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XXII CICLO

IDENTIFICATION, QUANTITATION AND ORIGIN OF SULFUR COMPOUNDS IN GRAPE PRODUCTS. AN APPRAOCH VIA HYPHENATED MASS SPECTROMETRY TECHNIQUES

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RIASSUNTO

Il contributo della Chimica Analitica nello studio delle matrici complesse, quali ad esempio quelle alimentari, è cruciale. In particolare lo sviluppo di metodi cromatografici avanzati può permettere di dosare importanti marker tecnologici, microbiologici e fisiologici e quindi consentire un approfondimento delle attuali conoscenze.

Questo lavoro è stato concepito allo scopo di definire gli strumenti per valutare il contributo di un'importante classe di molecole all'aroma dei vini.

Il vino riveste un ruolo di primaria importanza nelle tradizioni culturali ed alimentari Italiane, inoltre l'Italia risulta primo produttore e primo consumatore mondiale di vino. I prodotti enologici sono stati largamente studiati fin dai primi anni '70, anche se l'assenza di conoscenze biochimiche e microbiologiche e la mancanza di tecniche analitiche sufficientemente sensibili non ha consentito lo studio esaustivo della classe dei composti solforati.

Questa tesi, nata da esigenze legate alla chimica analitica e alla chimica enologica, ha avuto come punto centrale lo studio dei composti solforati nei vini.

Il primo problema che si è dovuto affrontare nello studio di matrici complesse come quelle enologiche è stato quello relativo agli effetti matrice. Il superamento o il contenimento di questo problema crea ogni giorno costi in termini di tempo e di preparazione di opportuni standard interni. In questa tesi si è proposto un metodo statistico basato sul modello a componente di varianza in grado di gestire questo problema. I vantaggi sono notevoli, a fronte di una incertezza leggermente maggiore e di una sensibilità di poco peggiorata.

Altro argomento trattato è stata la definizione dei metodi analitici in grado di quantificare e quindi di studiare i composti solforati. Questa materia è stata affrontata riconoscendo una prima distinzione all'interno del gruppo degli analiti indagati:

- composti solforati fermentativi: molecole derivanti dal metabolismo del lievito a partire da precursori amminoacidici
- composti solforati varietali: molecole presenti in specifiche varietà come coniugati della cisteina e del glutatione e rilasciati durante la fermentazione alcolica ad opera di specifiche attività enzimatiche.

Questa suddivisione è stata mantenuta anche nello sviluppo dei metodi analitici, in quanto sensibilità richieste e conseguenze metabolomiche erano anch'esse distinte.

Per quanto riguarda i composti solforati fermentativi, durante il dottorato di ricerca è stato ottimizzato e validato un metodo HS-SPME/GC-MS per l'analisi di un'ampia gamma di molecole tipicamente derivanti dal metabolismo del lievito. Tale approccio è poi stato la base per studi di natura più squisitamente metabolomica in cui è stato verificato come il *cultivar* (i.e. la varietà), l'invecchiamento, il ceppo di lievito e altre pratiche tecnologiche potessero influenze il livello e l'evoluzione degli analiti indagati.

I risultati ottenuti per quanto attiene lo sviluppo del metodo in spettrometria di massa hanno potuto confermare con la tecnica SPME con campionamento in spazio di testa sia una tecnica di elezione per l'analisi di molecole volatili. Inoltre questo approccio consente una gestione semplice ed *enviromental friendly* in confronto con i più obsoleti metodi di estrazione con solvente. Gli studi metabolomici hanno consentito di trarre importanti considerazioni su aspetti molto importanti nella definizione della qualità del prodotto finale. Tali risultati consentono anche una trasferibilità nei settori della ricerca applicata e dell'enologia.

Come ultima classe di analiti indagati si sono studiati i composti solforati varietali. L'assenza di metodi sensibili e sufficientemente rapidi da essere applicati per studi su un numero significativo di campioni ha spinto la nostra attenzione alla verifica delle potenzialità delle tecniche in spazio di testa nel dosare queste molecole. Si sono ottimizzati e validati metodi HS-SPME e purge and trap ed è stata definita una procedura non parametrica robusta per il confronto delle performance delle diverse procedure.

Visto il recente interesse della ricerca scientifica operante nel settore al controllo della formazione ed evoluzione di queste molecole, abbiamo focalizzato la nostra attenzione sui precursori dei composti solforati varietali.

Il primo stadio è stata la sintesi di queste molecole, seguito poi dall'ottimizzazione di metodi LC-MS/MS per il dosaggio di queste molecole. In questo contesto è stato possibile identificare per la prima volta un nuovo potenziale precursore di aroma, tramite esperimenti LC-MS/MS.

Da ultimo, attraverso l'isolamento dell'enzima responsabile della biosintesi dei precursori d'aroma è stato possibile proporre una preliminare spiegazione alla formazione di tali molecole durante la maturazione della materia prima.

In conclusione, grazie a questa tesi di dottorato, è stato possibile approfondire ed applicare alcune delle più moderne tecniche analitiche a disposizione, è stato possibile definire procedure statistiche per superare i più comuni ostacoli nello studio di matrici complesse, ed è stato possibile chiarire il contributo di una classe importante di molecole come i composti solforati nelle matrici enologiche.

ABSTRACT

The contribute of the Analytical Chemistry in the study of complex matrices, as for instance foodstuff, is crucial. In particular the development of chromatographic methods allows to quantify important technological, microbiological and physiological markers. The ensemble of all these information furthers a deeper and higher knowledge.

This Ph.D. project was designed to define the tools and evaluate the contribution of an important class of molecule on wine aroma.

Grape products are really important in the cultural and dietary Italian traditions; moreover Italy is both the first producer and the first consumer around the world. Oenological products have been largely studied since the early ’70, even if the lack of biochemical and microbiological knowledge and the poor sensitivity of the analytical techniques did not allow to carry an deep study on sulfur compounds.

This project, planned to bid analytical and oenological chemistry, focussed its attention on the study of sulfur compounds.

Dealing with complex matrices, the first problem we had to face was the matrix effects. The overcoming of such issue or the attempts in reducing it, costs to chemical laboratory, wasting of time and money to prepare suitable internal standards.

In this thesis we proposed a statistical approach based on the variance component model able to handle matrix effects. The benefits obtained by this approach definitely outweigh both the slightly worse sensitivity and uncertainty.

Another topic discussed in the thesis is the definition of analytical methods to quantify sulfur compounds. This subject has been studied distinguishing sulfur compounds into two sub-groups:

- fermentative sulfur compounds: molecules produced by yeast metabolisms from amino acidic precursors.
- varietal sulfur compounds: molecules present in specific variety, as conjugated with cysteine and glutathione. The free forms, which are

sensorially active, are released during fermentation by means of a specific enzymatic activity.

This further classification was kept in the development of the analytical methods because the two classes required different sensitivity as well as metabolomics study were separated.

Concerning fermentative sulfur compounds a HS-SPME/GC-MS method was optimised and validated. With this method a wide range of sulfur compounds were quantified. Such tool was applied to metabolomic studies, concerning the influence of variety, ageing, yeast strain and other technological practice on the level of 13 fermentative sulfur compounds.

The results obtained from the development of the analytical method permitted to highlight the potentiality of the HS-SPME technique in sampling volatile compounds in complex matrices. This approach allows an easier and safer lab conditions, avoiding the use of organic solvents. The metabolomic studies furnished important suggestion on the influence of important oenological variables on the level of important sulfur compounds.

The last topic discussed in this thesis concerns the study of varietal sulfur compounds. The lack of suitable analytical technique, sufficiently rapid and avoiding the use of mercuro-organic compounds stimulated our research in verifying the performance of headspace technique in extracting these analytes.

We optimised and validated HS-SPME and purge and trap methods and finally we defined a non-parametric robust approach to compare performances from different analytical methods.

Owing to the recent interest of the scientific research in trying to understand the formation and evolution of varietal sulfur compounds, we focussed our attention on their precursors.

The first step was the synthesis of the putative precursors, followed by the optimisation of LC-MS/MS methods to quantify them. It was possible to identify a new precursor by LC-MS/MS experiments.

Finally, by the isolation of the enzyme potentially responsible of the biosynthesis of the varietal thiols precursors, we gave a preliminary explanation of the formation of these glutathionylated precursors during grape ripening.

By this project it has been possible to study and apply the newest analytical techniques available and it has been possible to define statistical procedures to overcome the most common issues in studying complex matrices. Furthermore it was clarified the contribution of an important class of molecules, such as sullfur compounds, on oenological matrices.

GENERAL CONSIDERATIONS

Analytical chemistry is described as the area of chemistry responsible for characterising the composition of matter, both qualitatively (what is present) and quantitatively (how much is present). This description is limitative and quite misleading as almost all chemists routinely make qualitative or quantitative measurements. Therefore the argument has been made that analytical chemistry is not a separate branch of chemistry, but simply the application of chemical knowledge. The role of analytical chemistry is really not in performing a routine analysis on a routine sample (which is more appropriately called chemical analysis), but developing new methods for measuring chemical phenomena, improving established methods and extending existing methods to new types of samples.

In particular the challenge of developing and validating the method providing information is in the analytical chemist's responsibility and once the method is developed, the routine, daily application of the method, becomes the job of the chemical analyst.

Following these arguments the Division of Analytical Chemistry of the Federation of European Chemical Societies (FECS) defines "Analytical chemistry is a scientific discipline that develops and applies methods, instruments, and strategies to obtain information on the composition and nature of matter in space and time". Throughout its history, analytical chemistry has provided many of the tools and methods necessary for research in the other traditional areas of chemistry, as well as fostered multidisciplinary research in other fields, to name a few, medicinal chemistry, clinical chemistry, toxicology, forensic chemistry, material science, food chemistry, and environmental chemistry. Here is reported, we believe, one example of this distinction between analytical chemistry and chemical analysis.

1. INTRODUCTION

1.1 Food chemistry: the oenological matrices as a case of study

1.1.1 The contribution of the analytical chemistry

As stated above, analytical chemistry is a discipline which ranges among the other chemistry subjects. In particular, when dealing with real matrices, its contribution in unravelling complicated backgrounds can be crucial. Moreover with the recent "explosion" of *metabolomic*, the role played by analytical chemistry is growing as the development of robust, rapid, sensitive methods, able to measure important biomarkers and to correlate their presence with specific biological/technological conditions, gives a tool to gain a thorough knowledge of the matrix.

Foodstuffs represent an example of complex samples which can be investigated with a metabolomic approach. Up to day the food industry requires an higher contribution from scientific research to assure a quality product able to satisfy consumer expectations.

Among the several matrices that can be considered in the wide scenario of food chemistry, oenological products definitely play a fundamental role in Italy. According to the International Organisation of the Vine and Wine (OIV), Italy is the first producer and consumer of wine in the world. Moreover oenological market in Italy, represents a billionaire business, wine is on the basis of the Mediterranean diet and is one of the most important Italian brand exported around the world. The wine industry in its term requires a noteworthy effort from the chemical research to acquire a deeper knowledge on the matrix composition also from the metabolomic point of view, to define guideline able to drive possible product improvements, to asses the product quality and traceability and finally to furnish useful tool for the quality control.

In the case of wines, sparkling wines and spirits the first feature which undergoes the testing of consumer expectation is the aroma. A precise and thorough control of the volatile compounds composition gives a better knowledge of the sensorial

characteristics of the product, furnishes an objective tool to asses traceability and quality, permits to study the genesis and evolution of peculiar analytes and allows to track their interaction with other molecules.

The ensemble of all these potentialities, provided by the development of advanced analytical procedures, gives an idea on the role of analytical chemistry applied on food matrices and identifies a new branch of this discipline: flavour chemistry. The definition of the aromatic metabolome, i.e. the collection of all the relevant volatile metabolites in a biological system, will permit to identify the influence of technology, microbiology and physiology on the final product.

1.1.2 The wine matrices aroma: main volatile compounds

Production of wine matrices (wine, sparkling wine and spirits) in Italy is really important for its involvement in dietary habits and because of its traditional aspects. Grape – related beverages represent one of the most renowned products abroad and they contribute to the recognition and valorisation of the "Made in Italy" brand.

Modern analytical techniques allow to analyse a vast number of molecules able to define organoleptic characteristics influenced by metabolic and technological parameters.

Volatile compounds, i.e. the pool of molecules perceivable by smelling, contribute to the definition of the aroma of the products considered. The role of each molecule or class of molecules, depends on their concentration and on their chemical structure [1]. Due to the complexity of the analytical picture it is really hard to make correlation between sensory attributes and aromatic compounds content, or to correlate volatiles profile with specific biological conditions.

The ensemble of volatile compounds is influenced by several physiological, environmental, microbiological and technological variables. Many chemical-physical phenomena can affect volatile composition either before fermentation, during or afterwards. Finally technological procedures can provoke further strong modifications in the final product composition.

Aroma compounds can be distinguished in:

1. primary aroma compounds: molecules sensorially active, already present in grape;
2. pre-fermentative aroma: molecules originated during grape processing;
3. fermentative aroma: molecules generated by yeast metabolism;
4. ageing aroma: species generated via either chemical or enzymatic reaction during ageing.

The perception of a specific substance is related to its concentration, its chemical structure and its volatility. All these parameters contribute to the definition of a sensory threshold for every compound. Another important factor is the complexity of the matrix as some molecules can mask other compounds even when they are present at concentration higher than their sensory threshold [2, 3].

In wine matrices the most abundant organic compounds, other than ethanol, are higher alcohols, and ethyl ethers. Among all these substances the most appealing class, which until now has not been deeply investigated although presenting important metabolomic correlation with microbiology, technology and genomics, is the sulfur compounds one. In this thesis we focused our attention on these molecules aiming at retrieving connection with technology, microbiological practices and plant physiology and recognising objective biomarkers to asses product quality and traceability.

1.1.3 Sulfur compounds in oenological matrices

As stated above, sulfur compounds represent the most intriguing species present in oenological matrices both for their extremely low sensory threshold (**Table 1**) and for their implication in yeast microbiology, plant physiology and winemaking technology.

Table 1. Sensory threshold of the main sulfur compounds in grape products (from [4])

	Sensory threshold in several matrices		
Sulfur compound	**Wine**	**Water**	**Others ***
Hydrogen sulfide	1 ng/L - 150 μg/L	5 - 10 μg/L	0.8 μg/L (HS)
Methanethiol	-	0.02 - 0.2 μg/L	0.3 μg/L (HS)
Ethanethiol	1.1 μg/L	8 ng/L	1 - 10 μg/L (B)
Dimethyl sulfide	10 - 160 μg/L	0.3 - 10 μg/L	50 - 60 μg/L (B)
Diethyl sulfide	0.93 - 18 μg/L	-	1 - 30 μg/L (B)
Dimethyl disulfide	20 - 45 μg/L	0.06 - 30 μg/L	3 - 50 μg/L (B)
Diethyl disulfide	4.3 - 40 μg/L	-	0.4 μg/L (B)
2-mercaptoethan-1-ol	0.13 - 10 mg/L	-	0.1 - 10 mg/L (B)
2-Methylthioethan-1-ol	-	-	250 μg/L (HS)
3-methylthiopropan-1-ol	1.2 - 4.5 mg/L	-	500 μg/L (B)
4-methylthiobutan-1-ol	0.1 mg/L	-	0.08 - 1 mg/L (HS)
3-methylthiopropanal	-	0.2 - 50 μg/L	250 μg/L (B)
3-methylthiopropyl acetate	50 - 115 μg/L	-	50 μg/L (HS)
Benzothiazole	50 - 350 μg/L	-	50 μg/L (HS)
4-mercapto-4-methylpentan-2-one	0.8 - 3 ng/L	0.1 ng/L	0.6 ng/L (HS)
3-mercaptohexan-1-ol	-	12 - 15 ng/L	60 ng/L (HS)
4-mercapto-4-methylpentan-2-ol	-	-	55 ng/L (HS)
2-mercaptoethyl acetate	-	-	65 μg/L (HS)
2-mercaptopropyl acetate	-	-	35 μg/L (HS)
3-mercaptohexyl acetate	-	2.3 ng/L	4 ng/L (HS)
5-(2-hydroxyethyl)-4-methylthiazol	0.1 - 10 mg/L	-	-

* HS: Hydroalcoholic solution (12% v/v); B: Beer; A: Air

Sulfur compounds may have different sensorial characteristics according to their chemical structure (i.e. physical-chemical proprieties, position of the sulfur atom along the molecule, stereochemistry) and they represent the biggest unknown in wine chemistry at the moment [1].

In the wine products, only few sulfur compounds have been studied since the early ’70, and they were investigated mostly because of their connections with negative scents as “reduction, putrescence and rotten eggs” [5, 6]. More recently the improvement of the analytical techniques and the availability of more sensitive methods allowed to identify new species and permitted to revaluate sulfur compounds contribution to wine typicality and traceability. These two words are gaining a noteworthy importance in a world wide market, where it is important to be able to recognise specific products and to defend origin and authenticity. According to chemical structure, sulfur compounds can be classified in 5 groups:

- thiols (or mercaptans)
- sulfides
- polysulfides

- thioalcohol, thioketones and thioesters
- heterocyclic compounds;

This classification can be useful to discriminate sulfur compounds on the basis of the different chemical behaviours and to evaluate different chemical-physical characteristics in connection with the sensory threshold. Being interested in applying metabolomic approaches a more useful classification can be done considering the biological/physiological origin of these molecules, as follows:

- fermentative sulfur compounds (**Figure 1a**);
- varietal sulfur compounds (**Figure 1b**).

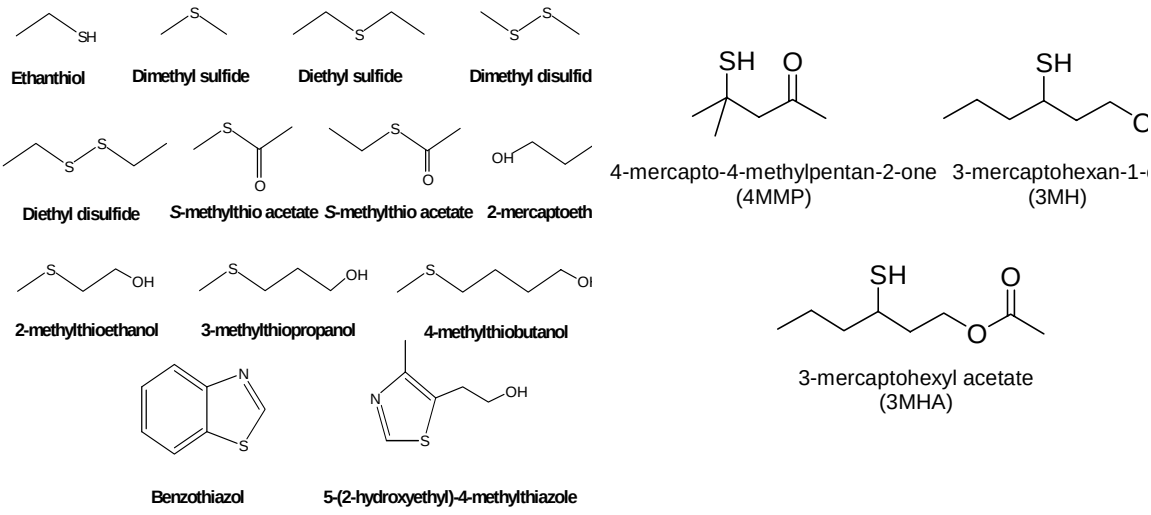


Figure 1. Considered sulfur compounds. **a)** fermentative sulfur compounds; **b)** varietal sulfur compounds

This latter classification is linked to origin of such molecules and allows to draw connections with either specific biological condition or physiological plant behaviours.

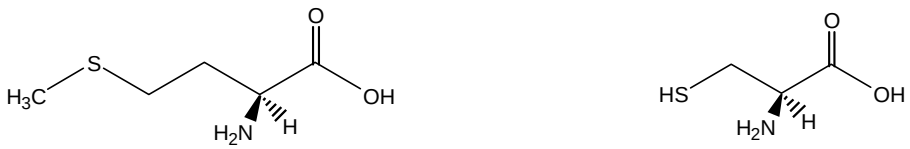
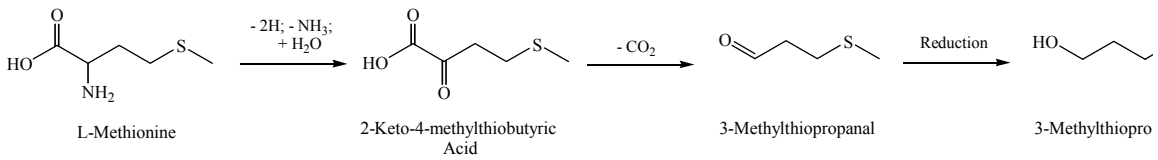


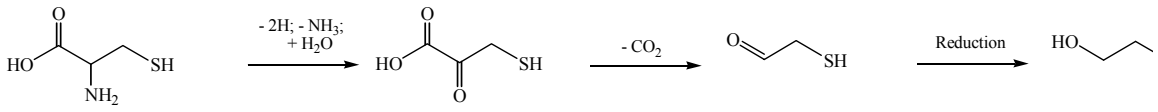
Figure 2. Amino acids involved in the biosynthesis of fermentative sulfur compounds. **a)** L-methionine and **b)** L-cysteine.

The molecules belonging to the former class (**Figure 1a**) derive from yeast metabolisms converting amino acidic precursors into the relevant sulfur compounds. In particular it is possible to recognise for all these molecules a common origin: L-methionine (**Figure 2a**) and L-cysteine (**Figure 2b**) play a primary role in fermentative sulfur compounds biogenesis.

A typical mechanism involving sulfured amino acids is the biosynthesis of 3-methylthiopropanol (methionol, MTP) and 2-mercaptoethanol (ME). The former pathway consists in the biotransformation, via Ehrlich mechanism, of methionine present in juice or made up in the yeast cell during fermentation (**Figure 3a**), the latter involves the reaction of cysteine, still according to Ehrlich mechanism (**Figure 3b**), to originate ME.



a)



b)

Figure 3. 3-Methylthiopropanol (**a**) and 2-mercaptoethanol (**b**) biosynthesis vie Ehrlich mechanism

MTP has a strong impact on white wine aroma determining unpleasant off-flavour whereas in sparkling wines its contribution is positive and permits an easy recognition of renowned products. The contribution of ME is predominantly negative and its presence in grape products should be limited. Being a thiol, this molecule is in equilibria with its dimer [7] and it seems to decrease during ageing [8].

Varietal sulfur compounds are predominantly present in specific grape varieties as conjugated forms with cysteine and glutathione (**Figure 4**). The biogenesis of varietal

thiols precursors is not clearly understood and many efforts have been done to understand their origin and enhance the capability of yeast strain to release the free forms from their conjugates [9-13]. At this stage, the other question microbiologists and geneticists are trying to solve is whether the actual precursor is the glutathionylated form or the cysteinylated one.

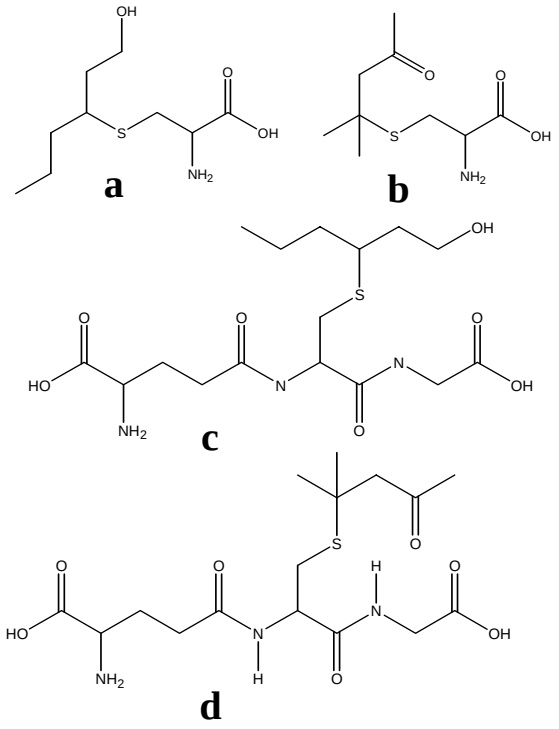


Figure 4. Varietal thiol precursors. **a)** 3-*S*-cysteinylhexan-1-ol **b)** 4-*S*-4-methylpentan-2-one **c)** 3-*S*-glutathionylhexan-1-ol **d)** 4-*S*-glutathionyl-4-methylpentan-2-one

Free thiols, which are sensorially active, are released during fermentation (**Figure 5**) by a *β*-lyase activity. These molecules (**Figure 1b**) have a strong positive impact on wine quality and are perceived as "grapefruit, tropical, guava" scents. Their extremely small concentration (ng/L) together with their very low sensory thresholds makes the definition of analytical methods able to quantify them highly challenging.

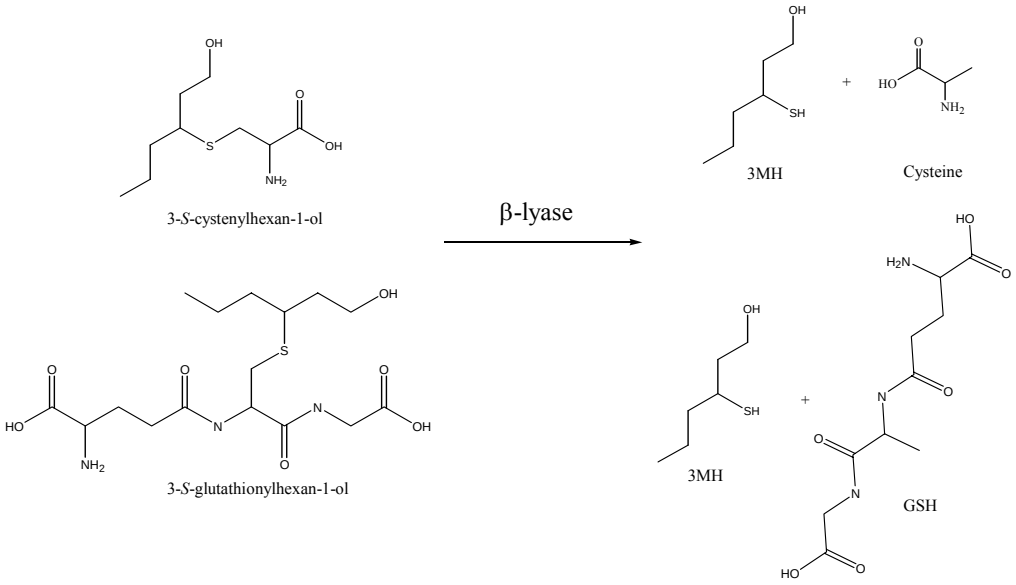


Figure 5. Biogenesis of varietal sulfur compounds

Finally, different pathways can be recognised in the formation of sulfur compounds; the three main mechanisms involved in the genesis of these molecules are:

- microbiological – enzymatic processes;
- chemical – physical reaction;
- phenomena induced by technological procedures.

