydrophobic probe in wine-like media. Displacement of the probe by addition of wine phenolics was measured as a way to compare and predict to some extent the efficiency of these proteins in wine fining. It was found that the binding of polyphenols was much more specific than that of the hydrophobic probe. Further analysis of the polyphenol pattern in protein-treated wine-like solutions pointed out two relevant facts: (1) proteins may interfere with the chemistry of the interactions between polyphenols and other wine components; (2) individual protein preparation having different surface hydrophobicities also have different specificities in binding different polymeric forms of the polyphenols and in their substitution products. These findings are related to the possible carry-over of transition metals and may be worth exploring for custom tailoring the fining process. Whether the practical application of the latter finding will call for production and/or screening of plant-derived proteins with features appropriate to this task remains to be investigated. However, the approaches presented in this study may be used for large-scale screening of protein suitability for fining application under laboratory conditions, providing guidelines for their use in actual winemaking applications.

KEYWORDS: Wine fining; plant proteins; anthocyanidins; protein hydrophobicity; mass spectrometry

33rd World Congress of Vine and Wine, **20-25 June 2010. Tblisi, Georgia**

Valorisation and characterisation of native Piedmont grape variety: the Uvalino

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The recovery of old native varieties can play an important role to preserve the biodiversity of wine area, allowing to obtain useful information about genetic, viticultural and enological knowledge. In this work, Uvalino, a Piedmont indigenous cv, was characterized by the analysis of grape and wine. The analysis of varietal glycosides aromatic compounds had shown a prevalence of benzenoids, particularly benzyl alcohol and 2–phenilethanol; moreover a relevant content in eugenol was observed. Within monoterpenic compounds, geraniol and its derivates were prevalent. Ocimenols, actinidols and Riesling acetal were the main molecules obtained by the chemical idrolysis reaction on the grape extract. These latter compounds, together with esters, charaterized also the wine aromatic profile. The malvidin and peonidin were prevalent monomeric anthocyanins in the Uvalino grapes. Moreover the content in resveratrol, a phenolic compound known for its remarkable antioxidant properties, was high both in grapes and wines.

ItPA, Italian Proteomics Association **Congresso Nazionale 2010. 9-12 Giugno 2010. Firenze, Italy**

Interaction between proteins of plant origin and wine components: molecular-based choice of protein fining agents for organoleptic improvement

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Gelatine, casein, egg albumin, and, more recently, proteins of from plant sources are commonly used in winemaking as fining agents to remove particles responsible for turbidity, to improve stability, and to control browning, over-oxidation, and bitterness during ageing. The formation of covalent and non-covalent interactions between the protein matrix and wine polyphenolics is the basis of the flocculation and of the consequent clarification which results in an overall improvement of wine quality parameters. In this work we studied the molecular basis of the interactions between plant proteins (soy, gluten, lentil and pea proteins) and polyphenolic compounds responsible for organoleptic as well as stability properties of wines, by using mass spectrometry methodologies (LC-ESI MS, MALDI TOF MS). Protein surface hydrophobicity was investigated in wine-like model system by spectrofluorimetric determination of changes in the binding properties of 1,8-anilinonaphthalenesulfonate (ANS), used as extrinsic fluorescent probe. Hydrophobic interactions between phenolic compounds and protein finings were evaluated by the study of competition of phenolic compounds with the ANS probe for the same binding sites. Structural characterization of phenolic compounds (polymer chain length and chemical structure and composition of individual chains), as well as their interactions with the plant proteins, essential for the definition of protein binding affinity, was performed by means of mass spectrometry techniques. Differences among interactions between polyphenols with the various protein matrices have been related with the quality parameters of the resulting wines.

***Congresso Internazionale sulla Viticoltura di Montagna*. 12/14 Maggio 2010. Castiglione di Sicilia (Catania) Italy**

Sensory profile and chemical composition of "Albarola" and "Bosco" white wines of "Cinque Terre" – vintage 2007