The former mechanism accounts for the biogenesis of both fermentative and varietal sulfur compounds by yeast metabolisms (amino acids conversion and/or conjugated forms degradation); beside these biochemical reactions other pathways can take place in the generation of sulfur compounds. In particular chemical reactions or processes photo-catalysed may occur during winemaking or ageing. The former case is particularly important for the generation of dimethyl sulfide(**Figure 6**).

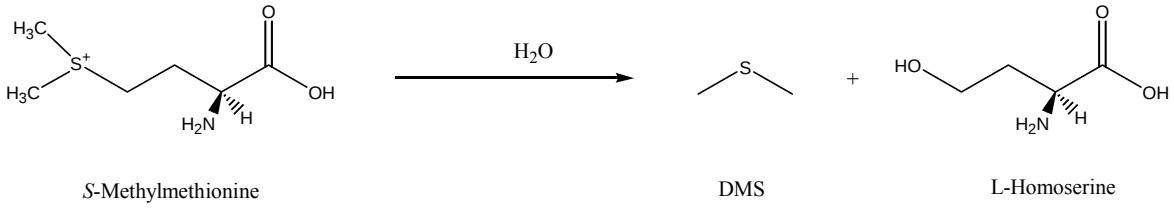


Figure 6. Formation mechanism of DMS

Due to its double contribution to wine aroma, this molecule represents the most important fermentative sulfur compound in grape products. Specifically this substance, when it is present at concentrations lower than its sensory threshold improves wine aroma [14-17], while, when it is present at higher level remarkably depreciates wine quality.

A typical example of photo-catalysed reaction is oxidation of riboflavin (**Figure 7**). This reaction leads to the formation of methional (MTPal), methanethiol (MetSH) and dimethyl disulfide (DMDS), causing unpleasant off-flavours, also known as "goût de lumière", and a general depreciation of the product quality [18, 19].

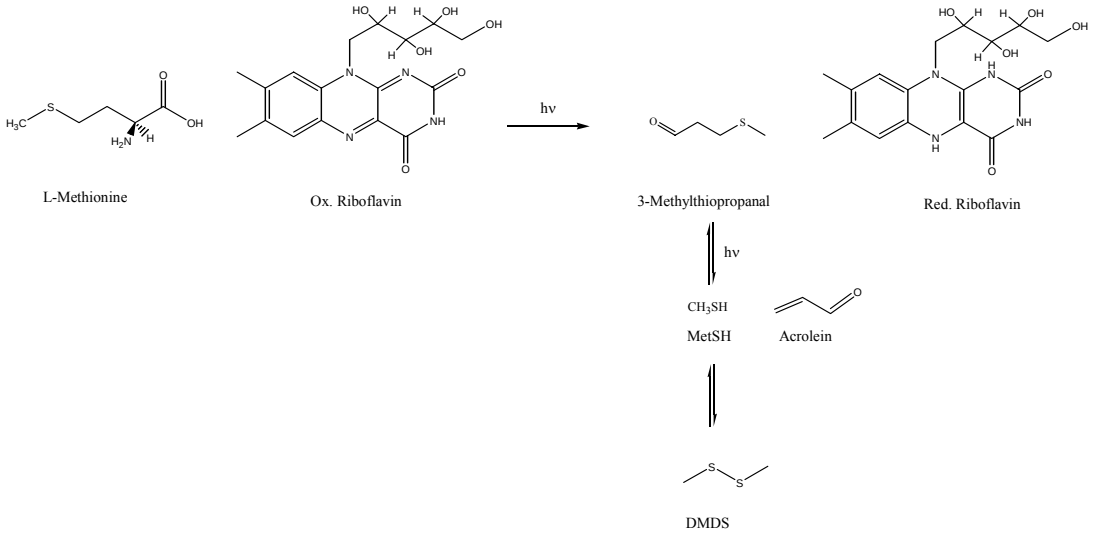


Figure 7. "Goût de lumière" production

Owing to their low sensory threshold and to their complex contribution both to positive and negative wine aroma, sulfur compounds are largely studied. In particular, due to their "bad reputation" wine sciences invested many resources in trying to limit sulfur compounds formation during the different phase of the winemaking.

The most commonly applied approach in the winemaking technology is the fining by means of $CuSO_4$; this treatment works well with thiols but has no effect on sulfides, disulfides and heterocyclic compounds. Moreover, in the literature, there is the evidence of the ineffectiveness of such treatment due to the hydrolysis of thioacetates, which regenerate their relevant thiols (**Figure 8**), with the consequent reappearance of the thiol off-flavours [20].

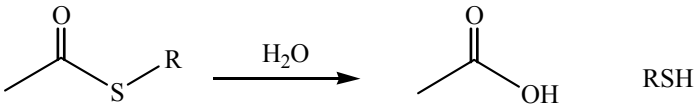


Figure 8. Thioacetates hydrolysis

1.1.4 Considered sulfur compounds

Aiming at defining markers able to assess wine typicality and traceability and at addressing wine technology, physiology and microbiology we decided to focus our attention on the design of analytical methods useful to investigate sulfur compounds and their precursors in grape products. The main goal we achieved is the definition of sensitive robust and easily applicable procedures to study oenological matrices. In the literature many methods have been described [4], but none of them satisfies the characteristics we were looking for. Furthermore very few informations about the biogenesis of both fermentative and varietal sulfur compounds is reported in the literature.

For the fermentative sulfur compounds the role of L-methionine and L-cysteine appears crucial, even if clear mechanisms describing the formation and the specific enzymes involved are given only for a small number of molecules.

Concerning varietal sulfur compounds, amino acidic conjugated forms seem to be their precursors even if no clear evidences regarding the biogenesis of such heterosides and the pathways leading to the free forms are reported in the literature.

1.2 Analytical Techniques

The low concentration of the investigated analytes requires the use of a preconcentration step before the instrumental analysis. Many techniques are available to sample and concentrate specific analytes; the most commonly applied in analytical chemistry are: solid phase extraction (SPE), solid phase microextraction (SPME) and purge and trap (PT).

Chromatography is an important tool of purification and mostly of selective analysis in complex matrices. Mass spectrometry (MS) can be used to detect the compounds coming out from a chromatographic column.

The on-line combination of gas chromatography (GC) and MS was developed in the mid 1960s, first for packed columns and soon after for capillary columns. The introduction of quadrupole mass analysers greatly facilitated GC-MS, because of their faster scanning and less stringent vacuum restrictions. The actual breakthrough of liquid chromatography mass spectrometry (LC-MS) as routinely applicable analytical technique is taking place in the mid 1990s.

1.2.1 Sampling Approaches

Quite often the analysis of the minor components in complex matrices requires both a pre-concentration and a clean-up step. The most commonly applied approaches involves liquid-liquid extraction (LLE), solid phase extraction (SPE) and more recently purge and trap (PT) and solid phase microextraction (SPME).

The LLE is generally discarded as it requires large volume of organic solvent and does not satisfy the general requirement of the *green chemistry*. SPE, and SPME are largely applied in different research fields. In particular the availability of different sorbent phase and the limited or no use of solvents made these techniques very appealing for analytical chemist.

The purge and trap approach, a solvent-free procedure, shows the same advantage of SPME and permits an higher enrichment of the analyte.

Solid-phase extraction (SPE) is a separation/enrichment process that is used to remove/extract analytes from a complex matrix taking advantage from their physical and chemical properties. This technique can be used to isolate analytes of interest from a wide variety of matrices, including urine, blood, water samples, beverages, soil, animal tissue, and consumer products.

SPE uses the affinity of solutes dissolved or suspended in a liquid (known as the mobile phase) for a solid through which the sample is passed (known as the stationary phase) to separate a mixture into required and unrequired components. The result is that either the analytes of interest or the impurities in the sample are retained on the stationary phase. The portion of solution that passes through the stationary phase is collected or discarded, depending on whether it contains the analytes or the impurities.

If the portion retained on the stationary phase includes the desired analytes, they can then be recovered from the stationary phase rinsing the stationary phase with an appropriate eluent.

The stationary phase is in the form of a packed syringe-shaped cartridge, which can be mounted on a commercially available extraction manifold. The manifold allows many samples to be processed simultaneously (**Figure 9**). A typical cartridge SPE manifold can accommodate up to 24 cartridges. Most SPE manifolds are equipped with a vacuum port where application of vacuum speeds up the extraction process by pulling the liquid sample through the stationary phase.

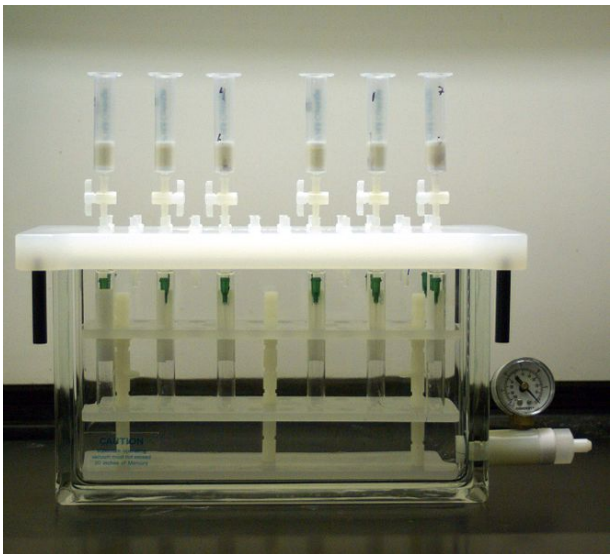


Figure 9. Example of manifold for SPE extraction

A typical solid phase extraction involves four basic steps. First, the cartridge is equilibrated with a non-polar solvent or slightly polar, which wets the surface and penetrates the bonded phase. Then the column is washed with water, or buffer of the same composition of the sample, to wet the silica surface. The sample is then added to the cartridge, and the analytes are retained on the sorbent while the solvent, salts, and other impurities pass through the cartridge. After the sample is loaded, the cartridge is washed with buffer or solvent to remove further impurities. Then, the analyte is eluted with a non-polar solvent or a buffer of the appropriate pH.

Solid phase microextraction, or SPME, is a sample preparation technique developed in the early 1990s at the University of Waterloo by Dr. Pawliszyn's group. It is a simple and inexpensive technique where the use of solvents is not necessary.

SPME can be thought as a very short chromatographic column turned inside out. SPME is a fibre coated with an extracting phase that can be a pseudo-liquid (polymer) or a solid (sorbent), which extracts different kinds of analytes, ranging from volatile to non-volatile, by different means. Thus, the analyte can be extracted from the liquid (direct solid phase microextraction) or gas phase (headspace solid phase microextraction). The quantity of analyte extracted by the fibre is proportional to its concentration in the sample, as long as equilibrium is reached or, in the case of short time pre-equilibrium, with the help of convection or agitation. After extraction, the SPME fibre is transferred to the injection port of separating instruments, like a gas chromatograph, where desorption of the analyte takes place and analysis is carried out (**Figure 10**).

The attraction of SPME is that the extraction is fast and simple and can be done without solvents, with detection limits which can reach parts per trillion (ppt) levels for certain compounds.

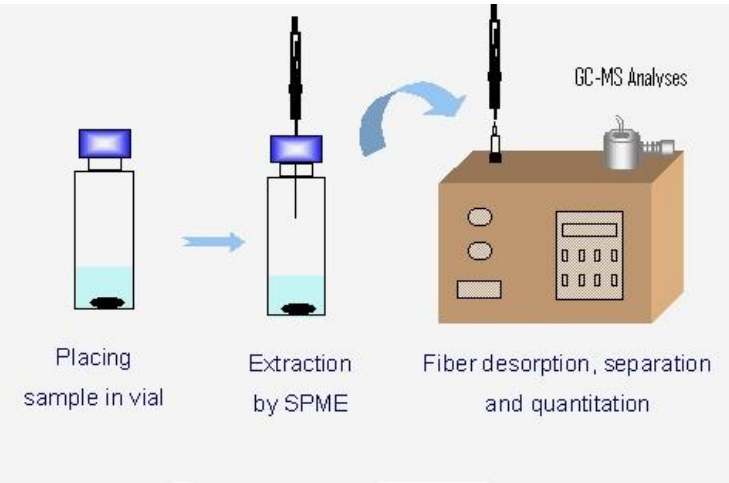


Figure 10. Manual HS-SPME procedure

The critical parameters in the HS-SPME are:

- ✓ **extraction time:** it is critical for the sample to establish equilibrium with the SPME fiber coating. Extractions typically take 15-20 minutes, but can be as short as 30 seconds. Headspace extractions are usually shorter than

immersion. The extraction time will depend on the size of the compounds, fibre coating, type of extraction used and sample concentration. In the absence of equilibrium the control of the time must be more stringent;

- ✓ **sample temperature:** it is critical for accurate quantitation of the sample. One must use a constant temperature for all extractions to obtain good precision. The use of heat during headspace extractions will help to release the analyte from the sample, to improve sensitivity, and to shorten the extraction time;
- ✓ **sample agitation:** it is important to reduce the equilibrium time and to improve accuracy and precision. This is crucial when analyzing semivolatile compounds by immersion sampling. A consistent agitation must be maintained for all extractions to gain good precision. Stirring, sonication, and vibration are all suitable methods to agitate the sample;
- ✓ **salt addition:** it can improve the extraction efficiency by changing the solubility of the analytes in the sample. The addition of 25-30% sodium chloride will increase the ionic strength of the sample, which reduces analyte solubility. The addition of salt is especially helpful when analyzing polar analytes in aqueous solution.

The purge and trap apparatus is used to gently extract volatile compounds from a liquid at room temperature. Typically, the liquid is an aqueous solution.

The purging tube is shown below.

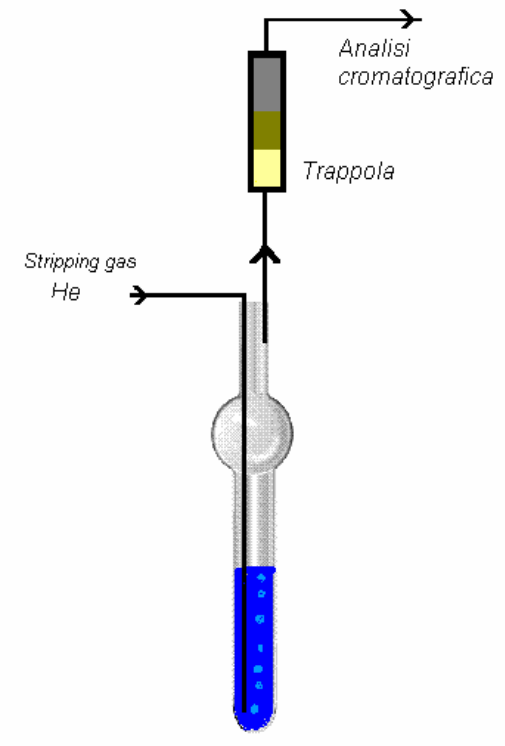


Figure 11. Representation of purge and trap sampling

Purge gas (e.g. helium) is bubbled through the sample in the purge tube and the analyte is collected on the trapping column above (**Figure 11**).

The trapping column contains a solid adsorbent (e.g. TENAX). After a predetermined purge time, the temperature of the trapping column is raised and the compounds that were trapped on it are driven off the trapping column into the chromatographic column.

1.2.2 Gas and Liquid Chromatography

Since its introduction in 1962, the technique of gas chromatography (GC), with packed and mostly open tubular column, has grown spectacularly. Any substance, organic or inorganic, which exhibits a vapour pressure of at least 60 torr (the column temperature may be raised to 350°C) can be eluted from a GC column. The major

limitation of GC is that samples, or their derivatives, must be volatile at the column operating temperature.

The range of operating modes is much greater in high-performance liquid chromatography (HPLC) because two immiscible phases, stationary and mobile, in contact with one another affect the separation. The variety of phase systems for LC allows for a wide range of selectivities, which can be adjusted to the components of interest in two ways: by varying the stationary phase or by modifying the mobile phase. Chromatographic separation in HPLC is the result of specific interactions of the sample molecules with both the stationary and mobile phases. With an interactive liquid mobile phase, another parameter is available for selectivity in addition to an active stationary phase. HPLC can be used whenever the sample can be dissolved in a liquid. HPLC comprises a number of different LC methods, covering a large number of applications.

1.2.3 Mass Spectrometry

Mass spectrometry probably represents the most versatile detector which can easily interfaced with a chromatographic apparatus. Its use is spread over several scientific disciplines and its applications may range from the recognition of new molecules in fundamental research till routine analyses in quality control process.

The two main parts of the MS instruments are the ionisation system and the mass analyser. While in GC electron impact (EI) is the widespread ionisation mode, in LC-MS the common way to introduce and ionise the sample is represented by the electrospray (ESI) approach.

The most extended analyser is the quadrupole which can be used as a single analyser or can be coupled with other quadrupoles to obtain the so called MS/MS techniques.

All ion sources are required to produce ions without mass discrimination from the sample and to accelerate them into the mass analyser. The usual source design has an ion withdrawal and focusing system. The ions formed are removed electrostatically from the chamber. Located behind the ions is the repeller, which has the same charge as the ions to be withdrawn. A strong electrostatic field between the first and second

accelerating slits of 400 to 4000 V, which is opposite in charge to the ions, accelerates the ions to their final velocities.

The electron ionisation source is a commonly used ionisation method when GC is coupled to MS. The ionising electrons from the cathode of an electron gun located perpendicular to the incoming gas stream collide with the sample molecules to produce a molecular ion. A source operating at 70 V, the conventional operating potential, also has sufficient energy to cause the characteristic fragmentation of sample molecules. Some compounds do not produce a molecular ion in an electron ionisation source.

When LC is interfaced to MS the common ionisation source is the electrospray. A voltage of 2 to 3 kV is applied to the metal capillary tip, which is typically 0.1 mm i.d. and located 1 to 3 cm from a large planar counter electrode. This counter electrode has an orifice leading to the MS sampling system. The very high electric field imposed causes an enrichment of positive electrolyte ions at the meniscus of the solution at the capillary tip. At a sufficiently high field, the cone is not stable and a liquid filament with a diameter of a few micrometers, whose surface is enriched on positive ions, is emitted from the cone tip. At some distance downstream the filament becomes unstable and forms a fine mist of positively charged droplets. The charged droplets shrink by solvent evaporation and repeated droplet disintegrations, leading to very small, highly charged droplets capable of producing gas-phase ions which enter the MS.

The function of the mass analyser is to separate the ions produced in the ion source according to their different mass – charge ratios. The analyser section is continuously pumped to a very low vacuum so that ions may be passed through it without colliding with the gas molecules. The energies and velocities V of the ions moving into the mass analyser are determined by the accelerating voltage (V) from the ion source slits and the charge z on the ions of mass m.

In the quadrupole mass analyser, ions from the ion source are injected into the quadrupole array, shown in **Figure 12**. Opposite pairs of electrodes are electrically connected; one pair at $+U_{dc}$ volts and the other pair at $-U_{dc}$ volts. An *rf* oscillator supplies a signal to each pair of rods, but the signal to the second pair is retarded by 180°. When the ratio U_{dc}/V_{rf} is controlled, the quadrupole field can be set to pass ions of only one *m/z* ratio down the entire length of the quadrupole array. When the *dc* and *rf*

amplitudes are changed simultaneously, ions of various mass–charge ratios will pass successively through the array to the detector and an entire mass spectrum can be produced. Registration of negative ions, as from a chemical ionization source, is possible with two electron multipliers, one for positive and one for negative ions. Scan rates can reach 780 Da s^{-1} before resolution is significantly affected. The quadrupole mass analyser is ideal for coupling with a gas chromatograph. Practical *m/z* limits are 4000 Da.

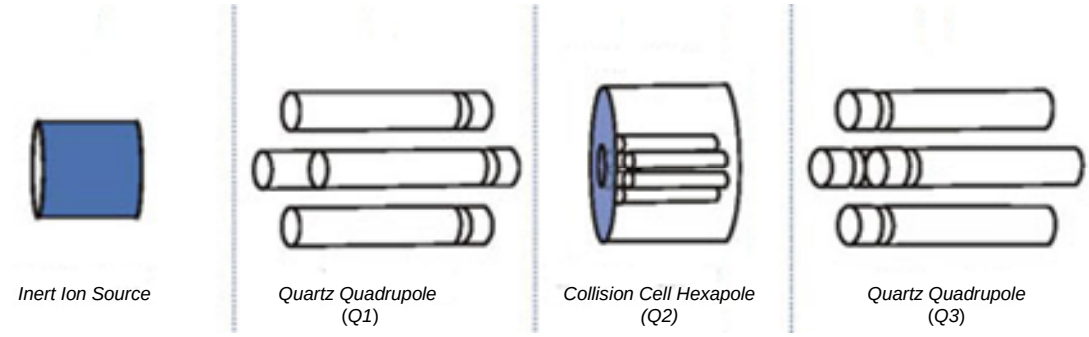


Figure 12. Triple quadrupole representation

The MS/MS technology is made up by coupling mass analyser and up to day it represents a quite common approach in analytical chemistry when high sensitivity and good selectivity are required. This approach furnishes more selectivity by picking the right parent and daughter ions and assures a better sensitivity by decreasing the noise. The MS /MS instruments utilised in this PhD were all triple quadrupole.

In the triple quadrupole, the first one (Q1) acts as regular mass filter and is exploited in selecting the parent ion which will be then fragmented in the second quadrupole (Q2) which operate as a collision cell (actually more commonly this segment is an hexapole or an octupole). The third quadrupole (Q3) works again as regular mass filer and it might work either scanning all the mass fragments produced in Q2 (Scan) or monitoring specific fragments generated in the collision cell (in MS/MS experiments called MRM).

According to the way the Q3 is exploited two different approaches have been used during this PhD.

Enhanced product ion mode (EPI): on a hybrid triple quadrupole/linear ion trap instrument such as all the new generation LC-MS/MS, this approach exploits the third

quadrupole (Q3) as a linear ion trap to enhance the sensitivity. A precursor ion (parent ion) is selected in the first stage (Q1), allowed to fragment in the collision cell (Q2) and then all resultant masses are detected in the second mass analyzer (Q3). This experiment is commonly performed to identify transitions used for quantification by tandem MS.

Multiple reaction monitoring (MRM) is based on a three step mass selection: in first stage, first quadrupole (Q1), the mass of the intact analyte (parent ion) is selected. In the second quadrupole (Q2) the fragmentation of the parent by collision with gas atoms (e.g. Helium) is carried out. In the third stage (Q3) a specific fragment of the parent is selected and then monitored (this approach represent the analogue of the SIM mode in the single quadrupole analyses). The two mass filters (Q1 and Q3) produce very specific and sensitive responses whereas the second quadrupole (Q2) acts like a collision cell.

The compound under scrutiny must be known and have been well-characterised previously before this type of experiment is undertaken. This methodology is used to confirm unambiguously the presence of a compound in a matrix e.g. drug testing with blood or urine samples. It is not only a highly specific method but also has very high sensitivity. Most assays use electrospray ionisation to introduce the sample into the mass spectrometer from the HPLC.

2. AIMS AND SCOPES OF THE PROJECT

This project has been formulated thanks to the requests advanced from both the scientific research and the productive environment. In particular it appeared evident how the lack of informations concerning sulfur compounds in grape products was affecting the completion of the understanding of oenological matrices aroma. Up to now the analytical procedures reported in the literature and the informations related to sulfur compounds formation, conversion and their effect on wine aroma appeared to be not sufficient. A demonstration of the importance and of the appealing originated from these molecules is represented from the efforts spent from many research groups all over the world, coming by many different scientific backgrounds, and from the number of scientific projects related to wine aroma.

The aim we are pointing at is quite ambitious and requires knowledge of analytical chemistry, organic chemistry, biochemistry and microbiology. This thesis represents a good example of multidisciplinary approach in facing a challenging scientific issue.

The final goal we tried to achieve is to supply robust tools able to improve the understanding of an important class of sensory-impact species such as the sulfur compounds.

Dealing with a research involving contributions from so many different disciplines and involving so many skills we have to design our project in autoconsistent macro subjects. Nonetheless it is clear that the *trait d'union* we keep in our mind in planning this project is to gain a better understanding on the contribution of sulfur compounds on grape products quality and typicality. The success of this research passes through the development of analytical methods able to study these matrices, and with the problems connected with that, the explanation of the influence of oenological practice on the studied species, the evaluation the formation and evolution of the considered sulfur compounds.

For these reasons the disclosure of this work has been organized in autoconsisten chapters. This decision has been taken to gain an easier way to discuss our results and to assure a better comprehension to our readers.

Considering the study of complex matrices the first problem an analytical chemist has to face is the occurrence of matrix effects. Such problems may interfere in the instrumental responses, falsing the results. For this reason we proposed a statistical approach to overcome this problem whenever the occurrence of matrix effect could affect the analysis and where the choice of suitable internal standards were not enough to assure the reliability of the analytical results.

The second topic we plan to deal with relates to the topic of the fermentative sulfur compounds.

We develop analytical methods able to study the concentration of some important sulfur compounds in grape products. The application of the methods we consider to develop will provide an useful tools to further consider ageing, variety and technological effects on the content of the studied analytes.

This study will furnish important indication on the formation, evolution and sensory contribution of some important sulfur compounds in several grape variety here taken into account.

The last topic we consider in our project is connected with the varietal sulfur compounds. This class of molecules has an important and positive effect on wine quality and knowledge on these molecules is highly required from both the productive and scientific world.

Also for these compounds we evaluate analytical approaches to quantify them and the we consider the genesis of the active form by studying the formation of their precursors

The information we can provide from these informations are crucial to drive technological and oenological practices.

This work exploits the collaboration of national and international institutions; in particular we collaborate with the IASMA Research Center (San Michele all'Adige, Italy), Unione Italiana Vini (Verona, Italy), the Institute Nationale de la Recherché

Agronomique (Montpellier, France) and the Australian Wine Research Institute (Adelaide, Australia).

3. THE PROBLEM OF THE MATRIX EFFECTS *

3.1 Preliminary considerations

The quantification of organic sulfur compounds is an important matter in the quality control of food and beverages as these molecules are heavy impact factor-sensory-substances. In particular in wines they can play the double role of off-flavours and of aroma-characteristics [8, 14, 15]. For these reasons different analytical methods have been reported to quantify sulfur volatiles in wines and recently a new method based on headspace solid-phase microextraction (HS-SPME) coupled with gas chromatography – mass spectrometry (GC-MS) was described [21].

When dealing with development of analytical methods for complex matrices, one of the hardest problem analytical chemists have to face is the occurrence of matrix effects. Matrix effect occurs when the unknown sample contains many impurities. If impurities present in the sample interact with the analyte to change the instrumental response, then a calibration curve based on pure analyte samples will give an incorrect determination.

Apart from a close matching between blank matrix and real matrix, two well established procedures are available in mass spectrometry analyses to correct for the matrix effect, the standard addition method and the use of isotopically labelled internal standards (IS) [22].

In the former one, standard solution (solution of known concentration of analyte) is added to the unknown solution so the effect of any impurities in the unknown are accounted for in the calibration. The operator does not know how much was in the solution initially but does know how much standard solution was added, and knows how the readings changed before and after adding the standard solution. Thus, the operator can determine by a suitable procedure the concentration initially present in the sample. Nonetheless the standard addition method is tedious and can suffer from interferences [23] while the use of labelled internal standards requires, in addition, the

* The contents of this chapter have been published in a peer reviewed paper

availability of an internal standard for each analyte considered [24] making it generally expensive and sometimes only able to reduce matrix-induced deviations [25].

In recent years the general problem of matrix- and calibration run-induced deviations in the context of the validation of an analytical method was treated using a statistical variance component model (VCM) [25-28].

Since previous results obtained during my PhD showed that the quantification of sulfur volatiles in wine matrices is affected by matrix effects we proposed and validates an alternative way to overcome the matrix effects problem by a statistical approach [21]. In particular the reliability of coupling HS-SPME and GC-MS analysis to VCM data treatment was tested using the dimethyl sulfide (DMS) as the target analyte. In this study the effectiveness of using d_6-DMS and ethylmethyl sulfide (EMS) as internal standards in reducing the matrix induced effect was compared [29].

3.2 Experiments

3.2.1 Chemicals and reagents

Dimethyl sulfide (DMS) and the internal standards dimethyl sulfide-d_6 (d_6-DMS) and ethylmethyl sulfide were purchased from Sigma-Aldrich (Milan, Italy) at purity > 98% and $MgSO_4 \cdot 7H_2O$ and ethanol anhydrous (> 99 %) were purchased from Carlo Erba (Milan, Italy). The wine matrices were kindly provided by the IASMA Research Center (Trento, Italy).

3.2.2 HS-SPME/GC-MS procedure

The SPME holder for manual sampling and the fiber used (CAR/PDMS/DVB; 50/30 µm × 2 cm) were purchased from Supelco (Bellefonte, PA, USA). Before utilisation the fiber was conditioned according to the producer instructions. 20 mL of wine were put into a 30 mL vials containing 5 g of $MgSO_4 \cdot 7H_2O$. The sampling was carried out at 35°C and the fiber exposed into the headspace for 30 mins. The solution was stirred at 500 rpm with a magnetic bar.

GC-MS analyses were performed with a Perkin-Elmer Autosystem XL gas chromatograph coupled with a TurboMass Gold mass spectrometer (Perkin Elmer, Boston, MA, USA) equipped with a 30 m × 0.32 mm i.d. × 0.25 μm film thickness Innowax capillary column (Agilent Technologies, Palo Alto, CA, USA). Helium was used as carrier gas (flow rate 1.2 mL/min). The transfer line and the ion source temperatures were 220°C and 150°C, respectively. The electron energy was 70 eV. All the analyses were carried out in single ion monitoring (SIM) mode using the ions reported in **Table 2**.

Table 2. Retention time and mass spectrometry fragments for the considered analytes

Analytes	RT (min)	Quantitation ion (*m/z*)	Qualifier ions (*m/z*)
DMS	4.81	62	45, 47
d_6-DMS	4.76	68	50, 66
EMS	6.36	76	61, 48

The chromatographic conditions were as follows: a) GC injector temperature 250°C, b) oven temperature program: 35°C (5 mins), 1°C/min to 40°C, 10°C/min to 250°C. Injection in splitless mode for 1 min.

3.2.3 Calibration curves

Calibration curves for the considered analyte (i.e. DMS) were prepared according to the internal standard method. Twenty two wines, red, white and sweet, were firstly deprived of DMS by bubbling nitrogen up to absence of the corresponding mass spectrometric signal obtained at m/z 62, as reported by Segurel et al. [14]. Afterwards, constant and increasing aliquots of the ethanolic working solution of both the internal standard and the analyte were respectively added to the deprived matrices to prepare the calibration solutions. The calibration curve for each wine was obtained using five concentration levels and three replicate solutions for level, namely $I = 15$ data points.

3.3 Statistical analysis

The data obtained for all wine matrices at a fixed concentration level were tested for the normality and for the presence of outliers using the Shapiro-Wilk test [30] and the Grubbs test [31], respectively. The ordinary least squares method was exploited to obtain the calibration curves for each wine matrix. The scedasticity of the experimental values at various concentrations for any wine matrix was established via Cochran test [32]. The homogeneity of the regression residual variances s_j^2, j = 1, 2, …, J, where J is the number of the matrices, was checked by the Cochran test. The presence of outlying calibration curves was examined using the Grubbs test on the intercept and on the slope data sets drawn from the regression. All tests were carried out at the 5% significance level.

3.3.1 Matrix-specific and matrix-comprehensive calibration functions

To account for matrix-induced deviations an averaged calibration straight line $y = \hat{a} + \hat{b}x$ was calculated, where $\hat{a} = \frac{1}{J}\sum_{j=1}^{J}\hat{a}_j$ and $\hat{b} = \frac{1}{J}\sum_{j=1}^{J}\hat{b}_j$; $\hat{a}_j$ and $\hat{b}_j$ are the intercept and the slope relevant to the j-th calibration line calculated using I data points.

Further the (1 - α)100% prediction interval at each x was calculated as [25]:

$$\hat{a} + \hat{b}x \pm s_0\, t_{\nu,\,1-\alpha/2}\left[1 + \frac{1}{J}\left(\frac{1}{I} + \frac{\left(x-\bar{x}\right)^2}{\sum\left(x-\bar{x}\right)^2}\right)\right]^{1/2} \qquad (1)$$

where $s_0^2 = \frac{1}{J}\sum_{j=1}^{J}s_j^2$ denotes the residual variance calculated pooling the residual variances s_j^2, and $t_{\nu,\,1-\alpha/2}$ is the 1-α/2 quantile of the central t-distribution with $\nu = J(I - 2)$ degrees of freedom.

As measurement values not in the (1-α)100% prediction interval may indicate the presence of additional sources of errors, their effective presence was checked by a significance test.

The proportion p of experimental data falling outside the (1-α)100% prediction interval was compared with the theoretical value α, at a chosen significance level, using the statistic

$$\frac{p-\alpha}{\sqrt{\frac{\alpha(1-\alpha)}{n}}} \tag{2}$$

where n is the total number of the experimental data. This statistic has an approximately normal distribution with zero mean and unit variance so that it asyntotically coincides with the normal standardised variate Z [33]. When the experimental proportion p appeared significantly larger than α, the procedure of the variance component model (VCM procedure) proposed by Juelicher et al. [25] was adopted.

This model assumes that the measurement value Y at a fixed x is a normally distributed variable with mean equal to $a + bx$ and variance given by

$$\sigma_Y^2 = \sigma_0^2 + \mathrm{var}(a_j + b_j x) \tag{3}$$

where σ_0^2 is the variance of the measurement error and $\mathrm{var}\left(a_j + b_j x\right)$ is the variance of the matrix/run-induced error. Under this hypothesis, the (1-α) 100% prediction interval at each x is constructed also with the contribute of the matrix/run-induced error and is given by

$$\hat{a} + \hat{b}x \pm t_{\nu,\,1-\alpha/2}\left[s_0^2 + s_0^2\left(\frac{1}{I} + \frac{\left(x-\bar{x}\right)^2}{\Sigma\left(x-\bar{x}\right)^2} \right) + \hat{\mathrm{var}}\left(a_j + b_j x\right) \right]^{1/2} \tag{4}$$

where s_0^2 and $\hat{\mathrm{var}}(a_j + b_j x)$ are used as estimates of σ_0^2 and $\mathrm{var}(a_j + b_j x)$, respectively. The term $\hat{\mathrm{var}}(a_j + b_j x)$, which represents the estimated scatter of the j-th calibration straight line around the overall calibration line, is calculated as

$$\hat{\mathrm{var}}(a_j + b_j x) = s^2_{\hat{a}_j + \hat{b}_j x} - s_0^2\left(\frac{1}{I} + \frac{\left(x-\bar{x}\right)^2}{\Sigma\left(x-\bar{x}\right)^2} \right) \tag{5a}$$

if this difference is positive, or as

$$\hat{\mathrm{var}}(a_j + b_j x) = 0 \tag{5b}$$

if the difference is negative [25, 26]. The term $s^2_{\hat{a}_j+\hat{b}_j x}$ denotes the empirical variance of the estimated responses at various matrices for any analyte concentration. The index *v* in equation 4 is now equal to *J*-1 [25].

3.3.2 Detection limits with and without matrix effects

The Hubaux-Vos approach [34] was used for the calculation of the critical and detection limits for any case considered. The basic idea of this procedure lies on the employment of the calibration curve and of the associated prediction band, which can be obtained considering the uncertainty of the measurements and the calibration function itself. The measurement variance is estimated by s_0^2 without matrix effect and by $s_0^2 + \hat{\text{var}}(a_j + b_j x)$ in its presence. Therefore, on the basis of the statistical model adopted for the measurement variance and of the availability of the overall calibration line $y = \hat{a} + \hat{b}x$, matrix-comprehensive critical and detection limits can be calculated as follows. In the signal domain the critical limit L_C is defined as the level which is exceeded by the signal *Y* at $x = 0$ with a very low probability α:

$$\frac{L_C - \hat{a}}{(s_0^2 + \hat{\text{var}}(a_j + b_j x)_{x=0} + \hat{\text{var}}(\hat{a}))^{1/2}} = t_{J-1,\,1-\alpha} \tag{6}$$

where the first two terms of the denominator on the left hand side of the equation represent an estimate for $\sigma^2_{L_C}$ and the last term is $\hat{\text{var}}(\hat{a}) = \frac{1}{J}\hat{\text{var}}(a_j + b_j x)_{x=0} + \frac{1}{J}\left(\frac{1}{I} + \frac{\bar{x}^2}{\sum\left(x - \bar{x}\right)^2}\right)s_0^2$ (see eq. E33 in ref. [26]). The corresponding critical concentration is $x_C = \frac{L_C - \hat{a}}{\hat{b}}$.

In the concentration domain the detection limit x_D is the concentration level which generates a response which can lie under L_C with probability β; that is where the power function is equal to 1-β. According to Juelicher et al. [25, 26] the power function is calculated by the equation

$p(x) = 1 - F_{J-1,\delta_x}\left[t_{J-1,1-\alpha}\left(\frac{s_0^2 + \hat{\mathrm{var}}\left(a_j + b_j x\right)_{x=0} + \hat{\mathrm{var}}\left(\hat{a}\right)}{s_0^2 + \hat{\mathrm{var}}\left(a_j + b_j x\right) + \hat{\mathrm{var}}\left(\hat{a}\right)} \right)^{1/2} \right]$, where F_{J-1,δ_x} is the distribution function of the non-central *t*-distribution with *J*-1 degrees of freedom and non-centrality parameter $\delta_x = \frac{\hat{b}x}{\left[s_0^2 + \hat{\mathrm{var}}\left(a_j + b_j x\right) + \hat{\mathrm{var}}\left(\hat{a}\right)\right]^{1/2}}$. The detection limit x_D is graphically determined putting $p(x) = 1-\beta$.

3.3.3 Estimation of the concentration uncertainty in the inverse regression

On the basis of the availability of the calibration line and of its (1 - α) 100% prediction interval, the obtainment of the discriminated value x_0 from an experimental response y_0 and of its confidence interval is straightforward using a graphical approach. The limits x_0^- and x_0^+ of the uncertainty interval on x_0 are obtained by intersecting the (1 - α)100% two sided prediction bands with the straight line $y = y_0$ [35].

In particular it can be noted that the graphical approach is a simpler alternative way to estimate the critical limit x_C and the detection limit x_D in the concentration domain. The critical concentration x_C is x_0 when the arbitrary value y_0 is L_C, upper limit of the prediction interval at $x = 0$, while x_D is the abscissa of the intersection of the parallel line to the x axis passing through L_C with the lower one sided (1 - β)100% prediction function.

3.4 Results and discussion

3.4.1 Effectiveness of the internal standard in reducing the matrix effects

Figure 13 shows the whole of the calibration data, collected from 22 matrices, in terms of peak area ratios of the analyte and of the internal standard d_6-DMS (**Figure 13a**) and EMS (**Figure 13b**) against the concentration levels.

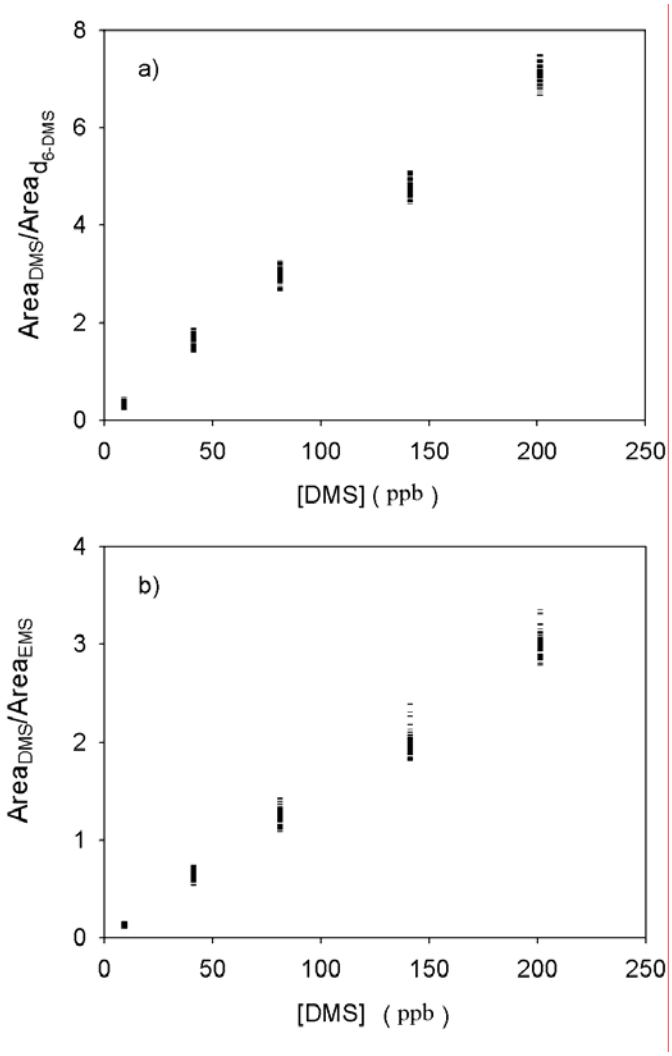


Figure 13. Calibration data using d_6-DMS and EMS as internal standards

The closeness of the experimental data at any concentration level suggests the possibility of using an unique overall calibration function for measurements relevant to different wine matrices. The measurement data at each concentration level were found to be normally distributed according to the Shapiro-Wilk test. The Cochran test indicated the homogeneity of variances with the concentration levels inside each matrix using both the internal standards and the inhomogeneity of variances induced by matrix effects when using EMS as IS.

Figure 14 shows twenty two calibration functions $\hat{a}_j + \hat{b}_j x$ obtained for each wine matrix with both internal standards. The Grubbs test for the intercepts $\hat{a}_j$ and for

the slopes $\hat{b}_j$, indicated that the matrix of the wine named Merlot was an outlier when using EMS as the internal standard and consequently it was no further considered.

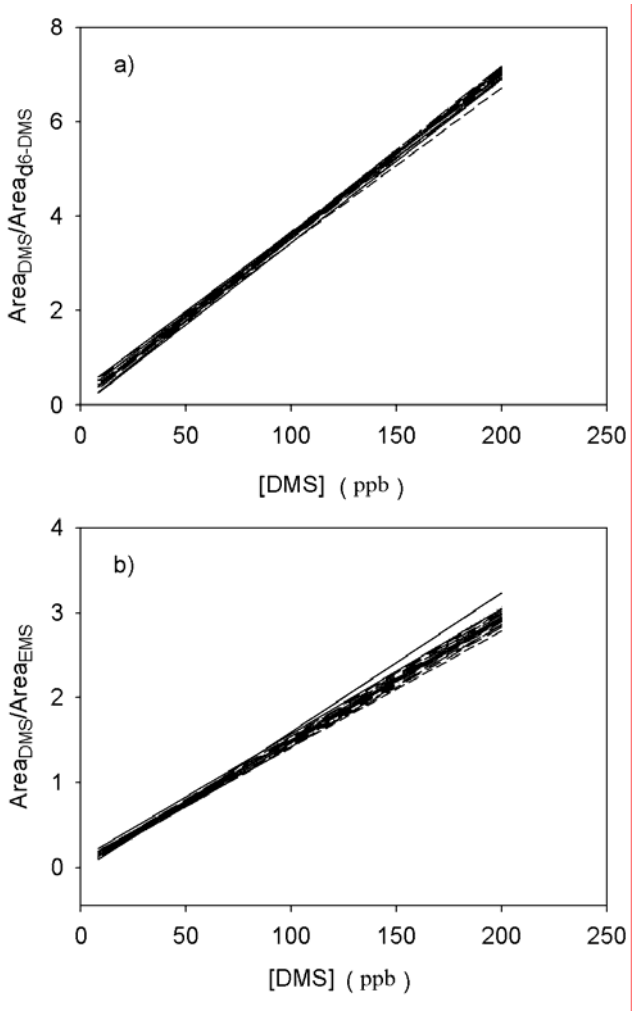


Figure 14. Calibration straight lines for the internal standards based on 15 experiments

The effectiveness of the overall calibration function calculated averaging the matrix calibration functions $\hat{a}_j + \hat{b}_j x$ was tested following the approach of Juelicher et. al. [25]. **Figure 15** depicts the overall calibration straight line together with the 98 percent prediction interval for the measurement values calculated by equation 1 (dotted line) which accounts for the averaged estimation error of the calibration functions.

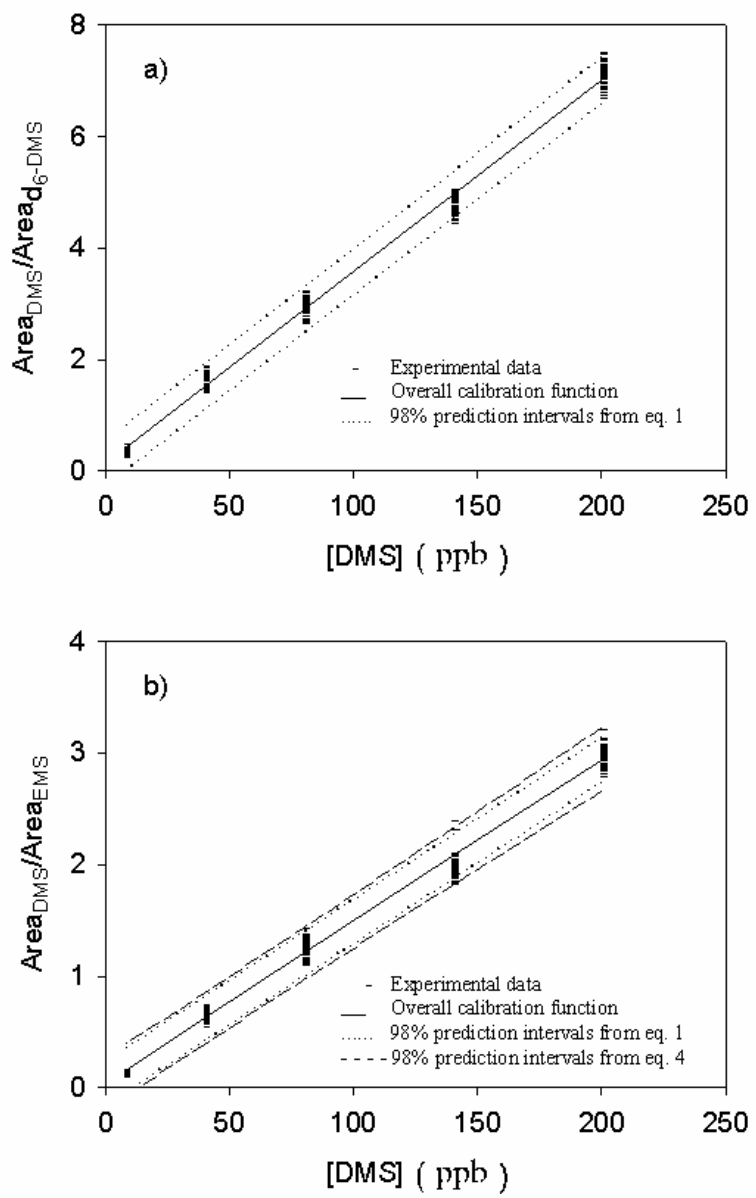


Figure 15. Overall calibration functions using d_6-DMa **(a)** and EMS **(b)** as internal standards

Ten out of the 22×(5×3) = 330 measurement values obtained with the use of the deuterated internal standard (i.e. 3%) were not in the interval (**Figure 15a**), while eleven out of the 21×(5×3) = 315 measurement values when using the EMS as the internal standard (i.e. 3.5%) were found outside (**Figure 15b**). The statistical test on the proportions (one tailed) indicated that the proportion of outside values was not significantly larger than 2 % for the d_6-DMS case and significantly larger than 2% for the EMS case. This means that no additional source of error induced by matrix/run effect was found using the isotopically labelled internal standard. Consequently the

overall calibration function together with its prediction band given by equation 1 can be used to calculate discriminated values, their uncertainties, and detection limits in place of single calibration straight lines for each matrix. When using the internal standard non-isotopically labelled, the results of the test on the proportion pointed out the inadequacy of equation 1 to account for the uncertainty introduced by the different matrices/runs. The VCM approach , which generated a larger prediction band via equation 4, appeared to properly handle the matrix-induced deviations (dashed line in **Figure 15b**). Actually the proportion of outside values was significantly lower than 2% (i.e. 0.3%).

3.4.2 Performance characteristics of the matrix-comprehensive approach

Table 3 reports detection limits, discriminated values and confidence limits of the discriminated values using the overall calibration function and two matrix-specified calibration functions for both the internal standards. The wine matrices reported gave the extreme values of the slopes $\hat{b}_j$, that is exhibited the greatest matrix induced effect.

Table 3. Detection limits and parameters obtained from the inverse regression using the two different internal standards.

	Critical limit	**Detection limit**	**Inverse regression**	
	x_c [a]	x_D [b]	x_0	(x_o^-, x_0^+)
		d_6-DMS		
			$y_0 = 3.6$	
Overall	13	25	101	(87, 110)
Teroldego	11	22	100	(89, 111)
Nero d'Avola	7	16	101	(95, 107)
		EMS		
			$y_0 = 1.5$	
Overall	16	30	101	(85, 116)
Teroldego	15	30	98	(84, 113)
Red cask wine	15	31	106	(91, 121)

[a] $\alpha = 0.01$

[b] $\alpha = 0.01$, $\beta = 0.01$

The uncertainty for a value drawn in the inverse regression was slightly greater in the EMS case as expected, both considering matrix-specific and overall calibration function. The choice of the y_0 values gives the opportunity of comparing the uncertainty at the same discriminated concentration using the overall lines. It is noticeable that the use of an overall calibration function can substitute profitably for the matrix-specific straight line working either with labelled or not labelled IS.

Analogously the presence of a variance contribution coming from the matrix effects raises the critical and the detection limits. The same result was found by comparing critical and detection limits in matrix-specific calibration curves. For instance, Nero d'Avola calibration curve gives a x_C of 7 µg/L and a x_D of 16 µg/L when d_6-DMS is used, while when EMS is employed the x_C and x_D are 10 µg/L and 18 µg/L, respectively.

In order to compare the parameters obtained either by combining the Hubaux-Vos approach with the VCM model or by the usual simple procedure based on the signal to noise ratio equal to 3, the chromatogram relevant to Nero d'Avola wine matrix, shown in **Figure 16**, is reported.

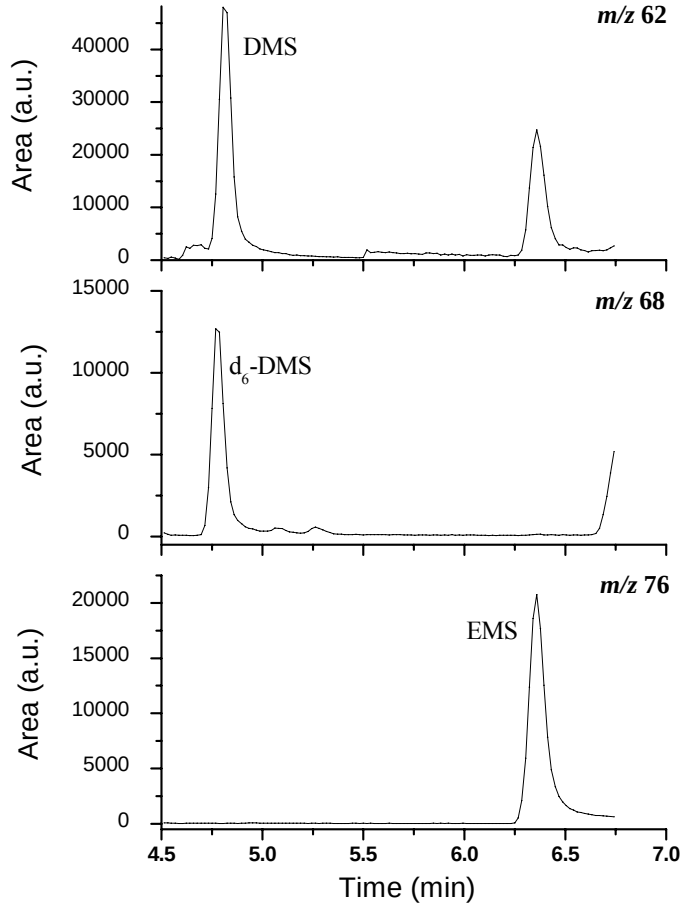


Figure 16. Example of GC-MS chromatogram recorded in SIM mode for a Nero d'Avola sample at concentration close to the detection limit.

This chromatogram refers to the lowest concentration considered in the calibration experiments. The peak height equal to 3 times the standard deviation of the background corresponds to a DMS concentration of 6 μg/L. As expected, this value resulted to be close to x_C obtained with the two IS and quite lower than the above reported x_D.

3.5 Conclusions

The use of internal standards in mass spectrometric quantitative analysis when different matrices are considered leads to the following general statements: i) the availability of an ideal internal standard cancels any matrix effect since the interaction between analyte and matrix and between internal standard and matrix are rigorously the same. This situation is approached using an isotopically labelled internal standard; ii)

often small deviations are found changing the matrices even with isotopically labelled internal standards. If these deviations are randomly distributed and can be attributed to calibration errors, the most suitable approach is the use of an overall calibration function averaged on the matrix-specified calibration functions. Sometime the deviations are still randomly distributed but their entity can be explained taking into account matrix effects in addition to calibration errors. If the primary objective is a screening analysis on several matrices, the overall calibration curve with its prediction band obtained by the VCM procedure is advisable; iii) the use of a non-isotopically labelled internal standard introduces not negligible matrix effects which sometime do not permit the use of an overall calibration curve.