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The DOC white wines of the "*Parco Nazionale delle Cinque Terre*", a region whose charm is well known, are blends produced with the following three cultivars "Albarola", "Bosco" and "Vermentino". Bosco, unlike the others, is mainly cultivated near the sea. This study was part of a project of valorisation of this "terroir", (LABTER, MIPAAF). Its aim was to compare Albarola and Bosco wines obtained in purity with grapes grown at two different altitudes (430 m and 240 m a.s.l.) in the 2007 vintage. Wine physical-chemical parameters, colour intensity and hue, volatile and phenolic compounds were analysed. A trained panel described the sensory profiles of the wines and evaluated their acceptability. The wines of the both varieties obtained with grapes grown at 430 m had a lower content of alcohol, a higher acidity and a lower pH. These parameters were also pointed out by the sensory analyses. Bosco wine obtained with grapes grown at 430 m had a lower colour intensity and resulted less agreeable. A higher content of isoamyl acetate (exotic fruit), 2-phenylethanol, 2-phenylethylacetate (flower) was detected in the Albarola wine produced with grapes grown at the lowest altitude. A higher sugar content and a lower acidity were pointed out in the Bosco grapes cultivated nearest the sea. This wine was also characterized by a greater harmony and body but no relevant differences were observed in the aromatic profile. The Albarola wine, obtained from vineyard grown at 240 m, showed a more intense aroma and was more acceptable.

Macrowine 2010 Third International Symposium on macromolecules and secondary metabolites of grapevine and wines. 16/18 June 2010. Torino, Italy

Uvalino wine: chemical and sensory profile

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Uvalino cv is a Piedmont native variety only recent admitted as licensed Italian grapevine. The wines needs two years of ageing. In the present work two experimental wines from different vintages (2004 and 2006) were evaluated in 2009. The chemical data were compared with the sensory profiles.
Uvalino wines showed a high alcohol content, a low acidity and a high pH. Moreover, they presented a remarkable content of phenolic compounds. The concentration of anthocyanins was particularly low, whereas the content of tannins, responsible for intense astringency, was high. Flavan-3-ols content was consistent during wine aging. In spite of low concentration of anthocyanins, the perceived intensity of color was high: this proved that the molecules giving the color to wines reached a high degree of stability. This color stability was also observed in the oldest wine demonstrating that co-pigmentation reactions took place.
The characterization of varietal and fermentative aromatic compounds showed a high content of benzenoids, particularly benzyl alcohol. Their presence, together with C13-norisoprenoid compounds, gave to the wines important sensory features correlated to the sensory analysis.
The evaluation of the wine sensory profile showed that fermentative aromatic compounds phenylethanol and 2-phenylethyl acetate, responsible for fruity and floral notes of wines, were most correlated with the sensory descriptors identified.
The chemical and sensory analysis showed how different molecules present in wines can contribute in a complex way to the formation of the sensory profile of a product.

Oeno 2011 9éme edition du Symposium International d'Oenologie. 15/17 June 2011. Bordeaux, France

Uvalino wine: chemical and sensory profile

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Numerous factors, such as exposition, soil composition, climatic conditions and growing-technique, influence the evolution of grapevine maturity in different locations. Moreover, also in the same vineyard and at a given date of harvest, the physiological characteristics of berries result related to the position of the cluster and/or the single berry, leading to wide heterogeneity. The wine making of heterogeneous grapes can modify the wine composition, altering its quality. Unripe berries from red grapes lead to low extractability of anthocyanins and proanthocyanidins from skins, while the galloylated proanthocyanidins from seeds result more easily extracted, contributing to increase bitterness and astringency. The distribution of berries, according to the concentration of total soluble solids at a given date, follows the trend of a Gaussian function: then a large number of unripe berries are harvested.
The aim of this work was to evaluate the differences of aromatic and polyphenolic patterns of berries harvested at the same date but characterized by different sugar concentrations.
Three Piedmont varieties were analysed: an aromatic (cv. Moscato Bianco), a white neutral (cv. Arneis) and a red neutral (cv. Barbera) grapevines. For each cultivar two different location were considered and analysed in two vintages (2008 and 2009). The berries of each cultivar were harvested and separated according to their percentage distribution in salt solutions. Barbera was characterized by high heterogeneity of berry density, independently from location and year; instead Moscato and Arneis showed a different degree of berries distribution according to the localization, independently from the year of harvesting. Different aromatic and polyphenolic patterns were observed for the cultivars, both for the comparison between groups of flotation and between groups and mass; these differences increased when the data were expressed per kg of grape than per number of berries, suggesting that the berries characterized by higher content of total soluble solids showed a slight dehydration.