The results achieved with the present work pointed out that the replacement of matrix-specific calibration curves with an overall one is totally satisfactory for HS-SPME analyses in an oenological context using an isotopically labelled internal standard. If an unlabelled internal standard is used, the procedure employing the overall calibration function can be maintained in the context of the variance component model which accounts for the increment of dispersion of the measurement values introduced by the different matrices. The drawback of a larger uncertainty of the concentration values calculated by the inverse regression for routine analyses is largely overcome by the benefits in terms of time and cost.

4. FERMENTATIVE SULFUR COMPOUNDS †

4.1 Preliminary considerations

Fermentative sulfur compounds (FSCs) have a primary influence on the perceived aroma of many foods and beverages [1, 36]. In the fermented drinks, they were mainly investigated in beer [37-39] and wine [4, 40, 41] mostly to justify possible off-scenting resembling onion, garlic, cooked cabbage, rubber and putrefaction, due to the presence at trace level of short-chain thiols, sulfides, disulfide, thioesters, and of heterocyclic compounds. By convention the usually considered off-flavour compounds in wine are divided into "light" (b.p. < 90°C) and "heavy" (b.p. > 90°C) compounds [4]. This difference implies different sampling methods for the analysis, e.g. static and dynamic headspace and headspace-solid phase micro-extraction (HS-SPME) for the lighter species and liquid-liquid extraction for the heavier [4, 42-44].

In wine, the aroma contribution of FSCs is often considered negative, due to their characteristics odours of rotten egg, putrefaction, onion, cabbage, garlic [4]. Nonetheless, FSCs are present in the vast majority of wines, and low concentration of these compounds can positively contribute to the aroma complexity of red and white wines [4]. In some cases FSCs appear to be involved in varietal and ageing-related differences between wines [8]: this statement is supported by the comparison of the aroma before and after treatment of wine with Ag salts.

Hydrogen sulfide (H_2S) is the most studied FSCs in wine, due to its association with reductive off-flavors often described as rotten egg and putrefaction [4]. Although H_2S can be formed from elemental sulfur and other fungicides often present on grapes [45], the major portion of H_2S arises as an intermediate in the biosynthesis of sulfur containing amino acids by yeast during fermentation [46, 47]. Yeast strain [47-49], must

† The contents of this chapter have been published in 3 peer reviewed publications

turbidity [50], availability of fermentation nutrients [51, 52], and presence of metal ions [53] can affect the amount of H_2S produced during fermentation.

Low molecular weight FSCs, such as mercaptans, sulfides and disulfides, have been also identified in wines. Among these, methylmercaptan (MetSH), ethylmercaptan (EtSH), dimethyl sulfide (DMS), diethyl disulfide (DEDS), have been indicated as potential contributors to wine aroma, due to their low odour threshold [4, 15, 54, 55]. The odour of these compounds is usually described with attributes such as reductive or putrefaction for the mercaptans, and cabbage, onion, or rubber for sulfides and disulfides [4]. Based on these descriptors, their contribution to wine aroma is generally considered negative. A positive effect of DMS on aroma was demonstrated by Spedding and Raut [16] and confirmed by Segurel et al. [15]: this compound increases by wine aging and its level can be variety-influenced [56].

Additionally, other FSCs, often referred to as "heavy", are produced during fermentation. Among these, the amino acid-related thioalcohol 3-(methylthio)-1-propanol (methionol), reported to have a boiled potatoes odour, is the most abundant FSCs in wine (around mg/L level). Heavy FSCs are generally characterised by odours often described as cooked vegetables, boiled potatoes, poultry, onions, but their contribution to wine aroma has still to be established. While a large number of studies have investigated, the effects of different fermentation conditions on H_2S formation, the studies on the factors affecting formation of other FSCs, particularly the low molecular weight ones, are relatively scarce. Particularly, to date, no study has explored the effects of different winemaking practices on the formation of FSCs in red wines.

Various nutrients that are essential to yeast metabolism are often suboptimal in grape must. Among these, yeast assimilable nitrogen (YAN), defined as the nitrogen contained in the ammonia and free α-amino acid (FAN) fractions of juice, provides nitrogen for protein biosynthesis of the cell, and is therefore of primary importance for correct functioning of cell metabolism (reviewed in [51]). Nitrogen supplementation in the winery, usually in the form of diammonium phosphate (DAP), has long been used for this reason. Previous work has indicated that DAP is a powerful modulator of various fermentation-derived aroma compounds, including H_2S [46, 50, 57-59], but the effects of its addition on other FSCs are still poorly understood.

Yeast strain is also known to play a fundamental role in determining wine aroma composition and characteristics. In regard to FSCs, the ability of strains of S. cerevisiae to produce different amounts of H_2S is well documented [60], and limited data on other FSCs are also available [60]. However, in recent years, the use of non-cerevisiae yeasts has received considerable attention, and strains of *Saccharomyces bayanus*, interspecies hybrids, and mixed starters of Saccharomyces and non-Saccharomyces strains are now available on the market in the form of active dry yeasts [61]. The effects of different winemaking practices, including nitrogen supplementation, on the formation of volatile compounds by non-conventional yeast strains have still to be investigated.

Several analytical approaches are employed to quantify FSCs in wine [4] and the headspace procedure, like the purge and trap and solid-phase microextraction methods, combined with gas chromatography coupled to different detectors, was shown to be quite effective [62-64]. As noted in recent papers [63, 65-69], the HS-SPME technique, with an improved choice of fibre coating phases, would seem to be one of the more promising approaches for the concurrent measurement of compounds with different boiling points, but to date none has allowed common quantification of compounds with relatively different physical and chemical properties.

During my thesis I developed a fast and sensitive HS-SPME/GC-MS quantification method [21] to measure 13 sulfur volatiles in wine characterised by a wide boiling point range (from 35 to 231°C)and chosen among those most frequently investigated in fermented beverages. Furthermore the content of these analytes have been quantified using our method and the obtained data were submitted to statistical treatments. Possible ageing and variety effects on such compounds were investigated in eighty wines of five vintages and of four varieties typical of Northern Italian wine-growing area of Trentino [8]. The varieties considered were the red berry native Teroldego (T) and Marzemino (Ma), the international Merlot (M) and the white berry Chardonnay (C), treated with standard vinification conditions in the different vintages.

Finally, the effects of DAP supplementation on FSCs of experimental Vitis vinifera cv. Shiraz wines obtained by fermentation of a low nitrogen must with

winemaking strains of *Saccharomyces cerevisiae* and *Saccharomyces bayanus* have been investigated, in order to provide a first characterization of the combined effects of yeast selection and nitrogen supplementation on the pool of FSCs in red wine made under typical winemaking conditions [70].

4.2 Experiments

4.2.1 Chemicals and reagents

The sulfur compounds utilised were: sodium sulfide, methylmercaptan (MetSH), ethylmercaptan (EtSH), dimethyl sulfide (DMS), diethyl sulfide (DES), dimethyl disulfide (DMDS), diethyl disulfide (DEDS), methyl thioacetate (MTA), ethyl thioacetate (ETA), 2-mercaptoethanol (ME), 2-(methylthio)-1-ethanol (MTE), 3-(methylthio)-1-propanol (MTP), 4-(methylthio)-1-butanol (MTB), benzothiazole (BT) and 5-(2-hydroxyethyl)-4-methylthiazole (HMT). Dimethyl sulfide-d_6 (d_6-DMS), dipropyl disulfide (DPDS), 3-(methylthio)-1-hexanol (MTH) and 4-methylthiazole (MT) were considered as possible internal standards (I.Ss.). All the purchased standards had a purity of >98%, and were supplied by Sigma-Aldrich (Milan, Italy) and Lancaster (Milan, Italy). Charcoal was supplied by Merck (Milan, Italy), inorganic compounds and anhydrous ethanol (> 99 %) by Carlo Erba (Milan, Italy).

Individual standard solutions for each sulfur compound in pure ethanol were prepared, and from these a working ethanolic solution containing all the analytes, sampling an aliquot of each standard solution; all the solutions were stored at -16°C. H_2S was obtained by dissolving sodium sulfide in water at pH 3.2. The same procedure was followed to prepare internal standard solutions at similar concentrations.

All compounds were identified by means of co-injection with pure reference compounds or comparison of their retention times and mass spectra with those of reference standards.

4.2.2 HS-SPME sampling

The SPME holder for manual sampling and the fibres used were purchased from Supelco (Bellefonte, PA, USA). On the basis of the compounds chosen and of the fibres used in previous works, those tested were: polyacrylate (PA; 85 μm x 1 cm); polydimethylsiloxane (PDMS; 100 μm x 1 cm); polydimethylsiloxane-divinylbenzene (PDMS-DVB; 65 μm x 1 cm); carboxen-polydimethylsiloxane (CAR-PDMS; 75 μm x 1 cm); carboxen-polydimethylsiloxane-divinylbenzene (CAR-PDMS-DVB; 50/30 μm x 1 cm); carboxen-polydimethylsiloxane-divinylbenzene (CAR-PDMS-DVB; 50/30 μm x 2 cm). Before sampling, all the fibres were conditioned according to the producer's instructions.

The performance of the fibres was tested by analysing aliquots of wines fortified with known amounts of the sulfur compounds and I.Ss. in triplicate using the headspace procedure, with experimental conditions close to those finally chosen.

The most promising fibre, in terms of the maximum signal for each compound, was chosen to optimise, by a univariate approach, the sampling procedure and the operating conditions (ionic strength, temperature of the sample, adsorption time).

4.2.3 GC-MS analysis

Chromatographic analysis was done with a Perkin Elmer Autosystem XL gas chromatograph coupled with a TurboMass Gold mass spectrometer (Perkin Elmer; Boston, MA, USA) equipped with a 30 m x 0.32 mm I.D. x 0.25 μm film thickness Innowax (PEG) fused-silica capillary column (Agilent Technologies; Palo Alto, CA, USA). Helium was used as carrier gas at a flow-rate of 1.2 mL/min. The temperature of the transfer line was 220°C. The electron impact energy was 70 eV, and MS source was set at 150°C. All the analysis was carried out in single ion recording (SIR) mode, using the NIST library to choose the fragments for quantification.

On the basis of the chromatographic conditions proposed by Segurel et al. (2004) [15], resolution tests on standard solutions and on wine matrixes spiked with the appropriate amounts of analytes were carried out to define the best working conditions. These were: (a) GC injector temperature 250°C, (b) injection in splitless mode for 1

minute, (c) oven temperature program: 35°C (5 mins), 1°C/min to 40°C, 10°C/min to 250°C.

4.2.4 Calibration curves and performance evaluation

Calibration curves for each analyte were prepared according to the internal standard method using the following as internal standards: d_6-DMS 25 µg/L, DPDS 25 µg/L, MT 10 µg/L and MTH 50 µg/L. The matrix was a white wine (WW; alcohol strength 10 % v/v.; sugar content: < 4 g/L; polyphenols content: 115 mg/L as (+) cathechine) treated twice with charcoal (3 g/L) to remove any sulfur compounds detectable by the proposed headspace SPME/GC-MS method and other main volatile compounds, except the most polar ones such as the higher alcohols. The aim of this pre-treatment of the sample was to obtain a matrix similar to wine, but free from volatiles potentially affecting adsorption.

Linearity and precision were verified in the concentration ranges typical of wines for each compound using 7 concentration levels and 5 replicate solutions per level.

For calculation of the critical (L_C) and detection (L_D) limits following the Hubaux-Vos method [34] four standard solutions at very low concentration values were prepared. The signals corresponding to these dedicated solutions, together with the signal corresponding to the blank solution, were used in regression. This procedure decreases the difference between the experimentally measured blank and the intercept of the regression line [71]. The quantification limit (L_Q) was estimated using the Eurachem approach [72], which states that the quantification limit is the analyte concentration for which the experimental relative standard deviation of the responses reaches a specific level, for example level 0.1.

4.2.5 GC-AED analysis of some fermentative sulfur compounds

The data relevant to hydrogen sulfide, methanethiol and carbon disulfide were provided by The Australian Wine Research Institute. The analyses of these compounds were performed according to Siebert et al. [ref] and they were carried out by people working at the Australian Wine Research Institute.

4.2.6 Sampling plan and basic quality data to test ageing and variety effects

Eighty single-variety wines produced in stainless steel tanks on a semi-industrial scale from about 100 kg of grape using traditional winemaking protocols in the experimental winery of the IASMA Research Center (Italy) were analysed. More precisely, four wines per four variety (three red-fruited, Teroldego, Marzemino, Merlot and a white-fruited Chardonnay) and per vintage year (1998, 2001, 2002, 2003, 2004) were sampled among wines produced with several *Saccharomyces Cerevisiae* yeast strains and referred to different grape-growing areas of Trentino. The yeast strain employed in this work are listed in **Table 4**. They were chosen among the most commonly yeasts marketed in the relevant vintages.

Table 4. Yeast strains employed to test ageing and variety effects

Number	Yeast Strains	Species and race	Producer
1	Blastosel Grand Cru	*Saccharomyces cerevisiae* (r. f. *bayanus*)	Pall Filtration & Separations, Verona, Italy
2	Blastosel Kappa	*Saccharomyces cerevisiae*	Pall Filtration & Separations, Verona, Italy
3	Blastosel Terroir	*Saccharomyces cerevisiae* (r. f. *bayanus*)	Pall Filtration & Separations, Verona, Italy
4	Blastosel VS	*Saccharomyces cerevisiae* (r. f. *bayanus*)	Pall Filtration & Separations, Verona, Italy
5	CAB90	*Saccharomyces cerevisiae*	INTEC, Verona, Italy
6	Collection cepage Merlot	*Saccharomyces cerevisiae*	Gist-Brocades, Delft, The Netherlands
7	Davistart	*Saccharomyces cerevisiae*	Gist-Brocades, Delft, The Netherlands
8	EC1118	*Saccharomyces cerevisiae* (r. f. *bayanus*)	Lalvin-Lallemand, Montreal, Canada
9	Enolevure K34	*Saccharomyces cerevisiae*	INRA, Montpellier, France
10	Fermiblanc Arom	*Saccharomyces cerevisiae*	Gist-Brocades, Delft, The Netherlands
11	Fermicru LS2	*Saccharomyces cerevisiae* (r. f. *bayanus*)	Gist-Brocades, Delft, The Netherlands
12	Fermivin Cryo	*Saccharomyces cerevisiae*	Gist-Brocades, Delft, The Netherlands
13	GAR26	*Saccharomyces cerevisiae*	Lalvin-Lallemand, Montreal, Canada
14	La Claire CGC62	*Saccharomyces cerevisiae*	Pall Filtration & Separations, Verona, Italy
15	La Claire EM2	*Saccharomyces cerevisiae*	Pall Filtration & Separations, Verona, Italy
16	Premium rouge	*Saccharomyces cerevisiae*	Vason, Verona, Italy
17	Fermicru VR5	*Saccharomyces cerevisiae*	DSM Food Specialties B.V., Delft, The Netherlands

The red wines were processed with a 7 day skin-contact and complete malolactic fermentation, while Chardonnay without skin-contact and malolactic fermentation. All sterile-filtered screw-topped bottled wines were stored at the same constant temperature of 16°C in the dark and analysed in the fall 2006. The minimal two years ageing of products should ensure the achievement of the equilibria between thioacetates and the relevant thiols [41, 73]. No wine was sulfur compounds off-flavouring at the sensory analysis, performed by a panel of five oenologists. Further no silver or copper fining was carried out.

The main basic parameters are reported in **Table 5** for each wine type at the bottling, showing similar variation ranges for the red wines group, but different from those of the white products for pH and total acidity.

Table 5. Main analytical data and SD (in brackets) for the four varieties considered

Variety	Alcohol Concentration (% Vol.)	pH	Titratable Acidity[a]
Teroldego	12.25 (0.69)	3.74 (0.18)	5.10 (0.84)
Marzemino	12.05 (0.47)	3.78 (0.11)	5.01 (0.32)
Chardonnay	12.23 (1.30)	3.27 (0.14)	6.91 (1.39)
Merlot	12.78 (0.80)	3.70 (0.18)	4.82 (0.57)

[a]Titratable acidity is expressed as tartaric acid in g/L.

4.2.7 Experimental plan for evaluating the effect of nutrient supplementation on fermentative sulfur compounds content

Shiraz grapes with low yeast assimilable nitrogen (YAN) were obtained from the Langhorne Creek winemaking district in South Australia during the 2007 vintage. No nutrient supplements had been applied to this vineyard block for 5 years. The grapes were picked and collected in 15 kg plastic bins. Once in the winery, different crates were pooled together to obtain an homogenous mass. Individual 30 kg lots were then destemmed and crushed, and the must was collected in 50 L stainless steel fermentation vessels. The analytical parameters of the must were as follows: 24°Brix, titratable acidity, 7.2 g/L as tartaric acid; pH, 3.3; YAN, 101 mg/L. Potassium metabisulfite was added at 100 mg/kg to each fermentation approximately 2 hours before inoculation. DAP additions were performed according to an experimental design consisting of three YAN concentrations, each one fermented in triplicate, for a total of nine fermentations. A control that did not receive any DAP addition represented the lowest nitrogen concentration (101 mg/L YAN), while in the two other treatments DAP was added to a final YAN concentration of 250 mg/L YAN and 400 mg/L YAN, respectively. All DAP additions were performed prior to inoculation. Following DAP additions, pH of the samples was measured and re-adjusted to 3.3 by means of 1N HCl. Then, the samples

were inoculated with either of two yeast *Saccharomyces Cerevisiae* D254 (Lallemand) and *Saccharomyces Bayanus* 1176 (Lallemand) at a rate of 1×106 cells/mL, following rehydration in water at 40°C for 30 min. Fermentations were carried out at 22 °C, with the cap plunged three times per day. Fermentation progress was monitored by enzymatic analysis of the residual sugars. Dominance of the inoculated strain in all the treatments was confirmed by transposon PCR analysis [74]. The wines were left to macerate on grape skins until the slowest treatment reached dryness (residual sugars ≤ 2 g/L), after which the fermented musts were pressed, the wines collected in 20 L stainless steel containers, and placed at 4 °C under inert nitrogen headspace to accelerate clarification. No malolactic fermentation was carried out. After 4 weeks 150 mg/L of potassium metabisulfite was added to the wines, which were then filtered through 0.45 μm membranes (Sartorius, Gottingen, Germany) and bottled under ROTE closures. Analysis of the sulfur compounds in the wines was carried out after 3 months of storage at 14 °C.

Titratable acidity, FAN, and ammonia were measured as previously described [74]. YAN was calculated as the sum of ammonia-derived nitrogen and FAN, and therefore did not include proline. Fermentation progress was monitored by daily analysis of residual sugar by means of an enzymatic kit [74].

4.3 Results and discussion

4.3.1 HS-SPME sampling optimisation

GC-MS analysis was carried out in SIR mode to recognise and quantify the analytes studied. **Table 6** gives the fragments chosen.

Table 6. Retention time and quantitation and qualifier ions

Analytes	RT (min)	Quantitation ion	Qualifier ions
DMS	4.76	62	45, 47
EtSH	4.61	62	47, 61
DES	8.52	75	61, 90
MTA	14.42	90	43, 47
DMDS	15.01	94	64, 79
ETA	15.40	104	43, 60
DEDS	18.00	122	66, 94
ME	22.19	78	47, 60
MTE	22.61	92	47, 61
MTP	24.72	106	58, 61
MTB	26.04	120	61, 102
BT	27.61	135	69, 108
HMT	30.58	112	85, 143
d_6-DMS	4.73	68	50, 66
DPDS	18.68	108	66, 150
MT	19.21	71	39, 99
MTH	26.57	148	61, 75

This data recording was shown to be totally successful in the complete resolution of all the analytes, also with regard to the high presence of ethanol. **Figure 17** shows a typical example of a chromatogram obtained following the procedure described.

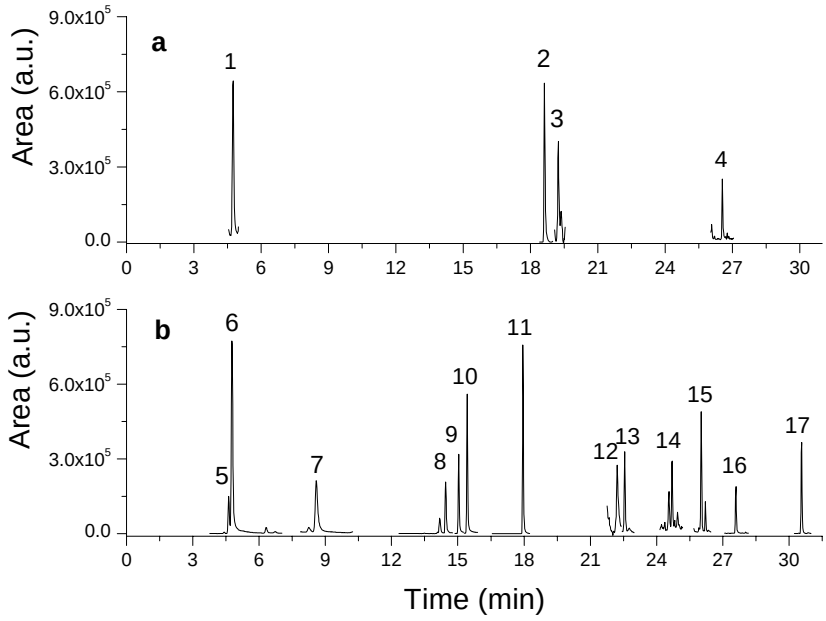


Figure 17. Chromatographic peaks obtained in SIR mode, in a commercial wine, for the investigated analytes 1: d_6-DMS, m/z 68; 2: DPDS, m/z 108; 3: MT, m/z 71; 4: MTH, m/z 148; 5: EtSH, m/z 62; 6: DMS, m/z 62; 7: DES, m/z 75; 8: MTA, m/z 90; 9: DMDS, m/z 94; 10: ETA, m/z 104; 11: DEDS, m/z 122; 12: ME, m/z 78; 13: MTE, m/z 92; 14: MTP, m/z 106; 15: MTB, m/z 120; 16: BT, m/z 135; 17: HMT, m/z 112. **Figure 17a.** Internal standards adopted; **Figure 17b.** analytes considered.

As shown in **Table 7**, the use of the 2 cm long DVB/CAR/PDMS fibre allowed the best results to be obtained, giving the biggest chromatographic peaks for each analyte, operating in conditions close to those finally chosen.

Table 7. Measured areas (arbitrary units) and standard deviation (in brackets) for the fibres considered on a spiked matrix. nd: not detected

	Fiber coatings					
Analytes	PDMS	CAR-PDMS	DVB-CAR-PDMS 1 cm	DVB-CAR-PDMS 2 cm	PA	DVB-PDMS
DMS	1011 (147)	4954 (486)	17186 (963)	37186 (1096)	5438 (884)	20597 (713)
d_6-DMS	324 (57)	1922 (369)	6535 (893)	21535 (1036)	3087 (237)	13944 (1069)
DES	247 (81)	8368 (1097)	9044 (1239)	17044 (1169)	1555 (151)	9479 (1483)
EtSH	787 (99)	nd	1888 (253)	7888 (536)	1928 (569)	2310 (127)
MTA	nd	nd	12728 (1176)	24728 (1493)	1239 (476)	1583 (229)
ETA	nd	nd	12151 (269)	25111 (830)	4196 (431)	6868 (2212)
ME	172 (51)	124 (24)	886 (96)	1486 (123)	144 (64)	521 (98)
DMDS	nd	1391 (410)	7251 (448)	13294 (937)	2024 (346)	6434 (179)
DEDS	15514 (1837)	99079 (1396)	205713 (10861)	445748 (10326)	34424 (1013)	69488 (1338)
DPDS	197091 (10837)	102419 (10397)	738315 (200697)	1538900 (249644)	142936 (9863)	407377 (12687)
MT	4868 (1442)	5750 (937)	2145 (569)	6145 (367)	682 (1068)	4048 (1007)
MTE	4876 (592)	25 (5)	10063 (489)	23063 (786)	4863 (1129)	3798 (547)
MTP	576 (81)	938 (207)	2284 (486)	7284 (1021)	4325 (1597)	2035 (636)
MTB	9562 (1361)	341 (97)	14496 (1239)	28496 (1486)	7219 (1837)	11879 (1191)
MTH	440 (100)	900 (116)	1297 (599)	3597 (617)	2238 (1093)	1168 (896)
BT	1696 (671)	1822 (207)	7672 (563)	11672 (1119)	6836 (1271)	7149 (764)
HMT	541 (118)	813 (109)	1947 (406)	2947 (419)	1571 (147)	720 (99)

To optimise ionic strength, different salt amounts were added to the sample we considered, as NaCl (2.0 and 5.0 M) and Na_2SO_4 (2.0 M) on the basis of previous works [14, 15, 63, 65-68, 75] and $MgSO_4 \cdot 7H_2O$, 0.5 and 1.0 M. The results are shown in **Figure 18**. $MgSO_4 \cdot 7H_2O$ 1.0 M, although realising a ionic strength slightly lower than some considered, showed the best salting-out effect for most compounds, thus allowing higher peak areas to be obtained (in the range from 10 % to 25 %).

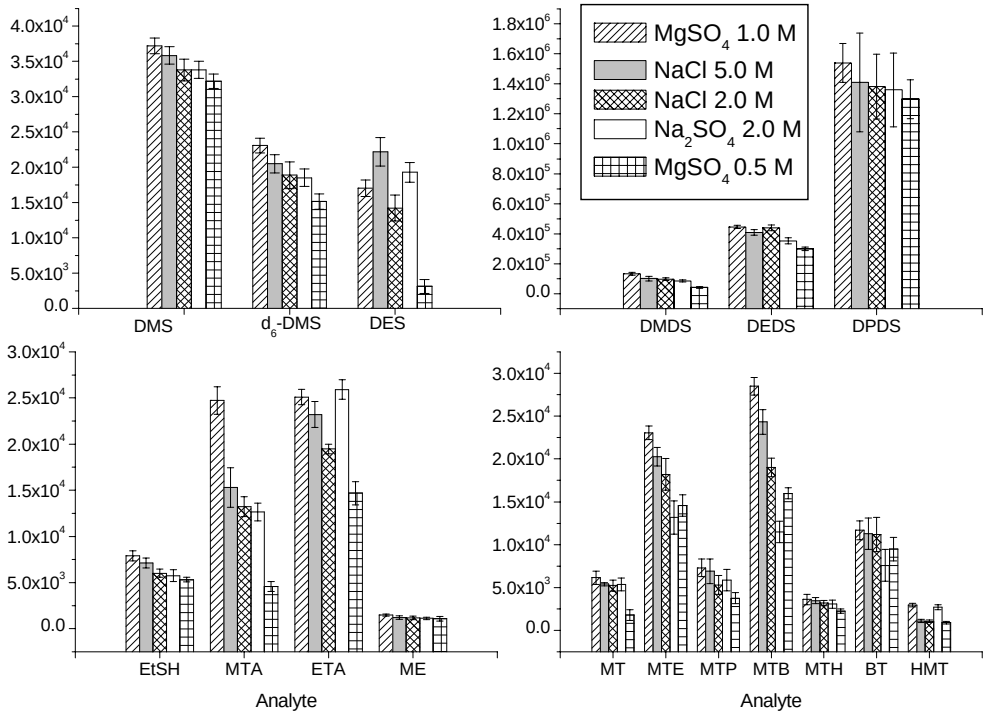


Figure 18. Salting-out effect of the tested salts. The different heights correspond to the different peak areas obtained for each compound. The error bars shown represents the SD of the mean.

Figure 19 shows the effects of solution temperature on the peak areas in the range of 25-65°C, demonstrating the different behaviour of different classes. A temperature of 35°C would appear to be the best compromise between a larger sampling of the less volatile compounds (**Figure 19d**) and a reduced desorption of the higher volatile compounds (**Figure 19a**).

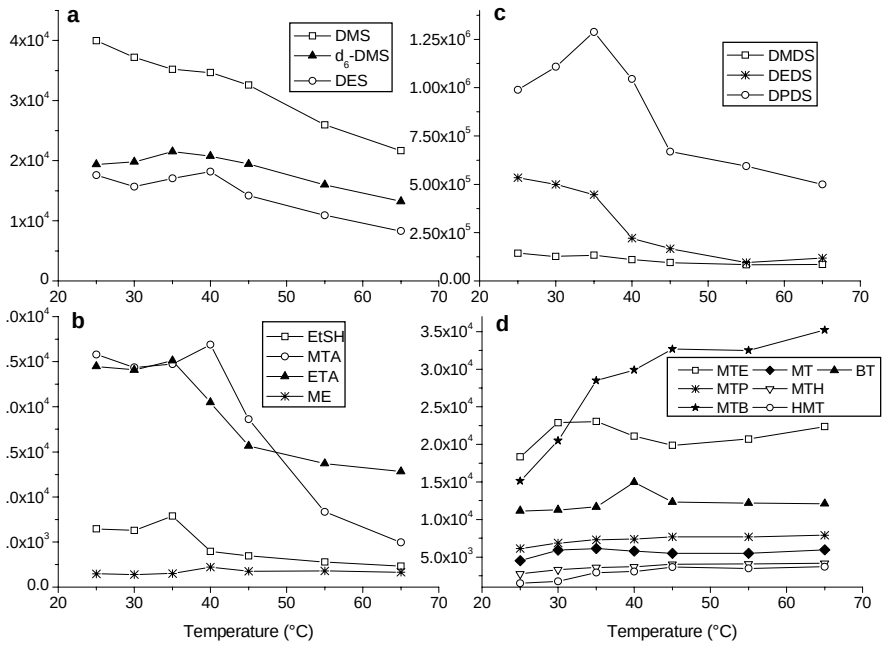


Figure 19. Dependence of the adsorption profiles on the temperature for the cosidered species.

Figure 20 shows the effect of adsorption time in the range 5-90 mins. **Figures 20a** and **20b** show that the more volatile compounds (e.g. DMS, ETA and MTA) reach an equilibrium more quickly than others, but then show decreasing signals as a consequence of competitive desorption mechanisms [76, 77]. On the contrary the heavier compounds, as shown in **Figure 20c** and **20d**, show an adsorption profile becoming constant at 30-40 mins, as theoretically expected [78]. Taking into account the aim of this work, i.e. the concurrent analysis of several compounds, the results obtained indicate a sampling time of 30 minutes as a good compromise for evaluating all the analytes considered.

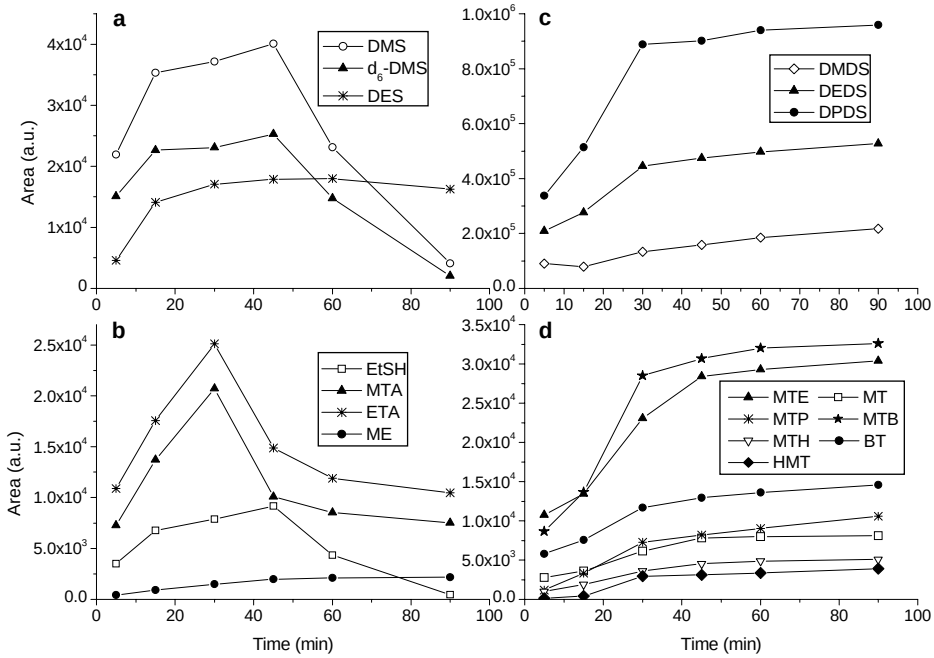


Figure 20. Dependence of the adsorption profiles on the time for the considered species.

Table 8 summarises the operating conditions adopted.

Table 8. Optimised operating conditions

Fiber coating	CAR-PDMS-DVB; 2 cm
Sample temperature	35 °C
Extraction	30 min with stirring (500 rpm)
Salt addition	5 g $MgSO_4 \cdot 7H_2O$
Sample volume	20 mL
Vial volume	30 mL

4.3.2 Validation

Table 9, first column, shows the concentration ranges of the sulfur compounds used for the construction of the calibration curves. These ranges correspond to those naturally occurring in wines. **Table 9**, second column, shows the concentration ranges dedicated to calculation of the limits

Table 9. Concentration ranges used for calibration curves and for detection limits

Analytes	Calibration Range (µg/L)	L_C, L_D and L_Q Range (µg/L)
DMS [a]	3.2 - 240.0	0.0 - 3.2
EtSH [a]	0.4 - 30.0	0.0 - 1.0
DES [a]	0.2 - 15.0	0.0 - 0.5
MTA [a]	0.8 - 60.0	0.0 - 2.0
DMDS [b]	0.2 - 15.0	0.0 - 0.5
ETA [a]	0.8 - 60.0	0.0 - 2.0
DEDS [b]	0.8 - 60.0	0.0 - 0.8
ME [c]	3.2 - 240.0	0.0 - 8.0
MTE [c]	0.8 - 60.0	0.0 - 2.0
MTP [c]	250 - 3250	0.0 - 20.0
MTB [c]	1.6 - 120.0	0.0 - 4.0
BT [d]	0.4 - 30.0	0.0 - 1.0
HMT [d]	0.4 - 30.0	0.0 - 1.0

[a] d_6-DMS as I.S.; [b] DPS as I.S.; [c] MTH as I.S.; [d] MT as I.S..

The proposed method gives linear responses in the explored concentration ranges as shown by the straight line model used in the regression analysis; **Table 10** reports the relative regression parameters together with the values of the detection limits.

Table 10. Parameters of calibration straight lines and values of the detection limits

Analytes	Slope	SD Slope	Intercept	SD Intercept	SD	R^2	L_C (µg/L)	L_D (µg/L)	L_Q (µg/L)
DMS [a]	1.03	0.01	0.045	0.047	0.07	0.997	0.078	0.156	0.358
EtSH [a]	1.19	0.02	0.042	0.012	0.05	0.994	0.067	0.134	0.254
DES [a]	7.28	0.06	0.053	0.010	0.12	0.994	0.057	0.114	0.336
MTA [a]	0.85	0.01	0.006	0.009	0.04	0.997	0.153	0.306	0.643
DMDS [b]	0.80	0.01	0.002	0.002	0.01	0.997	0.031	0.062	0.136
ETA [a]	3.27	0.37	0.000	0.004	0.11	0.996	0.102	0.204	0.429
DEDS [b]	1.02	0.01	0.044	0.016	0.07	0.994	0.037	0.074	0.156
ME [c]	0.82	0.01	0.011	0.029	0.09	0.993	0.383	0.766	1.563
MTE [c]	8.63	0.07	0.010	0.040	0.10	0.998	0.103	0.206	0.453
MTP [c]	0.54	0.00	0.102	0.020	0.07	0.996	0.826	1.652	5.862
MTB [c]	2.75	0.04	0.086	0.047	0.15	0.993	0.27	0.54	1.893
BT [d]	2.78	0.04	0.052	0.059	0.17	0.994	0.484	0.968	3.388
HMT [d]	0.83	0.01	0.119	0.018	0.08	0.993	0.436	0.872	3.806

[a] d_6-DMS as I.S.; [b] DPS as I.S.; [c] MTH as I.S.; [d] MT as I.S..

The detection limits found (**Table 10**) are lower than those found by other authors [62, 63, 65-68, 79] using the criteria 3 S/N.

4.3.3 Variation ranges of sulfur volatiles in the 80 considered wines

The sulfur compounds levels in the wines analysed together with the literature values are reported in **Table 11**. All the analyses were performed with two replicated samples.

Table 11. Variability range indexes of the thirteen sulfur volatiles analysed in the eighty wines and relevant ranges in the literature

Analyte	Mean (µg/L)	Min (µg/L)	Max (µg/L)	St.Dev.	Literature values (µg/L)
1	1.6	0.1	10.5	1.9	0 - 12
2	26.9	2.4	78.2	18.8	0 - 480
3	6.1	0.9	17.2	3.4	1 - 2
4	5.2	0.2	31.0	6.9	0 - 22
5	3.9	2.0	13.7	2.9	0 - 80
6	9.8	1.4	29.0	5.4	0 - 20
7	2.6	0.6	7.1	1.4	0 - 56
8	15.6	0.8	47.5	11.6	0 - 180
9	23.0	3.8	61.9	15.6	0 - 70
10	2551	862	4914	866	0 - 4500
11	38.7	5.3	107.6	24.9	0 - 180
12	5.2	1.0	14.1	3.5	0 - 14
13	2.6	0.9	6.1	1.1	5 - 50

Yeast strain effect coupled with yeast assimilable nitrogen (YAN) level in must could have a remarkable importance on different aroma compounds [80, 81] including some sulfur ones [41]. To point out a possible connection with the investigated sulfur compounds, we considered the different YAN level typical for the variety taken into account [82]. Marzemino and Merlot musts have similar mean YAN level (90 mg/L), Teroldego about 50% richer, and Chardonnay musts have 180 mg/L of YAN. Therefore, no clear association of this parameter with the mean varietal sulfur compound profile here measured seems to be possible.

Inspection of the data prompts some considerations. The contents found are in general in the ranges of German [41], French [14, 15], Spanish [66, 67], Swiss [83] and Greek [49] wines even if these refer sometimes to sub-ranges different for country and wine type [44, 84]. Diethyl sulfide only presents higher levels than those quantified by Mestres et al. [65] and Lopez et al. [85] in Spanish wines. The sulfides (dimethyl- and diethyl sulfide) contents are higher than those reported by several authors [65, 66, 85]. 2-(methylthio)-1-ethanol level is higher than that found in Spanish and Greek wines [65, 85], as well as benzothiazole content remarkably higher than in other Italian wines [86]. 5-(2-hydroxyethyl)-4-methylthiazole level is meanly about one tenth of that found by Rapp et al. [43].

4.2.4 Storage time and variety effects on the 80 considered wines

To recognise the presence of significant differences [87] due to ageing and variety effects on each sulfur compound, we applied the Tukey test, a pair-wise comparison of the means, to the data collected (**Table 12**).

Table 12. Mean values per vintage of the sulfur volatiles analysed. Tukey's test results.

Analyte (µg/L)	1998		2001		2002		2003		2004	
	mean	S.D.	mean	S.D.	mean	S.D.	mean	S.D.	mean	S.D.
1	0.7 ^{b}	0.26	1.5 ^{ab}	0.77	1.5 ^{ab}	0.48	1.8 ^{ab}	1.09	2.5 ^{a}	1.74
2	53.4 ^{a}	9.42	35.6 ^{b}	8.86	22.5 ^{c}	7.83	15.1 ^{d}	6.61	8.0 ^{c}	5.77
3	8.9 ^{a}	2.55	7.4 ^{ab}	2.26	4.9 ^{bc}	1.86	5.7 ^{bc}	2.38	3.5 ^{c}	1.79
4	8.7 ^{a}	8.23	4.0 ^{ab}	3.62	3.5 ^{b}	3.27	5.3 ^{ab}	4.33	4.5 ^{ab}	3.60
5	6.0 ^{a}	4.22	3.4 ^{b}	2.19	3.5 b	2.12	3.7 ^{b}	2.81	2.8 ^{b}	1.15
6	8.2 $^{n.s.}$	3.14	8.3 $^{n.s.}$	3.55	10.4 $^{n.s.}$	3.19	10.3 $^{n.s.}$	4.82	11.6 $^{n.s.}$	4.55
7	1.8 ^{b}	0.64	2.3 ^{ab}	0.82	2.8 ^{ab}	1.44	2.7 ^{ab}	1.49	3.2 ^{a}	2.09
8	4.8 ^{d}	2.39	10.9 ^{c}	8.30	13.8 ^{c}	4.36	21.0 ^{b}	12.04	27.7 ^{a}	11.24
9	26.4 ^{a}	14.14	25.5 ^{ab}	18.00	20.8 ^{ab}	15.58	23.2 ^{ab}	14.90	18.9 ^{b}	13.04
10	3386 ^{a}	840.5	2807 ^{b}	881.4	2543 ^{bc}	588.2	2170 ^{cd}	521.5	1850 ^{d}	596.9
11	38.7 $^{n.s.}$	24.84	42.6 $^{n.s.}$	32.53	41.0 $^{n.s.}$	22.29	41.1 $^{n.s.}$	26.81	30.2 $^{n.s.}$	16.00
12	6.6 ^{a}	3.21	4.4 ^{ab}	3.16	3.8 ^{b}	2.35	5.9 ^{ab}	4.24	5.1 ^{ab}	3.69
13	2.1 $^{n.s.}$	0.95	3.0 $^{n.s.}$	1.29	2.5 $^{n.s.}$	1.14	2.6 $^{n.s.}$	0.99	2.6 $^{n.s.}$	1.01

Values with the same letter do not differ significantly at the Tukey's test, $p < 0.05$. n.s. not significant.

A change in the concentration of sulfur volatiles with the storage time is reported in few papers [14, 56]. It is well known in fact that the DMS level increases with ageing [14, 17, 56], and that methyl- and ethyl thioacetates may hydrolyse in the first months of storage with the concomitant increase of the relevant thiols and disulfides [41]. The present research, on the basis of a balanced sampling plan and of a statistical approach adopted, shows that the level of some sulfur compounds is without doubt affected by ageing. This fact results to be quite important and useful in the definition and in the understanding of the wine aroma evolution.

Besides dimethyl sulfide, also 3-(methylthio)-1-propanol and 2-mercaptoethanol contents change in the course of time increasing and decreasing respectively. **Figure 21** shows the evolution of dimethyl sulfide (DMS) and 2-mercaptoethanol (ME), using a straight line model, confirming the data reported in the literature.

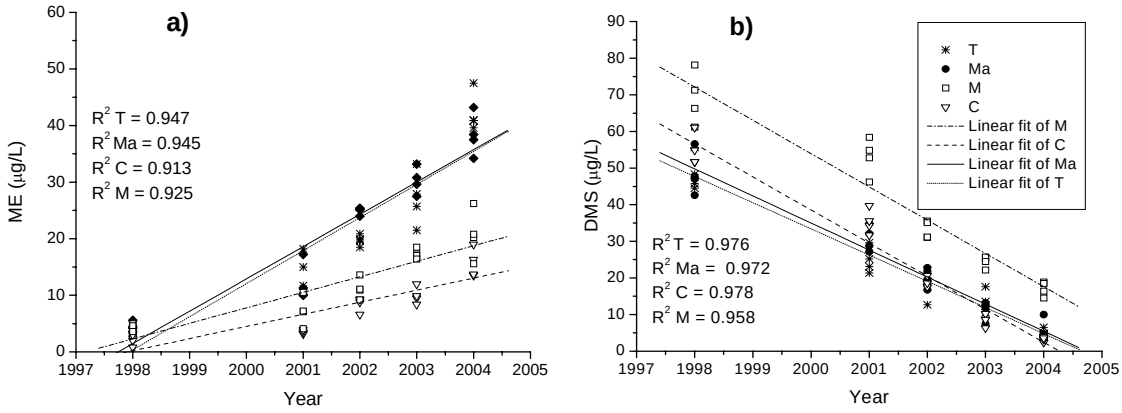


Figure 21. Influence of ageing and variety on the evolution profile of ME **(a)** and DMS **(b)**. Linear fitting and R^2. T: Teroldego; Ma: Marzemino; C: Chardonnay; M: Merlot.

It appears that for both the molecules considered, Merlot and Chardonnay show a parallel evolution profile with the storage time. Analogously ethyl mercaptan and diethyl sulfide vary in opposite ways with time, even if less significantly. Diethyl disulfide follows the increasing tendency with time shown by diethyl sulfide. This fact confirms the findings of Bobet et al. [88], regarding the shift with time towards the oxidised form in the redox equilibrium between thiols and disulfide.

The behaviours of the examined species with time can be rationalised on the basis of different arguments:

i)the level of dimethyl sulfide increases owing to the *S*-methylmethionine degradation [14];

ii)the decrease of 2-mercaptoethanol is due to the its oxidation [7, 89];

iii)the increase of 3-(methylthio)-1-propanol, until now never evidentiated, is supposed to derive either from the degradation of methionine via Strecker mechanism [90] or by decarboxylation of the 2-oxo-derivative of the amino acid obtained via Ehrlich mechanism to methional [91] and subsequent reduction.

To date, evidences of the dependence of sulfur compound concentrations on grapes variety have been never reported in literature with the exclusion of dimethyl sulfide [56] and of some tropical fruit scenting thiols [92], here not considered.

Our data in **Table 13** show that some sulfur compounds are more abundant in some wine varieties than in others.

Table 13. Mean values per variety of the sulfur volatiles analysed. Tukey's test results.

Analyte (µg/L)	Merlot		Teroldego		Marzemino		Chardonnay	
	mean	S.D.	mean	S.D.	mean	S.D.	mean	S.D.
1	0.7 ^{b}	0.35	3.2 ^{a}	2.00	1.5 ^{b}	1.05	0.8 ^{b}	0.32
2	39.9 ^{a}	17.67	22.1 ^{b}	14.83	21.9 ^{b}	14.20	23.8 ^{b}	15.62
3	7.2 ^{ab}	3.43	5.2 ^{bc}	1.95	7.5 ^{a}	4.68	4.3 ^{c}	2.05
4	9.0 ^{a}	6.96	0.7 ^{b}	0.18	1.1 ^{b}	0.49	10 ^{a}	8.35
5	4.9 ^{a}	2.40	2.2 ^{b}	0.13	2.3 ^{b}	0.22	6.0 ^{a}	4.08
6	8.5 ^{b}	3.21	13.7 ^{a}	6.57	7.6 ^{b}	5.01	9.2 ^{b}	2.94
7	2.1 $^{n.s.}$	0.66	2.7 $^{n.s.}$	2.03	2.7 $^{n.s.}$	1.58	2.7 $^{n.s.}$	1.10
8	11.5 ^{b}	6.27	20.7 ^{a}	12.91	22.3 ^{a}	12.82	8.0 ^{b}	5.61
9	44.7 ^{a}	8.90	11.5 ^{c}	3.70	24.2 ^{b}	9.40	11.4 ^{c}	7.05
10	3024 ^{a}	493	2569 ^{b}	755	2861 ^{ab}	1041	1749 ^{c}	453
11	75.1 ^{a}	20.02	28.9 ^{b}	7.27	26.0 ^{b}	7.85	24.9 ^{b}	13.58
12	9.0 a	3.29	2.9 ^{c}	2.40	3.2 ^{c}	2.14	5.5 ^{b}	1.82
13	2.3 ^{b}	0.83	2.0 ^{b}	0.56	2.4 ^{b}	1.08	3.6 ^{a}	1.16

Values with the same letter do not differ significantly at the Tukey's test, $p < 0.05$. n.s. not significant.

In particular, as shown by the Tukey test, the concentration of dimethyl sulfide and 4-(methylthio)-1-butanol in Merlot wines is significantly higher than in all the other varieties, thus supporting the important grassy/truffle-like scent for dimethyl sulfide [14, 15, 56] and the earthy-like scent for 4-(methylthio)-1-butanol [90], used commonly as descriptors for the Merlot aroma. Also the high level of 2-(methylthio)-1-ethanol and benzothiazole is discriminant of the Merlot variety. Ethyl mercaptan and methyl thioacetate concentrations, likely determining rotten eggs/garlic/onion-like scents when over the threshold levels, are on the average higher in Teroldego than in the other considered wines. Furthermore, a clear difference for dimethyl disulfide and diethyl sulfide between the groups Merlot/Chardonnay and Teroldego/Marzemino is found, showing the first group a higher content; the opposite for the 2-mercaptoethanol level. Diethyl sulfide is tendentially higher in Merlot and Marzemino wines, but yet at levels below the sensory threshold values. For 3-(methylthio)-1-propanol, boiled potatoes scenting, Merlot is again the leading variety, while Chardonnay has the lowest content, as expected for a white wine [84].

The box-plots of the content distribution of dimethyl disulfide (DMDS), diethyl disulfide (DEDS), 2-(methylthio)-1-ethanol (MTE) and benzothiazole (BT) as a function of the wine variety is the best way to show the spreading of the data inside each wine-type (**Figure 22**). This figure represents an example of highly significant content variations due to the variety.

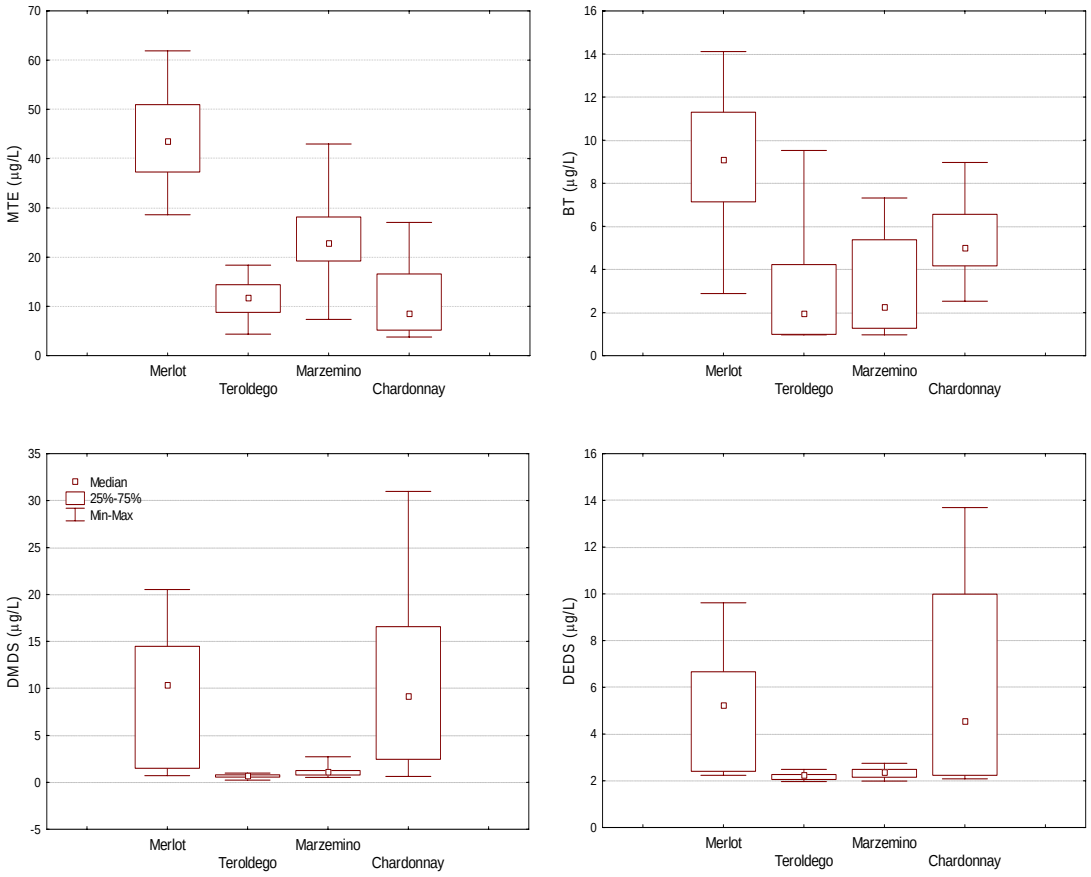


Figure 22. Box plot representation of MTE (top, left), BT (top right), DMDS (bottom, left) and DEDS (bottom, right)

Finally the data were submitted to the Principal Component Analysis (PCA) to allow a graphic representation of the scores and loading of the variables of the components. The first two components collect 50.9 % of the total variability of the system and the by-plot scattering of the scores indicates prevalent clustering according to three variety groupings, i.e. Merlot (△), Teroldego (♦) with Marzemino (○) and Chardonnay (□) (**Figure 23**). The first principal component (PC1) distinguishes Merlot from Marzemino and Teroldego, while the second principal component (PC2) separates the red wines from the white ones.