Enoforum 2011, 3-5 May 2011, (Arezzo, Italy)

Effetto del trattamento con proteine vegetali sulle componenti aromatiche e tanniche del vino

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La chiarifica mediante collaggio proteico consente di rimuovere dal vino le sostanze di natura colloidale responsabili della torbidità, migliorandone stabilità e caratteristiche organolettiche, controllando l'imbrunimento e la polimerizzazione ossidativa di composti polifenolici e riducendo la sensazione di astringenza. Le proteine animali sono state per anni le più utilizzate, ma le restrizioni normative rispetto ai coadiuvanti enologici di origine animale, rendono interessante l'impiego di proteine vegetali. Nel presente lavoro sono state studiate le interazioni non covalenti tra molecole idrofobiche responsabili di note organolettiche nei vini, quali polifenoli e molecole odorose, e chiarificanti proteici di origine vegetale da leguminose (soia, pisello e lenticchia) e da cereali (frumento). L'obiettivo della sperimentazione è comprendere il meccanismo di interazione di tali proteine per consentirne un impiego più selettivo e razionale, verificando l'ipotetica esistenza di interazioni preferenziali che possano essere sfruttate per rimuovere e/o trattenere nel vino classi specifiche di composti. L'indagine è partita da soluzioni idroalcoliche modello ed è stata estesa a vini bianchi e rossi. I composti coinvolti nell'interazione ed i complessi generati sono stati caratterizzati combinando tecniche separative e di spettrometria di massa. Nella fase successiva dello studio è stata valutata l'incidenza del trattamento di chiarifica sulle componenti sromatiche ed aromatiche di vini bianchi e rossi.

Enoforum 2011, 3-5 May 2011, (Arezzo, Italy)

Influenza della pacciamatura sui precursori aromatici di uve Arneis

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La gestione del vigneto influenza notevolmente la qualità dell'uva e del vino e molte sono le tecniche agronomiche utilizzate a tale scopo.
Recentemente è stato introdotto l'uso di teli riflettentie coperture del suolo che possono incrementare la luce solare nella zona dei frutti quindi migliorandone la maturazione e avere effetti positivi sulla qualità dei vini.
In questo lavoro si è valutata l'influenza della pacciamatura con telo bianco sul profilo dei precursori aromatici di uve Langhe Arneis DOC, in tre stadi di maturzione (preinvaiatura, invaiatura e raccolta) con tre ripetizioi biologiche.
Non sono state evidenziate differenze in nessuno stadio di maturazione sull'accumulo di solidi solubili, pH e acidità totale tra le due tesi. Relativamente ai composti aromatici glicosilati si osserva un ritardo iniziale nell'accumulo dei terpeni nella tesi pacciamata, ma alla raccolta le concentrazioni sono simili. Il geraniolo glicoside tende ad aumentare durante la maturazione ed è sempre un po' più elevato nella tesi non pacciamata. L'α-terpineolo sembra diminuire mentre il linalolo è rilevabile solo alla raccolta in entrambe le tesi, tuttavia nessuna differenza significativa è stata evidenziata dall'analisi statistica effettuata con XLSTAT.
Per i benzenoidi glicosilati si osserva un andamento simile a quanto già descritto per i terpeni mentre i norisoprenoidi aumentano in maturazione in entrambe le tesi e mostrano valori un po' più elevati all'invaiatura e alla raccolta nella tesi pacciamata.
Si ritiene pertanto che l'uso di teli per pacciamatura non abbia comportato alcuna perdita di qualità delle uve Arneis, né dal punto di vista tecnologico, né dal punto di vista dei precursori d'aroma, non si sono però riscontrati miglioramenti evidenti.

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Influence of Glutathione and Caffeic Acid on "Moscato d'Asti" Aroma

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Various aroma volatiles decrease during wine aging. These compounds include acetate esters, ethyl esters, terpenes, and others such as volatile thiols; losses in volatile components may be due to oxidation or other chemical reactions. For example ester concentration may change because of hydrolysis and esterification.

The typical aroma of the sweet-dessert wine Moscato d'Asti D.O.C.G. is due to low olfactory threshold terpenes and fermentation volatile compounds. The vulnerability of Muscat wine is also well-known problem imputed to the loss of their characteristic aroma during storage in bottle. Previous researches have revealed that both high acidity and conservation temperature as well as light exposure can accelerate terpenoid's chemical degradation and generally can shorten wine's "shelf-life". Terpenes as geraniol and nerol can interconvert and then form α-terpineol while linalool, the most important terpene for the Muscat aroma may be replaced by a α-terpineol too.