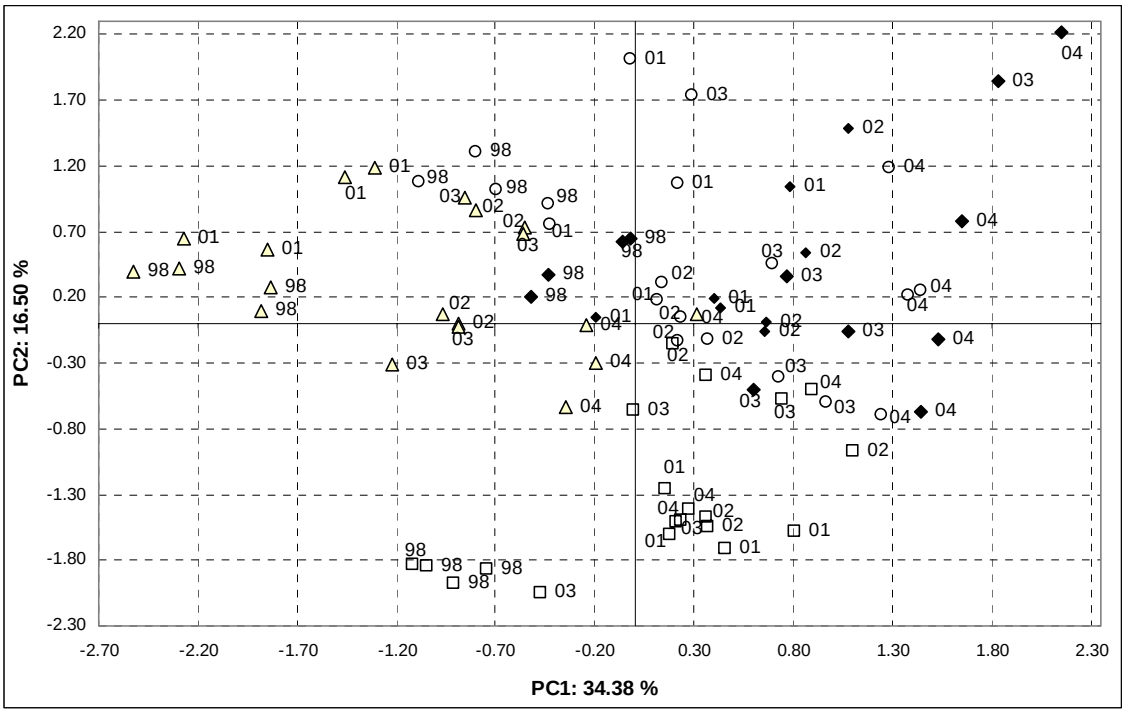


Figure 23. PCA score biplot for all the wine varieties analysed

The loadings plot (**Figure 24**) shows that the first component is positively related mostly with ethyl mercaptan , 2-mercaptoethanol, methyl thioacetate and ethyl thioacetate (group α) and negatively with 3-(methylthio)-1-propanol, diethyl sulfide, 2-(methylthio)-1-ethanol, 2-(methylthio)-1-butanol and dimethyl sulfide (group β). Also the vintage years shows a direct correlation with the PC1. Furthermore, the loadings analysis permits to observe an anticorrelation between ethyl mercaptan (group α) and diethyl disulfide (group γ), thus confirming a redox correlation between these compounds.

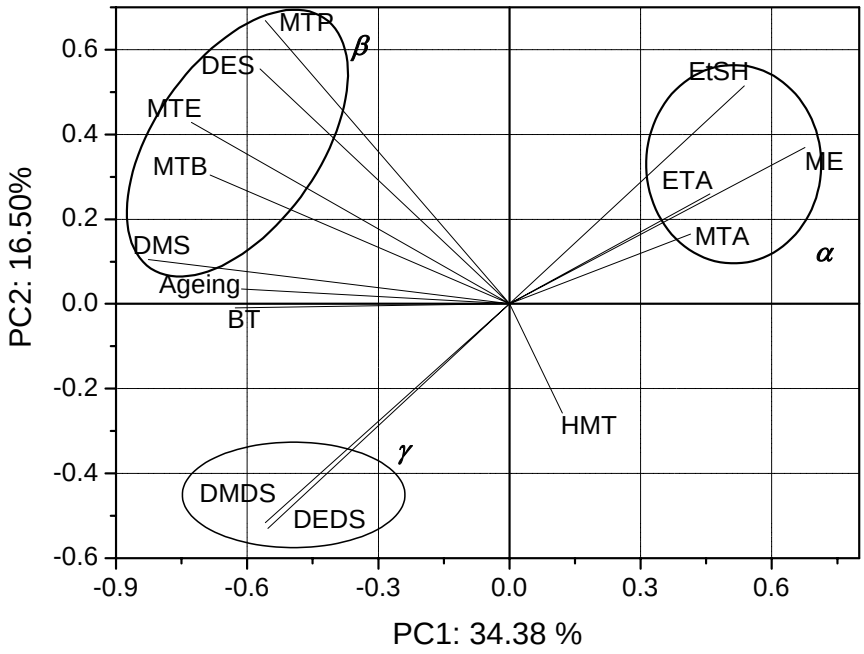


Figure 24. PCA loadings biplot for the sulfur compounds considered

The first component seems mostly connected with the ageing: a younger wine is more charged by the group α variables, while an aged one by those of group β. It also contributes to distinguish Merlot from Marzemino and Teroldego wines. The second function is positively related with quite all the variables, and distinguishes the white wines (Chardonnay) from the red ones. The group α of variables, above all represented by the classes of thiols and thioacetates, mostly model the wines of the variety Teroldego (♦) and in part also those of Marzemino (○), while the group β including sulfides, disulfides and most thioalcohols fits better the group of Merlot wines (△).

Merlot wines are particularly rich in such sulfur compounds and this fact supports the possibility that this class of aroma compounds can be responsible of important sensory differences among the red-grape varietal wines as above evidenced by Tukey test. Moreover, it appears difficult to distinguish Marzemino from Teroldego wines as both characterised by a remarkable variability of the level of thiols and thioacetates. These experimental results represent rather well the practical sensory situation by which it is often quite difficult to distinguish such wine-types, both well distinguishable from Merlot wines.

To point out differences linked to the variety, the temporal correlation of wines was eliminated performing a centring on each variable for each variety. In particular we

shifted every cluster of scores connected to a particular vintage for a quantity resulting from the difference between the total mean and the mean of each vintage for each sulfured variable and for each variety. Repeating PCA treatment the biplot represented in **Figure 25** was obtained. The variance explained by the first two functions increased to about 62 %. We recognise better the big difference existing among the scores of Merlot, Chardonnay and Marzemino plus Teroldego, these last ones resulting as two partially overlapped groups.

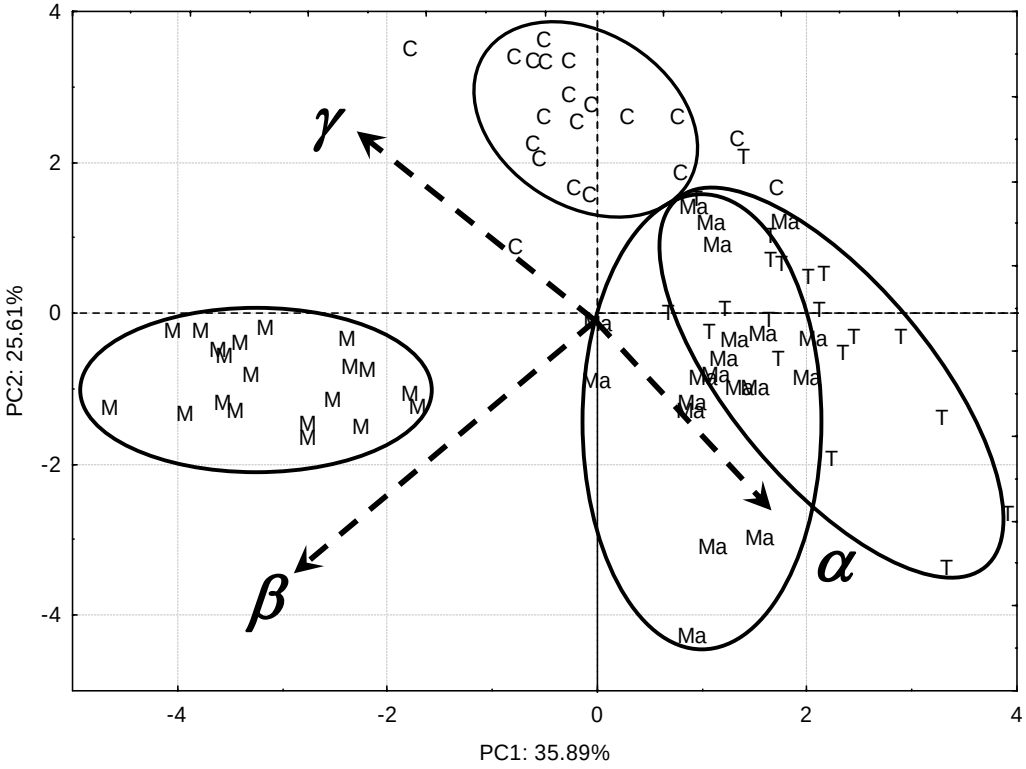


Figure 25. PCA scores and average loadings biplot. C: Chardonnay, M: Merlot, Ma: Marzemino, T: Teroldego. α: EtSH, ME, MTA and ETA; β: DMS, DES, MTE and MTP; γ: DMDS and DEDS.

In reason of the frequency per variety of yeasts employed and the rather large number of considered sulfur compounds, a possible "yeast effect" on wine aroma profile can be investigated examining the biplot distribution of the scores labelled according to the relevant inoculated yeast (**Figure 26**).

Number	Strain
1	Blastosel Grand Cru
2	Blastosel Kappa
3	Blastosel Terroir
4	Blastosel VS
5	CAB90
6	Collection cepage Merlot
7	Davistart
8	EC1118
9	Enolevure K34
10	Fermiblanc Arom
11	Fermicru LS2
12	Fermivin Cryo
13	GAR26
14	La Claire CGC62
15	La Claire EM2
16	Premium rouge
17	Fermicru VR5

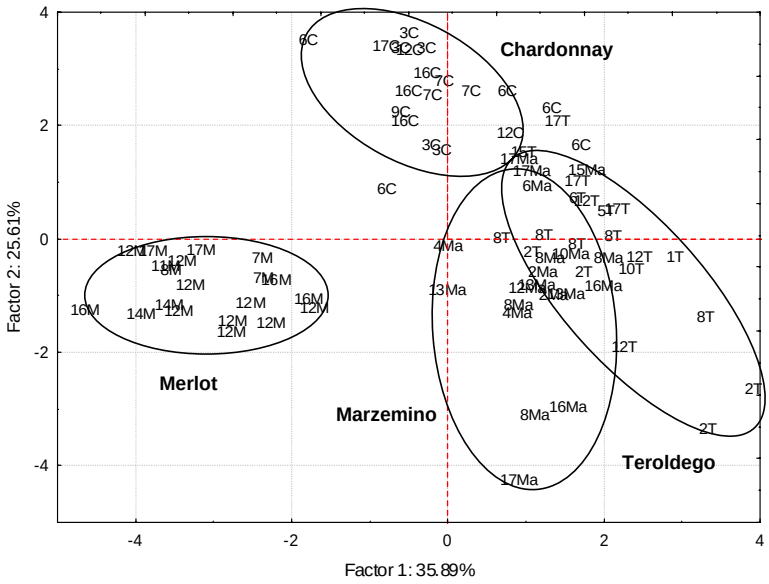


Figure 26. PCA score biplot for the considered wines and yeast strains employed

The scores of most used strains (i.e.: n. 6 for Chardonnay, n. 8 for Teroldego and Marzemino and n. 12 for Merlot) resulted to be spread in the whole space of the variety's group. Further, among the most spread yeasts for all varieties, we found the n. 12 and 17: evidence of relevant scores is signed by segments connecting each score per variety or indicated with a circle in the case of the presence of only one score. It did not occur any evidence for particular area associated to those or other strain types. Moreover, the scores group of different yeasts per variety are rather far each to the other, this fact indicating the possible absence of an yeast-effect in determining the wine discrimination according to the variety.

4.2.5 Nitrogen supplementation effects on fermentative sulfur compounds

Fermentations of Shiraz musts with low (100 mg/L YAN), medium (250 mg/L YAN) and high nitrogen (400 mg/L YAN)level were carried out with *Saccharomyces Cerevisiae* D254 and *Saccharomyces Bayanus* yeast AWRI 1176 by maceration on grape skins at 22 °C.

For both yeasts, nitrogen supplementation increased fermentation rate, with the high YAN musts completing in 10 days, the medium YAN musts in 13-14 days and the low YAN musts in 20 days. YAN became undetectable after 2 days of fermentation for

the low YAN musts, 3 days for the medium YAN musts and 4 days for the high YAN musts.

The progression of H_2S during fermentation is shown in **Figure 27** as a function of fermentation progress, expressed as residual sugars.

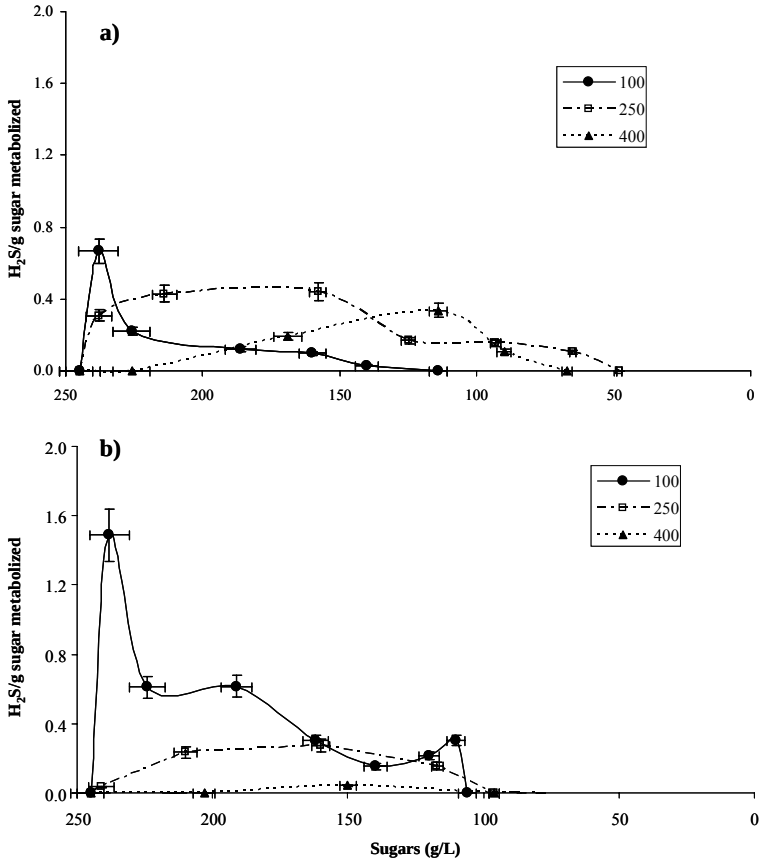


Figure 27. H_2S development as a function of the sugar content. **a)** D254 yeast strain **b)** 1176 yeast strain

Nitrogen supplementation had a strong impact on the stage of fermentation at which H_2S production started. For both yeasts, in the non supplemented fermentations, production of H_2S commenced upon depletion of YAN, whereas in the other treatments occurrence of H_2S was already observed before YAN was completely depleted. Nitrogen supplementation also had a strong influence on the stage of fermentation at which cessation of H_2S production occurred. In the case of yeast 1176, production of H_2S ceased for all treatments around 100 g/L of residual sugars.

The total H_2S developed during fermentation was affected by both yeast and nitrogen concentration (**Table 14**).

Table 14. H_2S formation during the several fermentation steps

	Total H_2S released (μg/L must)	Fermentation step when H_2S production ceased (g/L of residual sugars)	Final wine (μg/L wine)
D254 100	102	120	nd
D254 250	284	50	2.3
D254 400	121	70	0.5
1176 100	326	100	nd
1176 250	116	100	nd
1176 400	9	100	nd

In the case of *Saccharomyces Bayanus* 1176, a remarkable decrease in the total H_2S released occurred when the fermentations were supplemented with DAP. These results indicate that, for this strain, DAP supplementation was a powerful modulator of H_2S production during fermentation. Conversely, when fermentations were carried out with yeast D254, the range of H_2S concentrations observed across the various treatments was much smaller. Noteworthy, although maximum H_2S production per gram of sugar metabolised was observed for the control fermentations (**Figure 27**), the highest total H_2S was observed in the 250 mg/L YAN fermentation. **Table 14** also shows the concentration of H_2S in the wines 2 months after bottling, measured with GC-AED. In spite of the higher H_2S production observed for yeast 1176, no H_2S was detected in the wines made with this strain.

The results of the analysis of the different FSCs compounds are reported in **Table 15**.

Table 15. Volatile sulfur compounds concentration and Tukey's test results*

	D254						1176					
	100		250		400		100		250		400	
MetSH	nd	b	0.7	a	0.8	a	nd	b	nd	b	0.8	a
EtSH	0.8	a	0.8	a	0.9	a	0.7	b	1.1	a	1.0	a
CS_2	6.0	b	8.0	a	9.0	a	5.0	a	5.0	a	6.0	a
DES	2.0	b	8.6	a	10.8	a	1.8	c	5.2	b	8.7	a
DEDS	2.6	b	4.5	a	6.1	a	1.9	c	3.3	b	4.3	a
DMS	2.5	b	8.6	a	10.8	a	2.0	c	4.0	b	5.9	a
DMDS	nd	c	2.3	b	3.8	a	nd	c	0.7	b	1.0	a
MTA	5.0	b	7.3	ab	8.5	a	3.9	b	4.7	b	6.4	a
ETA	1.1	a	2.2	b	2.8	b	1.2	b	1.9	a	1.8	a
ME	38.4	a	38.7	a	39.8	a	40.3	a	39.4	a	41.1	a
MTE	33.7	a	37.7	a	40.3	a	33.2	b	39.3	a	40.8	a
MTP	2900	a	3130	a	3003	a	3054	a	2994	a	3005	a
MTB	22.4	a	21.7	a	20.7	a	20.1	a	20.8	a	21.8	a
BT	15.6	a	15.7	a	14.7	a	16.0	a	15.4	a	16.8	a
HMT	0.8	a	0.5	a	0.8	a	0.9	a	0.8	a	1.0	a

* Value with the same letter do not differ significantly according to the Tukey's test, $p < 0.05$

For both yeasts, sulfides and disulfides increased with nitrogen additions, with yeast D254 wines showing generally higher concentration values compared to yeast 1176. MetSH was only found in wines from nitrogen supplemented fermentations, but no difference was observed between the two yeasts for this compound. As for EtSH, nitrogen supplementation stimulated an increase in the concentration of this compound when fermentations were carried out with 1176, while no treatment effect was observed for D254. The two thioesters MTA and ETA showed a general increase in wines obtained from nitrogen supplemented fermentations. Finally, with the exception of 2-(methylthio)-1-ethanol, which showed a small increase with nitrogen supplementation, no significant difference was observed for the heavy FSCs 2-mercaptoethanol, 3-(methylthio)-1-propanol, 4-(methylthio)-1-butanol, benzothiazole, and 5-(2-hydroxyethyl)-4-methylthiazole with respect to yeast and nitrogen supplementation.

Principal component analysis (PCA) was used to identify the FSCs that best discriminated between the different treatments. The results are given in **Figure 28**.

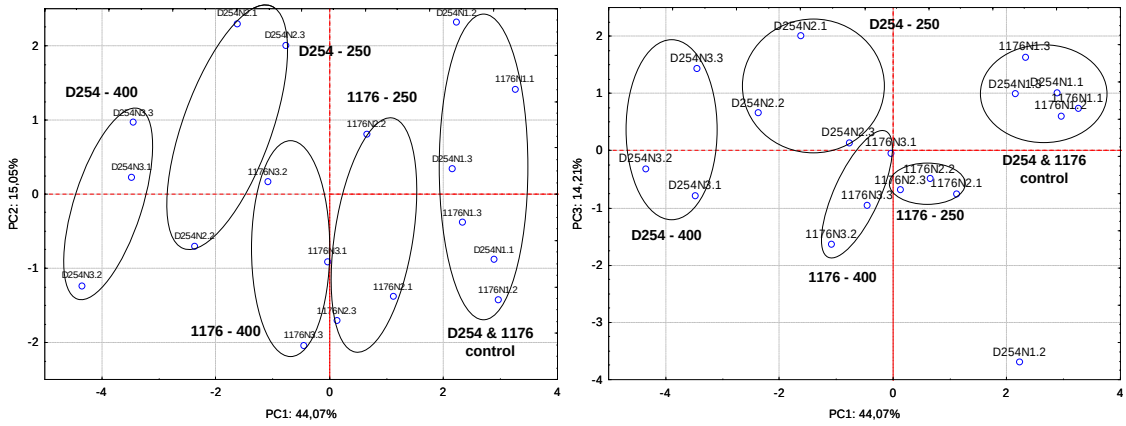


Figure 28. PCA scores biplot. PC1 vs PC2 and PC1 vs PC3 was represented

The first principal component (PC1) explained 44% of the total variance, and was mainly characterised by MetSH, DES, DEDS, DMS, DMDS, MTA and ETA, with positive loadings. PC2, which accounted for 15% of the total variance, was characterized by H2S with positive loadings and by EtSH, 2-mercaptoethanol and 5-(2-hydroxyethyl)-4-methylthiazole with negative loadings. PC3 accounted for 14% of the total variation, and was mainly characterised by 4-(methylthio)-1-butanol with positive loadings and for 2-mercaptoethanol and 3-(methylthio)-1-butanol with negative loadings. At low nitrogen concentration, the wines obtained with the two yeasts could not be clearly separated by the two first principal components. As nitrogen was increased by means of DAP addition, a clear distinction between the two yeasts became apparent, with 1176 wines being mainly associated with EtSH, 2-mercaptoethanol and 2-(methylthio)-1-ethanol. Differences between medium and high initial YAN concentrations for this yeast were however moderate. Conversely, in the case of D254, a further separation was observed between the two nitrogen additions, with wines obtained from an initial YAN of 250 mg/L being strongly characterised by H_2S, and wines from the 400 mg/L YAN fermentation being mainly associated with MetSH, DES, DEDS, DMS, DMDS, and the two thioesters.

Although various biochemical mechanisms can account for production of H_2S by yeast, it is generally accepted that the major portion of the H_2S formed during fermentation derives form the sulfate/sulfite assimilation pathway that leads to the formation of the amino acids cysteine and methionine [45, 50]. Nitrogen availability

regulates the balance between H_2S sequestration and excretion by determining the intracellular concentration of the carbon-nitrogen precursors [46]. In particular, it has been postulated that under conditions of nitrogen deficiency the low rate of formation of carbon-nitrogen precursors results in H_2S accumulation in the cell, and consequent excretion in the fermentation medium [46]. Furthermore, sulfite reductase activity appears to remain stable after nitrogen depletion, leading to prolonged H_2S formation [51]. The results obtained in the present study are only in partial agreement with these observations. In fact, while the data obtained for *Saccharomyces bayanus* AWRI 1176 strain were consistent with the expected effect of nitrogen supplementation lowering H_2S formation, increased concentrations of initial nitrogen did not decrease in H_2S formation for *Saccharomyces cerevisiae* D254, but instead increased total H_2S when DAP was added to reach an initial YAN of 250 mg/L. This is in agreement with the recent observation that shortage of *O*-acetylhomoserine is not always responsible for increased production of H_2S [93]. Rauhut et al. [60] reported that wines made with certain yeast strains had increased H_2S when DAP was added at 500 mg/L.

Other nutrient deficiencies are known to stimulate H_2S production, such as lack of the vitamins pantothenic acid and pyridoxine [59]. However, the grapes used in this study had a concentration of pantothenic acid of 600 µg/L, which markedly exceeds the minimum pantothenate concentration of 250 µg/L needed to suppress H_2S formation in model media [59].

Variability in H_2S production between yeast strains has long been known [46, 47] but the biochemical mechanism, regulating H_2S production are poorly understood. Strain differences have been attributed to many factors but are now generally understood to involve genetic mutations in the sulfate assimilation pathway and *S*-amino acid metabolism [93].

The concentration of H_2S in the finished wines was affected by both yeast and nitrogen supplementation. Surprisingly, no correlation was found between total H_2S produced during fermentation and final concentration of H_2S in the wines. H_2S has low solubility and high volatility and can be largely removed by the CO_2 evolving during fermentation [60]. However, the final concentration of H_2S appeared to be correlated with the stage of fermentation at which H_2S production ceased. According to Henschke and de Kluis [94], H_2S formed at the end of fermentation could be of greater

technological importance than that present in the vigorous part of the fermentation, due to the reduced purging off effect of CO_2, which is responsible for the removal of most of the H_2S produced during fermentation. Therefore, fermentations characterised by late formation of H_2S can potentially result in wines with higher H_2S concentrations. The results of this study, although still limited, seem to confirm this suggestion, as fermentations showing late H_2S formation were also characterised by higher H_2S concentration in the final wines (**Table 14**) [94]. Considering the primary importance of H_2S on wine aroma quality, the relation between total H_2S produced, timing of production, and concentration of H_2S in finished wines are worth of further investigation.

In addition to H_2S, a total of 15 FSCs were measured in the wines investigated in this study, with the aim of obtaining a comprehensive evaluation of the effect of the fermentation conditions studied on the volatile sulfur profile of Shiraz. In spite of the large number of reports describing the effects of fermentation conditions on H_2S formation, only a limited number of studies have investigated the relation between fermentation management and formation of FSCs, in particular mercaptans, sulfides, and disulfides, potentially affecting wine aroma. In a previous study, we observed no correlation between nitrogen supplementation and final DMS concentration in Shiraz wines. Conversely, in the current study, for both yeast strains nitrogen supplementation induced a general increase in the concentration of sulfides and disulfides, including DMS. Various authors have proposed that sulfides and disulfides can be formed by yeast as a result of the catabolism of the sulfur amino acids cysteine and methionine [90, 95], but an involvement of sulfur amino acid biosynthetic pathways has also been suggested [36]. In the case of DMS, there is evidence that formation of this compound by the yeast during grape must fermentation might be linked to cysteine, cystine, methionine or glutathione metabolism [51, 54]. However, a chemical pathway can be also involved in its formation, with *S*-methylmethionine as a possible precursor [14]. De Boer and Wilson [96] proposed that the S-methylmethionine synthesised by the yeast can then be chemically transformed into DMS. In beer fermentations it has been shown that yeast can also form DMS through enzymatic reduction of dimethylsulfoxide (DMSO) [97], and that inactivation of the gene encoding for the reductase enzyme

suppress the formation of DMS from DMSO [97]. Furthermore, the concentration of DMS at the end of fermentation correlated with increasing additions of initial DMSO. However, while in wort DMSO is formed in large amounts during malt kilning [97], its occurrence in grape juice has not yet been demonstrated. As for the other sulfides, the origin of these compounds in wine fermentations remains largely unknown. In a recent study, Buzzini et al. [98] suggested that methionine is the essential precursor to DMDS in *Basidiomycetous* yeasts. Finally, both DMDS and DEDS have been identified as products of the reaction between H_2S and mercaptans in the presence of copper [99]. This might have also contributed to the higher concentrations observed here, considering the increased formation of ethanthiol and methanethiol at higher nitrogen treatments.

Two mercaptans, namely EtSH and MetSH, as well as their corresponding acetate esters, MTA and ETA, were detected and quantified in the experimental wines. MetSH showed a trend similar to that of sulfides, that is, concentrations of this compound increased in conjunction with nitrogen supplementation. This seems to confirm the observation, reported by other authors, that sulfides and MetSH derive from interrelated metabolic pathways, sharing methionine as common precursor [54]. An increase with increasing nitrogen was also found for EtSH in the case of *Saccharomyces Bayanus* 1176, while no effect with increasing nitrogen was observed for this compound in the wines produced with *Saccharomyces cerevisiae* D254. Ethanethiol can also be formed from the reaction of H_2S and acetaldehyde [20], which was formed in higher concentrations by the 1176 yeast (data not shown). As for the two mercaptoacetates, these compounds are formed by the yeast through esterification of the corresponding mercaptans [100], most likely catalysed by an alcohol acetyltransferase. The relative proportions observed in this experiment are consistent with those reported by Leppanen et al. [101]. The generalized increase of MTA and ETA observed here is likely to be due to the increased acetate esters biosynthesis resulting from nitrogen supplementation, as previously observed in red fermentations for other volatiles[102].

Nitrogen supplementation did not cause any variation in the concentration of the high molecular weight sulfur compounds measured in this study, except for a moderate increase of 2-(methylthio)-1-ethanol when 1176 fermentations were supplemented with nitrogen. The concentrations found for this compound in this study are similar to those

reported in Merlot [8]. Grape variety has been shown to be a major source of variation in the concentration of this compound [49]. Based on our results, this might be due to differences in nitrogen concentration. Various authors have reported that increased nitrogen availability is negatively correlated with production of 3-(methylthio)-1-propanol (methionol) by the yeast [103], as this compound derives from methionine via the Ehrlich pathway [104]. However, it has been also indicated that the presence of high levels of solids can stimulate the production of methionol [49], which might have counterbalanced the effects of nitrogen supplementation in the current study. Among the other compounds detected, it has been suggested that homomethionine and cysteine could be the precursors to 4-(methylthio)-1-butanol and to 2-mercaptoethanol respectively [43], but variations in nitrogen availability had no effects on these two compounds under our experimental conditions.

The effects of nitrogen supplementation on the FSCs composition of the experimental wines are summarized in **Figure 28**. *Saccharomyces cerevisiae* D254 showed a strong response to DAP addition, with patterns of FSCs that were also dependent on the initial nitrogen concentration. In particular, the 250 mg/L YAN and 400 mg/L YAN wines were characterised respectively by increased concentrations of H_2S and of MetSH, sulfides and disulfides. Conversely, the effects of DAP additions on the pool of FSCs produced by *Saccharomyces bayanus* 1176 were less pronounced, although increased production of low molecular weight sulfur compounds and of EtSH were generally associated with nitrogen-supplemented fermentations. Interestingly, nitrogen supplementation before fermentation gave a much clearer distinction between the VSCs profiles of the two yeasts compared to non-supplemented treatments (see **Figure 28**). In a recent study, the highest similarities in fermentation-derived volatiles produced by two *Saccharomyces cerevisiae* yeast were found at initial nitrogen concentrations of 250 mg/L YAN, although in that case FSCs were not considered [58]. Altogether, these results indicate that DAP addition has the potential to modulate the differences between yeast in regard to their characteristic volatile patterns.

The sensory impact of sulfur volatile compounds in wine is well documented [4]. However, there is an extremely wide variation in the odor threshold values reported by different authors. Moreover, certain sulfur compounds are known to contribute positively when they are present in sub- or peri-threshold concentrations, but they can

be responsible for off-flavors at higher concentrations. For this reason, the use of preference threshold instead of odour thresholds have been recommended when assessing the potential impact of sulfur compounds on wine aroma on the basis of compositional data [8], although no study has reported these values to date. In general, based on the values recently reviewed [4] for thresholds in hydroalcoholic solutions, it appears that H_2S, CS_2, DMS, DEDS, MetSH, EtSH, and methionol were present in the experimental wines in concentrations that suggest a possible contribution to the aroma of these wines. As for the thioesters MTA and ETA, no threshold values have been reported for these compounds in wine-like matrices. However, the concentrations observed for these two compounds were much lower than the threshold reported in beer , suggesting a negligible contribution to the aroma of the experimental wines. However, it has been postulated that MTA and ETA can be hydrolyzed to their corresponding mercaptans during aging. The changes in thioesters concentration observed in this study in response to nitrogen supplementation might therefore become of sensory relevance with aging.

4.4 Conclusions

Headspace solid phase microextraction, a technique requiring very simple equipment and avoiding the use of organic solvents and multiple working steps, was shown to be a successful approach for the analysis of organic sulphur compounds in a wide boiling point range.

The proposed sampling method, in conjunction with a GC-MS apparatus operating in SIR mode, allowed the simultaneous quantification of many sulphur volatile species of high interest from an oenological point of view, with different physico-chemical characteristics. The comparison of analyte responses in wine matrices treated either with charcoal or with $AgNO_3$ indicated that the use of suitable internal standards can satisfactorily compensate for matrix effects in red and white wines even taking into account the possible influence of ethanol or sugar contents.

The described procedure, fast, precise and accurate, gives a L_D value for each analyte which satisfies oenological requirements, being below the sensory threshold in wine and in most cases also the L_D values reported in the literature. For all these reasons

the method described is potentially suitable for easy transfer to the control of wine production processes.

It was put in evidence that, besides dimethyl sulfide, the level of some sulfur volatiles among the thirteen analysed can depend on the aging, this fact enlarging the knowledge of compounds connected to the aroma development in wine. Some of the thirteen resulted also interestingly correlated, this fact improving the knowledge on sulfured compounds intervariability. On the basis of all volatiles investigated, it was also possible, for the first time, to discriminate different variety-pure wines produced in several years, mostly from red grapes that were characterised by chemically different groups of volatiles. The relevant contents when related to the variety, gave useful information to justify possible particular scents associable to the variety itself, even in connection with the aging. These compounds, belonging to the groups of substances usually considered as wine off-flavouring, were found at levels lower than their commonly accepted perception thresholds in wine and however at concentrations not off-flavouring the wine matrices. At the same time, it appears that occasional higher levels of some sulfur volatiles basically induced either by particular nutritional situations for the yeast or by a wrong technological conduction of the vinification, could easily transform a typical wine in an off-flavouring product.

In conclusion, this study has demonstrated that DAP supplementation can strongly affect the pool of FSCs of Shiraz wine. Genetically different Saccharomyces yeasts were shown to respond differently to DAP addition prior to alcoholic fermentation in regard to production of H_2S. In particular, for one of the two yeast tested, DAP addition for increasing YAN to a concentration of 250 mg/L resulted in increased formation of H_2S compared to non-supplemented fermentations. For this yeast, DAP-supplemented fermentation were also characterised by extended production of H_2S, which appeared to be associated with increased H_2S in the final wines. In general, DAP supplementation corresponded to higher concentrations of organic FSCs in the finished wines. These results raise concern about the widespread use of DAP supplementation to reduce the formation of reductive off-flavors in wines. Sensory evaluation of the wines is currently

in progress with the aim of shedding some light on the sensory differences associated with the changes in sulfur volatile compounds observed in this study.

5. VARIETAL SULFUR COMPOUNDS ‡

5.1 Preliminary considerations

Varietal sulfur compounds (VSCs) (**Figure 29**) represent, together with the FSCs, one of the most appealing topics in wine sciences [4, 9-11].

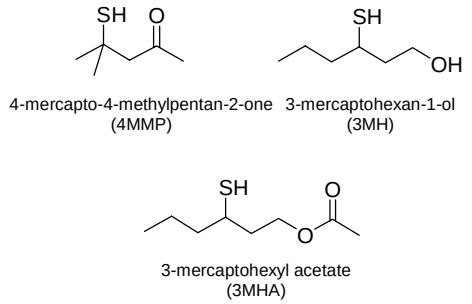


Figure 29. Varietal sulfur compounds

These molecules were firstly recognised as impact character in tropical fruits [105, 106] (passion fruits and guava), and afterwards they were found also in Vitis vinifera grape variety [92, 107-110]. Varietal sulfur compounds are responsible of sensory notes describes as "tropical, passion fruit, grape fruit" or as "Sauvignon Blanc aroma". As all the thiols they have really low sensory thresholds [111] and this characteristic make them really important in defying sensory attributes.

In grape VSCs are present as conjugated form with cysteine and glutathione (**Figure 30**)

‡ The contents of this chapter have been published in 3 peer reviewed publications

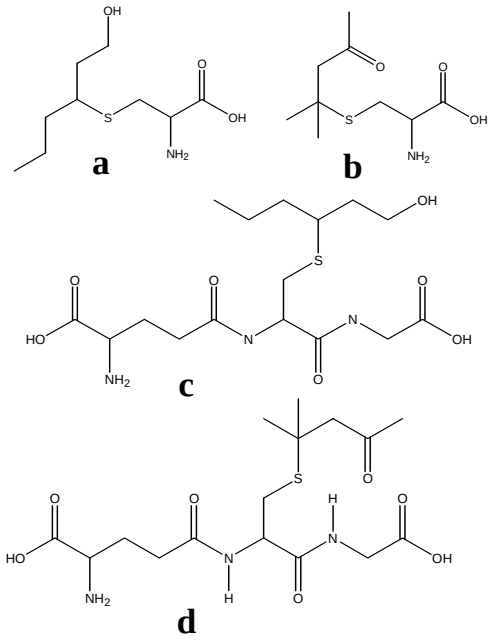


Figure 30. Varietal sulfur compound precursors

and they are released during fermentation by a β-lyasic activity [112-118]. This particular enzymatic activity is not highly expressed in Saccharomyces Cerevisiae strain, as only 5 % of conjugated precursors are cleaved during alcoholic fermentation [11, 115, 119].

The study of varietal thiols and of their precursors has only recently started [111, 120, 121]; the low concentration of these molecules, their reactivity and the lack of analytical methods able to quantify them are the main reasons of this absence. Nonetheless, due to the highly positive impact of these molecules, this topic quickly obtained the title of one the most investigated field in wine science and is currently the subject involving the most copious number of skills in this area. The influence of technological practices have been also taken into account; in particular the effect of variable like yeast strain [9, 10, 115, 119, 122], skin contact and pressure applied to the grapes [123] and noble rot infection [124-127] seem to deeply influence the level of free thiols.

Recent attention has been also paid to varietal thiols precursors [112-114, 116, 117, 124, 128], even if the biological pathway/s leading to the formation of this molecule has not been clearly demonstrated yet.

The explanation given so far, relates to the enzymatic activity of the glutathione *S*-transferase present in grape berries [129]. The formation of glutathione conjugates in plants and animals can be ascribed to processes related to detoxification of xenobiotic and endogenous substrates [130-135]. The enzymes responsible for glutathione conjugation have been identified as glutathione *S*-transferases [132, 133]. These enzymes catalyse the reaction of electrophiles, such as alkylating agents, with the -SH group of glutathione, thereby neutralising the reactive sites and rendering the products more water-soluble. Glutathione conjugates are usually unstable, and are thought to be metabolised further by cleavage of the glutamate and glycine residues, followed by acetylation of the resultant free amino group of the cysteinyl residue [136]. A further proof of the crucial role of the glutathionylated precursors in the formation of the free thiols has been provided from the Subileau et al [137]; this work proved that the depletion of the gene responsible of the transportation of glutathione (GSH) originates a lower production of the relevant free thiols after fermentation.

The analyses of the volatile thiols are quite cumbersome because they are present at extremely low contents (up to few ng/L) and because they may react with oxidant agents (molecular oxigen, quinones, etc) so affecting the validity of the results [138-140]. Two approaches to quantify these analytes are reported with separative and pre-concentration steps giving the needed sensibility [111, 141]. In both the approaches the recovery of the thiols from the wine is carried out by a preliminary liquid-liquid extraction with methylene chloride, followed by a trapping with either *p*-hydroxymercuribenzoate [111] or with the Affi-Gel 501 resin (a cross-linked agarose gel containing phenylmercuric chloride) [141]. A third method, characterized by an on-fibre derivatisation headspace solid phase microextraction (HS-SPME) sampling, was recently described [142] with good results in the quantification of 2-furanmethanethiol and 3-mercaptohexyl acetate at concentrations below 0.1 ng/L, but with very poor results in respect to 3MH.

The analysis of varietal thiol precursors has been mainly carried out by means of derivatisation followed by GC-MS analysis [112-114, 116, 128, 143], only recently a LC-MS/MS method has been proposed but only for the Cys-3MH, without differentiation of the two diastereoisomers [144].

During my thesis I developed analytical GC-MS methods to analyse the free thiols, using different sampling approaches. In particular we tested the effectiveness of SPE, HS-SPME [42] and Purge & Trap [145] procedures in recovering the analytes. We exploited mono- and multivariate experimental design to optimise extraction conditions and we used a statistical method to compare the performances of the three different sampling techniques studied.

Finally we focussed our attention on thiol precursors: we developed LC-MS/MS methods to quantify cysteinylated and glutathionylated species, we identified a new glutathionylated precursors, we studied the biogenesis of varietal sulfur compounds precursors in grape berries and their accumulation in grape and grape vine tissues by isolating the putative enzyme responsible for their synthesis and by colorimetric and MS experiments [118].

Additionally, since none of these molecules is commercially available we had to synthesise and purify them together with the relevant internal standards.

5.2 Experiments

5.2.1 Chemical and reagents

The free thiol compounds analysed were: 3-mercaptohexan-1-ol (3-MH), 3-mercaptohexyl acetate (3-MHA). The internal standard (I.S.) used was 6-mercaptohexan-1-ol (6-MH). 3-MH and 3-MHA (purity > 98%) were purchased from Interchim (Montluçon, France) and 6-MH from Sigma-Aldrich (Milan, Italy). Inorganic compounds and methanol, dichloromethane, *n*-pentane and anhydrous ethanol (> 99 %) were purchased from Carlo Erba (Milan, Italy).

A standard solution for each compound was prepared in pure ethanol and stored at - 16°C. Starting from these solutions a working ethanol solution containing each analyte at 0.2 mg/L concentration was prepared.

All chromatographic solvents were HPLC grade; all chemicals were analytical reagent grade unless otherwise stated and water was obtained from a MilliQ purification

system (Millipore, Milan, Italy). Solvents and chemicals were obtained from either Sigma-Aldrich (Milan, Italy) or BDH (Milan, Italy). All prepared solutions were % v/v with the balance made up with MilliQ water.

5.2.2 HS-SPME sampling

The SPME holder for manual sampling and the fibres used were purchased from Supelco (Bellefonte, PA, USA). The fibre coating selected to sample the two analytes and the I.S. was carboxen-polydimethylsiloxane-divinylbenzene (CAR-PDMS-DVB; 50/30 µm x 2 cm); the amount of salt added to 20 mL of solution was 5 g of $MgSO_4 \cdot 7H_2O$ [21]. This choice gave the best results in terms of the maximum signal for each compound. The operative conditions (temperature of the sample, adsorption time and solution pH) were optimised [78]. Before sampling, the fibre was conditioned according to the producer's instructions.

5.2.3 SPE procedure

The ENV+® cartridges (1 g of highly cross-linked styrene-divinylbenzene polyhydroxylated polymer) for SPE extractions were supplied by Isolute (IST Ltd., Mid Glamorgan, U.K.). Extraction of 3-MH and 3-MHA with the addition of the internal standard (6-MH) was carried out according to Boido et al. [146]. The sample pH was adjusted to 7.0 to reduce the absorption of free fatty acids on the resin, and so limiting possible interferences in GC-MS analysis. A 100 mL sample volume (wine/distilled water with a ratio of 1:1 v/v) was percolated through the cartridge; the recovery of the analytes was carried out by means of 30 mL of CH_2Cl_2 and then the eluted solution was added to 60 mL of *n*-pentane to obtain an azeotropic pentane-methylenechloride mixture, 2:1 v/v with a lower boiling point of 31°C. The solution obtained in this way was anhydrificated with Na_2SO_4 and concentrated up to about 100 µL by distillation with a Vigreux column [147].

5.2.4 Purge and trap extraction

The Purge and Trap (PT) sampling was carried out with a Tekmar 3000 (Tekmar Teledyne, Mason, OH, USA) equipped with a trap packed with Tenax TA (Tenax Chrompack, Bergen op Zoom, The Netherlands).

To optimize the purge and trap procedure a multivariate approach, based on the central composite design (CCD), was followed [148]. The parameters affecting the sampling and the operative range of interest were recognized performing a preliminary monovariate study on the following experimental parameters: sample temperature (St), flow rate (Fr), purge flow time (Pft), the trap temperature (Tt) and desorption time (Dt).

The CCD approach comes from the combination of a complete factorial design with a star design; the number of experiments required (n_{ex}) is given by the formula $n_{ex} = 2^k + 2 \cdot k + r_0$ where k is the factor number (4) and r_0 is the number of experiments made in the centre of the experimental design (12). Since the time optimization experiments required 4 analysis days, the tests were randomized repeating 3 times a day the centre point to check any possible bias.

To quantitatively correlate responses and factors the response surface was described by a quadratic polynomial model with interactions:

$$S = a_0 + \sum_{i=1}^{k} a_i x_i + \sum_{i=1}^{k} a_{ii} x_i^2 + \sum_{1 \le i < j}^{k} a_{ij} x_i x_j$$

where S represents the signal (peak area) and the " a " coefficients represent the regression parameters: zero order (a_0), first-order (a_i), second order (a_{ii}) and interaction (a_{ij}) term respectively. The best regression models were obtained by a forward search step-wise variable selection algorithm with a $F_{\text{to enter}} = 2.0$ using the Minitab software package.

The response given by each set of parameters was obtained by analysing a white wine spiked with 0.1µg/L of each compound. As reported in a previous work [42] all the analyses were carried out adjusting the solution pH to 7.0 to reduce the stripping of the free fatty acids and to avoid the hydrolysis of 3-MHA.

5.2.5 GC-MS analyses

Chromatographic analysis was carried out with a Perkin Elmer Autosystem XL gas chromatograph coupled with a TurboMass Gold mass spectrometer (Perkin Elmer; Boston, MA, USA). The separation module was made up by connecting two fused-silica capillary columns with different polarity: a 30 m × 0.32 mm I.D. × 0.25 μm film thickness Innowax (Polyethylene glycol) column (Agilent Technologies; Palo Alto, CA, USA) and a 10 m × 0.32 mm I.D. × 0.25 μm film thickness HP-1 column (Agilent Technologies; Palo Alto, CA, USA). Helium was used as the carrier gas at a flow-rate of 1.2 mL/min. The temperature of the transfer line was 220°C. The electron impact energy was 70 eV and the MS source was set at 150°C. All the analyses were carried out in single ion recording (SIR) mode, using the NIST library and the literature [111, 141, 149] for the choice of fragments to be quantified.

On the basis of the resolution tests carried out on standard solutions and on wine matrices with appropriate amounts of analytes added, the chosen working conditions were: (a) GC injector temperature 250°C, (b) injection in splitless mode for 5 minutes, (c) oven temperature programme: 35°C (5 mins), 1°C/min to 40°C, 10°C/min to 250°C.

5.2.6 Validation

Calibration curves for each analyte were prepared following the internal standard procedure. The matrix was a white wine (WW; alcohol strength 10 % v/v.; sugar content: < 4 g/L; total polyphenol contents: 155 mg/L as (+) cathechine) treated twice with charcoal (3 g/L) to remove any sulphur compound, detectable using the proposed headspace SPME, SPE and Purge and Trap methods, and other main compounds, except the most polar ones such as higher alcohols. The aim of this sample pre-treatment was to obtain a matrix relatively similar to wine, but without the volatiles which may influence the HS-SPME and Purge and Trap adsorption [21]. After this treatment, the SO_2 total level was reset to 100 mg/L to ensure protection in relation to redox processes favoured by oxygen and pH was adjusted to 7.0.

This matrix allowed the preparation of “reference” calibration curves, as the charcoal treatment strongly reduces possible matrix effects. They therefore permitted us to highlight these effects in dedicated HS-SPME measurements, as shown below.

The scedasticity of the data collected was established using the F_{max} test [150] and the adequacy of either a linear or a quadratic model was checked using the Mandel test [87]. Linearity and precision were evaluated in the concentration ranges typical of 3-MH and 3-MHA in wine, using 7 concentration levels and 5 replicate solutions per level.

The Hubaux-Vos approach [34] was followed for the calculation of the critical (L_C) and the detection (L_D) limits. The signals corresponding to four standard solutions at very low concentration values together with the signal corresponding to the blank solution were used in regression, thus reducing the difference between the measured blank and the intercept of the regression line [71].

5.2.7 Matrix effect on recovery in the HS-SPME procedure

To test for possible matrix effects in HS-SPME analysis and to verify the suitability of the internal standard chosen, we followed an approach already described [21, 65, 79]. The recovery of each compound measured using the HS-SPME procedure was established as the percentage ratio between the thiol compound level calculated by discrimination on the particular calibration curve and the analyte concentration of a spiked sample.

For this purpose two dry wines, a white wine (WW) and a red wine (RW) at about 10 % v/v alcoholic strength, characterised by different amounts of ester content (6281 µg/L and 3016 µg/L [146], respectively) and polyphenol content (140 mg/L and 2830 mg/L as (+) cathechine [152], respectively), were deprived of thiols by adding an excess of $AgNO_3$ (about 5 mg/L). This procedure ensures the complete removal of sulphur compounds from the solution. The excess of Ag ions was eliminated by adding an appropriate amount of NaCl followed by filtration at 0.22 µm, resetting the Ag concentration to the normal level (< 20 µg/L by ICP-MS analysis). Afterwards the SO_2 total content was reset to about 100 mg/L. Unlike the wine treatment described for the construction of the calibration curves, this aims at obtaining a matrix deprived only of thiol compounds.

The different wine matrices considered, with different analyte content added, were prepared taking into account the nature of the wine considered (red and white wines),

the alcoholic strength in WW (10, 12 and 15 % v/v) and the sugar content in WW at 3 levels: < 4 g/L (dry wine), 50 and 100 g/L of sugar (glucose:fructose, 1:1). Each measurement was repeated three times.

5.2.8 Wine analysed

Fifty-two single-variety wines produced in different wineries in northern Italy from white grapes (Sauvignon Blanc, Traminer, Verdicchio and Mueller Thurgau) from the 2006 vintage and stored in wine cellars at a temperature of 16°C were analysed using the three proposed procedures.

5.2.9 Method comparison

Since the concentration values obtained with the different sampling methods were affected by comparable errors the regression comparisons were carried out via the Theil's non-parametric procedure [153, 154]. Briefly the slope β was estimated by *b*, the median of all the possible pairwise slopes between points, and the intercept α by *a*, the median of all $a_i = y_i - b\,x_i$, where (x_i, y_i) represent the coordinates (concentrations) of the *i*-th wine sample. A non parametric test for the hypothesis $H_0 : \alpha = 0$ and $\beta = 1$ has been performed [154]. In analogy with the classical least square regression, the effectiveness of the fitted line was evaluated by the coefficient of determination defined now as

$$R^2 = 1 - \left(\frac{med|r_i|}{\underset{i}{med}\left(\left| y_i - \underset{j}{med}(y_j) \right| \right)} \right)^2$$

The abbreviation "*med*" stands for the median, r_i is the *i*-th residual and y_i is the *i*-th observed [155]. The term $med_j(y_i)$ represents the median of the *n* observed data.

5.2.10 Varietal thiol precursors synthesis

γ-L-Glutamyl-S-(1,1-dimethyl-3-oxobutyl)-L-cysteinylglycine (**Figure 31**). To glutathione (2.00 g, 6.5 mmol) in water (13 mL), was added pyridine (1.03 g, 13.0 mmol) and mesityl oxide (0.64 g, 6.5 mmol). The mixture was stirred at 25°C for 45 hours before being diluted with water (40 mL) and rinsed with dichloromethane. The aqueous layer was finally concentrated under reduced pressure at 50 ºC. The residue (2.53 g) was recrystallised from a mixture of ethanol (80 mL) and water (6 mL) and filtered to obtain a white powder (2.13 g, 79%); m_{pt} 160-162 ºC; α_D -9.5 (c. 0.63, H_2O).

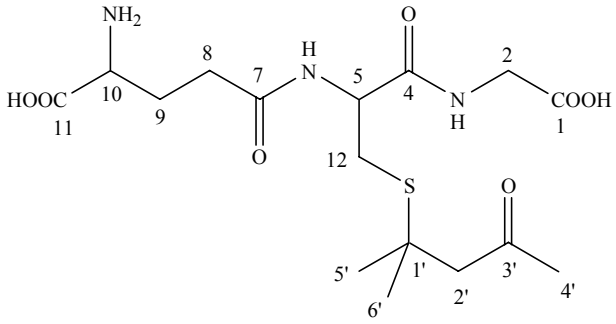


Figure 31. Chemical structure Glut-4MMP

^{1}H NMR: (δ ppm, D_2O):4.52 (1H, dd, J = 7.9, 5.2 Hz, H_5); 3.90 (2H, s, H_2); 3.76 (1H, t, J = 6.3 Hz, H_{10}); 3.11 (1H, dd, J = 13.0, 5.2 Hz, H_{12a}); 2.90 (1H, dd, J = 12.9, 7.6 Hz, H_{12b}); 2.78 (2H, s, $H_{2'}$); 2.60-2.45 (2H, m, H_8); 2.20 (3H, s, $H_{4'}$); 2.15 (2H, app. q, J ~ 6.5 Hz, H_9); 1.35 (6H, s, $H_{5',6'}$). ^{13}C NMR: (δ ppm, D_2O):213.0 ($C_{3'}$); 175.5, 174.1, 172.9, 172.0 ($C_{1,4,7,11}$); 53.1 (C_{10}); 52.9 ($C_{2'}$); 52.7 (C_5); 44.0 ($C_{1'}$); 41.4 (C_2); 32.0 ($C_{4'}$); 31.2 (C_8); 29.1 (C_{12}); 28.3 ($C_{5',6'}$); 26.1 (C_9).ESI-MS (m/z): 406.0 $(M+H)^+$; 428.0 $(M+Na)^+$; 444.0 $(M+K)^+$.

(1'R and 1'S)-γ-L-Glutamyl-S-(1-(2-hydroxyethyl)-n-butyl)-L-cysteinylglycine (**Figure 32**). The synthesis of this compound has been based on the synthesis of the previous glutathionyl conjugate. Glutathione (0.5 g, 1.2 mmol) in aqueous acetonitrile (15 mL of 50% (v/v)) was treated with pyridine (0.2 g, 2.5 mmol) and (*E*)-2-hexenal (0.12 g, 1.2 mmol). After stirring for 72 hrs at room temperature, the reaction was worked up as described above. The aqueous layer was injected into a preparative LC-DAD column and the 3-*S*-glutathionylhexanal was recovered, obtaining a yellow solid (0.34 g, 70%). A portion of this product (0.14 g, 0.34 mmol) in ice water (10 mL) was treated with $NaBH_4$ (0.03 g, 0.68 mmol) for 2 hrs before being acidified to pH 3.1 with

HCl 1N. The mixture was loaded onto a C18 reverse phase preparative LC-DAD column and eluted wit a gradient starting from 1% aqueous ethanol to 30% aqueous ethanol. In this way we have been able to separate the unreduced aldehyde from the target compound which gave a white powder (0.18 g, 36.6%); m_{pt} 106-108 ºC. ESI-MS (m/z): 408.0 $(M+H)^+$; 430.0 $(M+Na)^+$; 446.0 $(M+K)^+$.