The oxidative spoilage of white young wines, from an aromatic point of view, is also a phenomenon that leads to a loss of floral and fruity aromas with subsequently formation of atypical notes associated with the deterioration of the product.

Currently in the market, sweet-dessert aromatic wines like Moscato d'asti D.O.C.G. contain high levels of free sulfur dioxide, even if under the EU law limit. As a result of the sulfur dioxide disadvantages on human healt, the trend is to limit this use.

Moreover, Roussis and co-workers (2007) found that sulphur dioxide gives limited protection to wine volatile after bottling while caffeic acid and glutathione or their mixture, natural constituents of wine, slow the decrease of several esters and terpenes during white wine storage.

Consequntly, the aim of this work was to examine the effect that glutathione and caffeic acid have on the characteristics of a particular and vulnerable wine as Moscato d'Asti D.O.C.G.

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Evaluation of stilbenes content in grapes (cv. Uvalino) during ripening and drying

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The chemical compounds belonging to the stilbenes have recently received a particular attention due to the role they play in plant physiology (phytoalexins) and for their anti-oxidant properties. The Uvalino is a red-berry grape variety typically growth in Piedmont, Italy. This cultivar is characterized by its ability to synthesise large amounts of resveratrol glucoside (*trans* piceid). This explains its high resistance to *Botrytis cinerea*. The aim of the work is the evaluation of stylbenes and other phenolics during ripening and drying of Uvalino grapes. After accumulation, values for trans-piceatannol and trans-pterostylbene remain constant during the whole ripening period, while the piceide isomers continue to be synthesized until harvest. At the harvest time, the most abundant stylbene appears to be piceide (*trans* and *cis*) and *trans*-pterostylbene, while *trans*-piceatannol is the lowest. The amount of total stylbenes found is remarkably higher than other Piedmontese, Italian and international wines. Due to the highlevels of resveratrol also in the Uvalino wines, the study of the different stylbenes found in grapes, both in their glucoside and free forms, could be interesting for nutraceutic purposes, or alternatively, be used as varietal marker. The determination of the stylbenes during ripening, suggests that their synthesis begin at veraison, as for anthocyanins, but the stylbenes accumulation in berries happens suddenly. The high content in stylbenes and the low quantity of anthocyanins are a varietal character. The amount and evolution of stylbenes and other phenolic compounds have been monitored during drying in a drying room and over –ripening on the plant. As expected, the berries drying process appears to be more intense in grapes placed in a drying room, while all compounds are reduced when drying directly on the plant. When drying takes place under optimal temperature and humidity conditions, as in the drying room, the values of the main phenolic indices are higher than that found at harvest. On the other hand, on the plant, the reductions are far more important, with the exception of stylbenes and hydroy-cynnamic acids.

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The sensory profile of grapes for variety characterization

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Grape tasting gives a global characterization of the product with the evaluation of flavour and texture parameters. It is more and more used by professionals as an instrument to decide the harvest time and to allow the winemakers to adapt the vinification techniques. Nevertheless, the definition of the sensory profile of grapes can be also important to characterize a cultivar. In this experience the grape sensory profile of grapes can be also important to characterize a cultivar. In this experience the grape sensory profiles of some important Italian varieties have been realized: the white and aromatic cv. Moscato Bianco di Canelli, the white cv. Arneis and Manzoni Bianco, the red cv. Barbera, Croatina and Nebbiolo. At the technological maturity the berries were collected in different locations in north-western Itay (Piedmont) on the same vineyards in two vintages – 2008 and 2009- with different climatic conditions. They were calibrated according to their density estimated by flotation in salt solutions. Only the largest classes were chosen for the sensory analysis. The grape sensory profile was realized by a trained panel of CRA-ENO (9 assessors) using a method set up from previous experiences. A common vocabulary and a tasting sheet were developed using table grapes. The final descriptors of grape were twenty-seven and allowed a characterization of berry, skin, pulp and seeds. Their intensity (except for 4 qualitative descriptors) was evaluated in duplicate on an unstructured scale. The largest classes were not the same in 2008 and 2009, but they were representative of the grape condition at the moment of the technological maturity. The results showed that the sensory profile of berries can discriminate and characterize a cultivar despite the vintage and the origin of grapes. Only in the case of the "Moscato bianco di Canelli" the sensory profile was influenced by the different location of the vineyards.