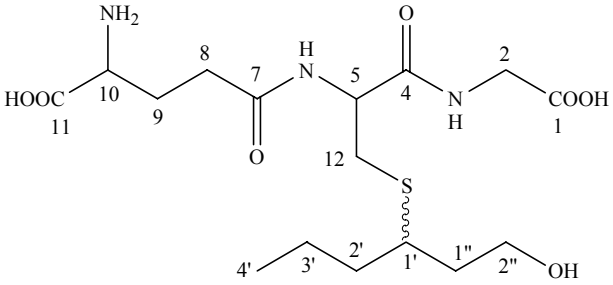


Figure 32. Chemical structure Glut-3MH

(3'R and 3'S) d_8-γ-L-Glutamyl-S-(3-(3-hydroxy-n-propyl)-3-n-propyl)-L-cysteinylglycine. Glutathione (0.5 g, 1.2 mmol) was treated with d_8-(*E*)-2-hexenal (0.13 g, 1.2 mmol [156]) exactly as described above. Reduction of the carbonyl was effected with $NaBH_4$ (0.03 g, 0.68 mmol) and preparative liquid chromatography as before provided the d_8-analogue (100 mg, 20%). As was the case with the 4-MMP conjugate, the ^1H NMR spectrum was consistent with that of the unlabelled compounds. **ESI-MS (m/z):** 416.2 $(M+H)^+$; 438.0 $(M+Na)^+$; 454.0 $(M+K)^+$.

3-*S*-cysteinylhexan-1-ol were kindly donated from prof. R.L. Baumes (INRA, Montpellier, France).

5.2.11 Thiols precursors SPE extraction

The grape juice was adjusted to pH 9.0 with 1 M sodium hydroxide. The solution was percolated through an Oasis® MAX mixed mode SPE cartridge (6 cc, 150 mg, Waters, Rydalmere, NSW, Australia) which had been previously conditioned with methanol (3 mL), water (3 mL) and 50 mM sodium acetate (3 mL). After loading the sample, the cartridge was rinsed with 50 mM sodium acetate (3 mL) and the analyte was then recovered with 2% formic acid in methanol (2 mL). The eluate was brought to dryness under a stream of nitrogen and the analytes were finally dissolved in MilliQ

water (0.8 mL). This solution was used for HPLC-MS/MS experiments. The Oasis® MAX cartridge resulted to give the best extraction performances amongst all the cartridges tested.

5.2.12 LC-MS/MS method to analyse thiols precursors

HPLC-MS Instrumentation. All HPLC-MS analyses were carried out on an Agilent 1200 instrument (Agilent, Milan, Italy) equipped with a binary pump and diode array detector (DAD) connected in series to a 4000 Q Trap hybrid tandem mass spectrometer with a TurboIonSpray source (Applied Biosystems/MDS Sciex, Concord, ON, Canada). Data acquisition and processing were performed using Applied Biosystems/MDS Sciex Analyst software (version 1.4.2).

HPLC Conditions. The column was a 250 × 2.1 mm i.d., 5 μm, Alltima C18 (Grace Davison Discovery Sciences, Milan, Italy) operated at 25 °C and protected by a 4 × 2 mm i.d. guard column of the same material (Phenomenex, Milan, Italy). The solvents were 0.1% aqueous acetic acid (Solvent A) and 0.1% acetic acid in acetonitrile (Solvent B) with a flow rate of 0.300 mL/min. The gradient for solvent B was as follows: 0 min, 5%; 10 min, 7%; 15 min, 7%; 32 min, 80%, 35 min, 90%. The column was equilibrated with 5% B for 10 min prior to an injection. A 10 μL injection volume was used for each sample.

Mass Spectrometer Conditions. All mass spectrometric data were collected in positive ion mode. Nitrogen was used for curtain gas (CUR), 103.4 kPa, nebulising gas (GS1), 344.7 kPa, drying gas (GS2), 344.7 kPa and collision gas (High). The ion spray voltage, declustering potential, source temperature and collision energy were set at 5500 V, 60 V, 500 °C and 30 V, respectively. For Enhanced Product Ion (EPI) experiments, Q1 had unit resolution, the scan rate was set at 1000 amu/s, dynamic fill time was selected for the ion trap, and mass spectra were recorded between m/z 100 and 500 for a parent ion of m/z 406.4. For Multiple Reaction Monitoring (MRM), Q1 and Q3 had unit resolution, and the transitions chosen were m/z 406.4→331.3, m/z 406.4→259.3 and m/z 406.4→174.2, 408.4 → 261.1, 408.4 → 171.1, 408.4 → 161.9, 222.1 → 205.1, 222.1 → 101.1, 222.1 → 83.0 for Glut-4MMP, Glut-3MH and Cys-3MH respectively. MRM parameters were optimised with infusion MS/MS experiments of a pure synthetic

reference compound (2 mg/L) dissolved in water, using an infusion pump operating at 5 μL/min.

5.2.13 Validation of the HPLC-MS/MS method to analyse Glut-3MH and Cys-3MH

Calibration curves for each analyte were prepared following the internal standard procedure. The matrix was a Chardonnay juice which did not show any trace of Glut-3MH and Cys-3MH, detectable using the proposed HPLC-MS/MS method. The juice pH was adjusted to 9.0 according to the method previously discussed.

We decided to use a real juice instead of using a synthetic one to reduce the presence of matrix effects.

Linearity and precision were evaluated in the concentration range typical for Cys-3MH while no information concerning the level of Glut-3MH has been ever reported in the literature. We used 7 concentration levels and 3 replicate solutions per level. Detection limit was evaluated following the statistical procedure indicated in the previous chapters.

5.2.14 Preparation of a Sauvignon Blanc juice crude extract

Six litres of Sauvignon Blanc juice (Tasmania, 2008 vintage) were percolated batch wise through a sintered glass funnel (Ø 100 mm, 50 mm bed height) packed with 150 g of C18 sorbent. The C18 phase was previously activated with 100 mL of methanol and then washed with 100 mL of water. After loading the juice (300 mL), the column was rinsed with water (100 mL), and eluted with methanol (100 mL). The C18 stationary phase was re-equilibrated with water (100 mL) and another batch of juice was passed through, with the process being repeated until all the juice was eluted. The methanol eluates were pooled and the methanol was removed on a rotary evaporator at 40 mbar with a 30 ºC water bath to yield a final volume of ~200 mL of aqueous juice extract.

This crude aqueous extract (~200 mL) was diluted with water (50 mL) and ~30 mL was applied to a C18 column (250 × 35 mm, 100 mm bed height) previously

equilibrated with 100 mL of methanol followed by 100 mL of water. After loading the crude extract, the column was rinsed with water (200 mL) and eluted with methanol (100 mL) using nitrogen gas to provide flow. The colourless water fraction was discarded while the yellow/orange methanol fraction was retained. The remainder of the crude extract was processed batch-wise in the same manner as above, with the column being re-equilibrated with 100 mL of water after each elution with methanol. The methanol fractions were pooled and concentrated with a rotary evaporator as above, giving a total volume of ~20 mL of a syrupy, light orange, juice extract. This extract was diluted 1:9 with water and filtered through a 0.45 µm filter for HPLC-MS analysis.

5.2.15 Isolation of glutathionylated precursors in leaf blades

The leaf blades (50 g) were frozen with liquid nitrogen and then were ground in a coffee grinder. The powder was transferred to a Schott Bottle (1 L volume) and soaked in 30 mL of synthetic wine (10% Ethanol, 90 % MilliQ water, pH 3.2). The mixture was put on a stirring plate and left there for 48 hours. The mixture was filtered through 2 layers of Miracloth to remove the leaf matter and then we passed the solution through different filters up to 0.45 µm.

The solution was loaded onto a C18 reverse phase column and eluted with, successively, 1% aqueous ethanol, 5% aqueous ethanol, 15% aqueous ethanol and 40% aqueous ethanol. The presence of thiol precursors was checked by testing an aliquot of the fractions collected with ninhydrin. All the fractions which were showing a pink coloration were submitted to LC-MS/MS analysis. The fractions containing thiol precursors were combined and concentrated on rotary evaporator to 0.5 mL.

5.3 Results and discussion

5.3.1 HS-SPME sampling

For HS-SPME approach, **Figure 33** shows the effects of the solution temperature on the analyte signals in the 20-60°C range, operating at pH 3.8 with a sampling time of 40 minutes.

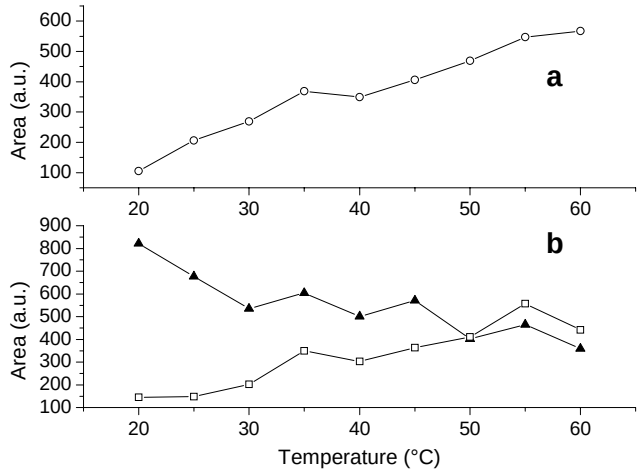


Figure 33. Influence of sample temperature on the measured signal. **a)** 6-MH; **b)** 3-MHA (▲), 3-MH (□)

Figure 33a shows internal standard behaviour which follows 3-MH profile. A temperature of 40°C appears to be the best compromise between a larger sampling of the thioalcohols (3-MH and 6-MH), characterised by a lower affinity to the fibre coating, and a reduced thermal desorption of the thioester (3-MHA). **Figure 33b** shows the behaviour of the analytes: 3-MHA shows a decrement, due to possible hydrolysis in the wine matrix or to desorption of the analyte as the consequence of the temperature increase, while the 3-MH signal increases steadily with the temperature.

Figure 34 displays the sampling time effect in the range 15-90 mins, operating at 40°C with a pH solution equal to 3.8.

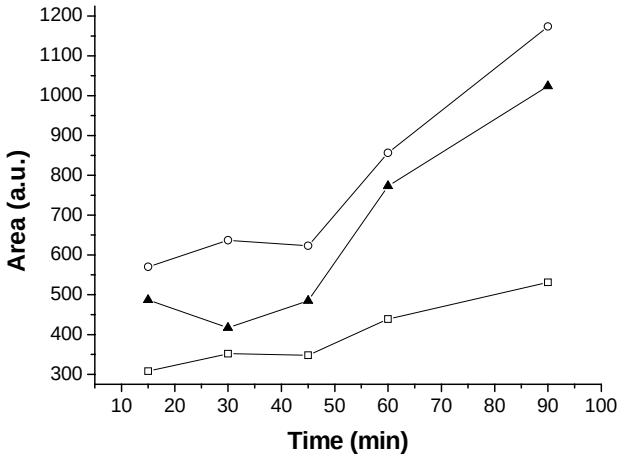


Figure 34. Sampling time effect on signal measured. 3-MH (○); 6-MH (▲); 3-MHA (□)

All the compounds studied display the same profile, as expected from the literature [78]. Taking into account the aims of this work, i.e. a good sensitivity and a short execution time, a sampling time of 40 minutes is a good compromise for evaluating the analytes considered.

In **Figure 35** the influence of solution pH on the adsorption profile is illustrated. A pH increment can reduce both the hydrolysis of 3-MHA and the antioxidant protection owing to a shift in the equilibrium between SO_2 and HSO_3^- towards the monoacidic form. Working with oxidizable compounds, a lowering of antioxidant protection favours their oxidation with loss of signal. Moreover, by increasing the pH to 7, phenolic acids can easily be transformed into quinones [158], which can in their turn oxidize the thiols [159], even though with a delayed time as highlighted by Blanchard et al. [139].

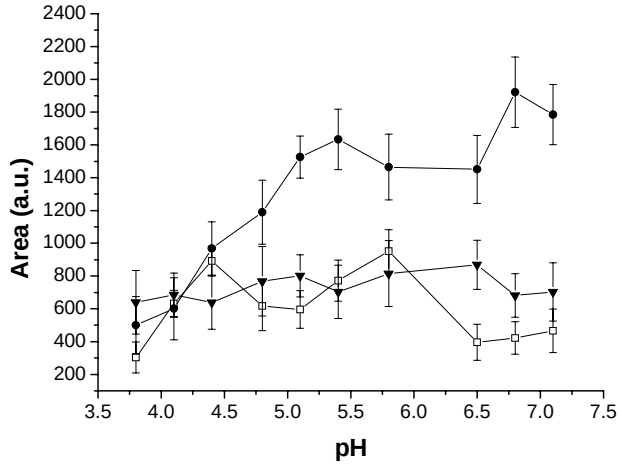


Figure 35. Solution pH effect on the measured signal. 3-MHA (●); 6-MH (▼); 3-MH (□).

The hydrolysis extent of 3-MHA at pH values higher than 4.5 is found negligible (**Figure 35**). In contrast to the experimental evidence, this reaction should lead to a lowering in the trend in the peak area values with a concomitant increase in the associated 3-MH signal. Below pH 4.5 3-mercaptohexyl acetate effectively decreases, but a concomitant decrease of 3-MH occurs. Therefore hydrolysis still plays a negligible role in this range. The lowering of 3-MH and the raising of 3-MHA at higher pH values should be attributed to different matrix effects. Analogously, oxidation also practically does not occur as 6-MH remains relatively constant, owing to the presence of a suitable amount of SO_2 which considerably limits the direct oxidation and the mediated oxidation via polyphenols [138, 139]. A pH value of 7 was chosen, to maximize the signal of 3-MHA, which is in a lower content in wine.

Table 16 summarises the operating conditions adopted.

Table 16. Optimised HS-SPME sampling conditions

Fiber coating	CAR-PDMS-DVB; 2 cm
Sample temperature	40 °C
Extraction	40 min with stirring (500 rpm)
Salt addition	5 g $MgSO_4 \cdot 7H_2O$
Solution pH	7.0
Sample volume	20 mL
Vial volume	30 mL

5.3.2 SPE procedure

The complete effectiveness of the ENV+® cartridges in retaining the analytes was tested comparing peak areas of the two thiols before and after the SPE step. This ratio was very close to unity by working with the same volume conditions. The solution introduced into the cartridges was an alcoholic solution, while for the recovery CH_2Cl_2 was employed.

5.3.3 Purge and Trap sampling optimization

The considered ranges for each variable are reported in **Table 17**.

Table 17. Variables submitted to the multivariate optimisation

Instrumental parameter	Min	Max
Sample temperature	20 °C	60 °C
Trap temperature	20 °C	60 °C
Purge time	10 min	16 min
Flow rate	20 mL/min	70 mL/min

On the basis of preliminary tests, a desorption time of 2 mins was found to be enough to the complete desorption of all the analytes; consequently only the other four parameters, systematically affecting the sampling recovery, i.e. sample temperature, purge flow rate, purge flow time and trap temperature, via a randomized central composite design were optimized. An example of a optimised response surface obtained from the multivariate procedure was reported in **Figure 36**.

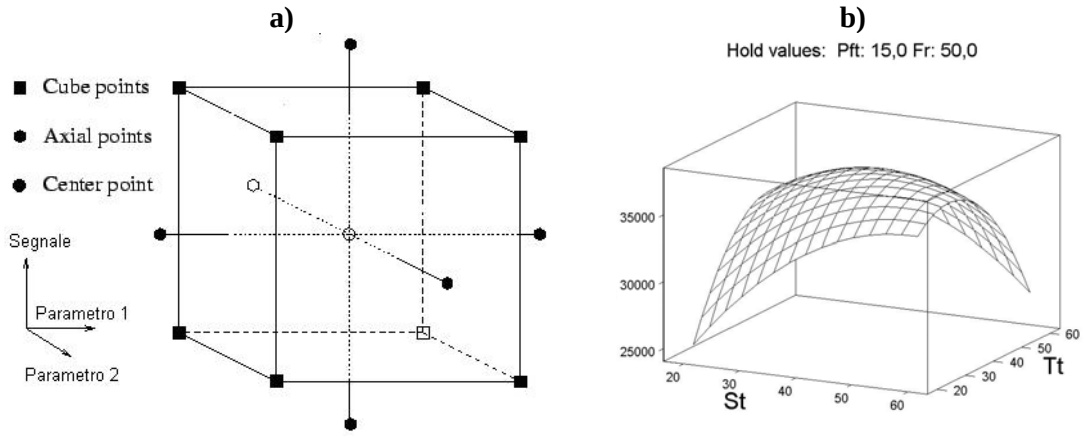


Figure 36. a) Adopted experimental design. **b)** Example of response surfaced for 3-MHA. Variables: Sample temperature (St) and Trap temperature (Tt)

A F-test [148] to check the goodness of the quadratic model was performed. The regression parameters showed in **Table 18** point out that the interaction terms have a poor influence on the model, while the first- and second-order terms show an opposite effects.

Table 18. Parameter of the response surface

3-MH	y = - 78941.0 + 1325.5 St + 895.8 Tt + 7125.2 Pft + 453.7 Fr - 7.1 St^2 - 11.8 Tt^2 - 165.7 Pft^2 + 3.7 Fr^2 - 9.5 St · Tt + 8.0 St · Pft - 8.4 St · Fr + 2.7 Tt · Fr + 7.4 Tt · Fr - 45.5 Pft · Fr R^2 = 0.82
3-MHA	y = - 152124.0 + 1240.7 St + 91.5 Tt + 22384.1 Pft + 253.5 Fr - 10.2 St^2 - 0.3 Tt^2 - 750.2 Pft^2 + 1.5 Fr^2 - 8.3 St · Tt + 3.2 St · Pft - 0.1 St · Fr - 4.8 Tt · Fr + 5.4 Tt · Fr - 36.8 Pft · Fr R^2 = 0.97
6-MH	y = - 76872.5 + 15076.6 St + 484.5 Tt + 6820.7 Pft + 429.6 Fr - 7.7 St^2 - 6.5 Tt^2 - 102.7 Pft^2 + 1.5 Fr^2 - 8.4 St · Tt - 20.7 St · Pft - 4.7 St · Fr + 0.01 Tt · Fr + 5.6 Tt · Fr - 36.6 Pft · Fr R^2 = 0.82

As already reported [160] increments of the flow rate, of the sample temperature and of the purge flow time increased the amount of analyte sampled, owing to an increase of the analyte amount purged from the solution, while an increment of the trap temperature decreased the signal measured owing to a lowering of the trapping efficiency. The negligibility of interaction terms in the regression model explains the closeness between the values of the parameters found by the multivariate approach and those given by the simple monovariate procedure. This fact, together with the absence of a marked peak in the response surface, grants a high ruggedness to the Purge and Trap approach for the analytical problem in hand, making easy its applications. **Table 19** summarise the chosen operative conditions.

Table 19. Optimised Purge and Trap conditions

Solution pH	7.0
Sample volume	5 mL
Sample temperature	45°C
Purge flow time	15 min
Purge flow rate	50 mL/min
Trap temperature	38°C
Desorption time	2 min

5.3.4 GC-MS analysis resolution

Preliminary experiments indicated that in our matrices the column used in previous works [21, 141], even working with the SIM mode, gave incomplete

resolution. The use of the above described hyphenated column permitted totally satisfactory results. The SIM mode was employed to recognise thiols, using the fragments shown in **Table 20**.

Table 20. GC-MS identification and quantitation parameters for the considered analytes

Analytes	RT (min)	Quantitation ion	Qualifier ions
3-MHA	25.72	116	67, 83, 88
3-MH	26.78	134	67, 82, 100
6-MH (I.S.)	28.11	87	55, 67, 116

Typical HS-SPME chromatogram relevant to a real wine with the proposed I.S. is reported in **Figure 37**.

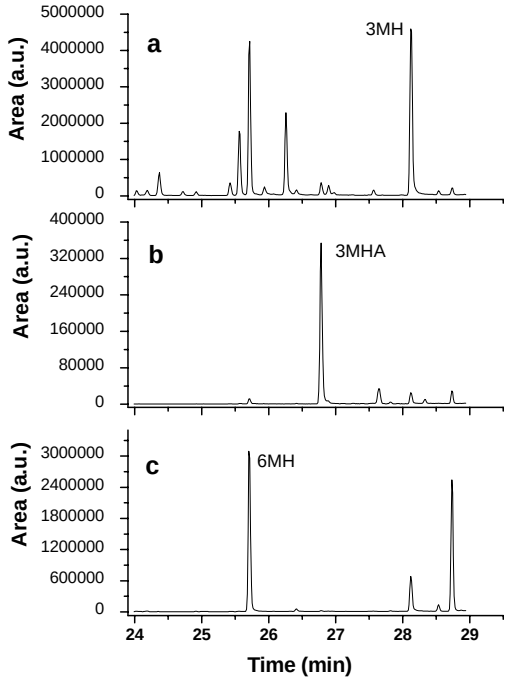


Figure 37. Example of HS-SPME chromatogram.

5.3.5 Validation

Linearity was studied with 7 calibration levels between 0.25 and 4.00 µg/L for 3-MH (calibration points: 0.25, 0.50, 0.75, 1.00, 2.00, 3.00, 4.00) and between 0.10 and 1.50 µg/L (calibration points: 0.10, 0.20, 0.30, 0.50, 0.75, 1.25, 1.50) for 3-MHA, with

five replicates for each level. These ranges correspond to those naturally occurring in wines. Critical and detection limits were calculated via the Hubaux Vos approach, using the following concentrations for both analytes: 0.00, 0.02, 0.05, 0.15, 0.25 µg/L.

Table 21 reports the results of the Hartley and Mandel tests for the HS-SPME, SPE and P&T sampling data.

Table 21. Results of statistical tests for the three considered samplig techniques

	Analyte	*F_{max}	Scedasticity	**F_{mandel}	Model
HS-SPME	3-MHA	10.90	homoscedasticity	2.75	Linear
	3-MH	12.08	homoscedasticity	1.75	Linear
SPE	3-MHA	17.83	homoscedasticity	2.94	Linear
	3-MH	14.96	homoscedasticity	3.92	Linear
P&T	3-MHA	15.40	homoscedasticity	2.75	Linear
	3-MH	16.12	homoscedasticity	1.75	Linear

*$F_{4,7}^{\alpha=0.05}$ = 29.9 **$F_{(1,3)}^{\alpha=0.05}$ = 4.16

The former test proves the homoscedasticity of the data, thus allowing the use of the unweighted regression, the latter one shows that a linear model is the best way to fit the experimental data. The regression parameters of the straight line model used and the value of the detection limits are given in **Table 22**.

Table 22. Regression parameter and detection limits for the considered sampling techniques

	Analytes	Slope	SD Slope	Intercept	SD Intercept	SD	R^2	L_C (µg/L)	L_D (µg/ L)
HS-SPME	3-MHA	1.30	0.01	0.028	0.011	0.04	0.992	0.029	0.057
	3-MH	1.12	0.02	0.041	0.010	0.03	0.993	0.037	0.069
SPE	3-MHA	0.79	0.01	0.033	0.006	0.02	0.995	0.042	0.083
	3-MH	0.86	0.03	0.018	0.007	0.02	0.993	0.037	0.074
P&T	3-MHA	2.63	0.03	0.015	0.009	0.05	0.992	0.019	0.036
	3-MH	1.12	0.02	0.021	0.010	0.03	0.993	0.023	0.048

5.3.6 Effectiveness of the internal standard adopted

Tests carried out on the two wines (WW and RW) treated with $AgNO_3$, and suitably added with the factor species (sugar and ethanol) and the two thiols to realise

specific analyte concentrations in specific matrices, showed that the matrix strongly influences the apparent partition constant between the liquid phase and the fibre coatings [78].

Table 23. Matrix effect on the HS-SPME signal. SD reported in brackets.

Matrix	Wine Composition	Level	3-MHA		3-MH	
			Area	Recovery	Area	Recovery
WW	Sugars 3 g/L Alcohol 10% v/v	0.75	1743 (5.4)	100.4 (5.7)	563 (5.0)	103.9 (5.9)
		Middle	863 (5.6)	99.4 (4.9)	222 (7.5)	99.3 (6.6)
		Low	354 (5.5)	103.0 (5.9)	131 (7.3)	100.3 (7.9)
WW	Sugars 3 g/L Alcohol 12% v/v	High	1563 (4.2)	99.4 (3.1)	521 (6.0)	103.2 (4.5)
		Middle	762 (5.2)	103.8 (5.5)	220 (6.1)	98.3 (4.9)
		Low	302 (6.1)	98.4 (5.5)	124 (6.2)	99.8 (5.7)
WW	Sugars 3 g/L Alcohol 15% v/v	High	1342 (3.3)	101.6 (2.7)	482 (6.0)	103.7 (5.8)
		Middle	703 (3.8)	100.5 (3.3)	212 (6.2)	104.4 (6.1)
		Low	286 (4.1)	101.8 (1.9)	115 (7.2)	98.3 (7.2)
WW	Sugars 50 g/L Alcohol 10% v/v	High	1864 (4.0)	104.8 (3.7)	612 (6.6)	104.3 (6.6)
		Middle	966 (5.1)	99.5 (5.3)	251 (7.0)	98.1 (6.6)
		Low	363 (5.0)	99.7 (4.7)	112 (7.5)	97.6 (7.1)
WW	Sugars 100 g/L Alcohol 10% V/v	High	2001 (4.2)	100.1 (3.9)	653 (5.5)	100.1 (4.9)
		Middle	1002 (5.8)	103.8 (6.0)	243 (5.7)	100.4 (5.9)
		Low	352 (7.1)	102.3 (7.0)	122 (6.8)	99.6 (6.2)
RW	Sugars 3 g/L Alcohol 10% v/v	High	1982 (4.7)	102.8 (3.4)	601 (5.5)	106.5 (4.3)
		Middle	876 (6.0)	99.5 (6.1)	221 (5.6)	107.2 (4.9)
		Low	351 (5.5)	98.7 (5.1)	117 (6.0)	103.2 (6.1)

3-MHA: high level = 0.75 µg/L; middle level = 0.35 µg/L; low level = 0.15 µg/L
3-MH: high level = 2.00 µg/L; middle level = 0.75 µg/L; low level = 0.30 µg/L

Peak areas reported in **Table 23** demonstrate that the thiols considered show an inversely proportional dependence between the ethanol concentration and the signal measured. Besides ethanol, many other compounds may potentially affect sampling. An

increase in sugar concentration causes a rise in the signal measured. The data relative to red and white wine highlight the effect of different polyphenol and ester content, in connection to possible competitive adsorption [76] and redox reactions [138, 139]. It is indeed well-known that a typical red wine has a higher content of polyphenols and a lower content of fruity esters.

To conclude, analyte recoveries in HS-SPME experiments on wines vary widely owing to matrix effects and experimental conditions. For these reasons reliable quantification requires the use of methodologies involving suitable spiked internal standards. The recovery values close to 100% reported in **Table 23** prove that the internal standard chosen (6-MH) successfully corrects the matrix effects highlighted by different peak area values in the HS-SPME analysis.

5.3.7 Comparison of the methods PT vs. HS-SPME and vs. SPE

Figure 38 shows the accordance of results obtained using the three different sampling methods. Each point on the graph represents a single wine sample with coordinates equal to the concentrations obtained from the two techniques compared.

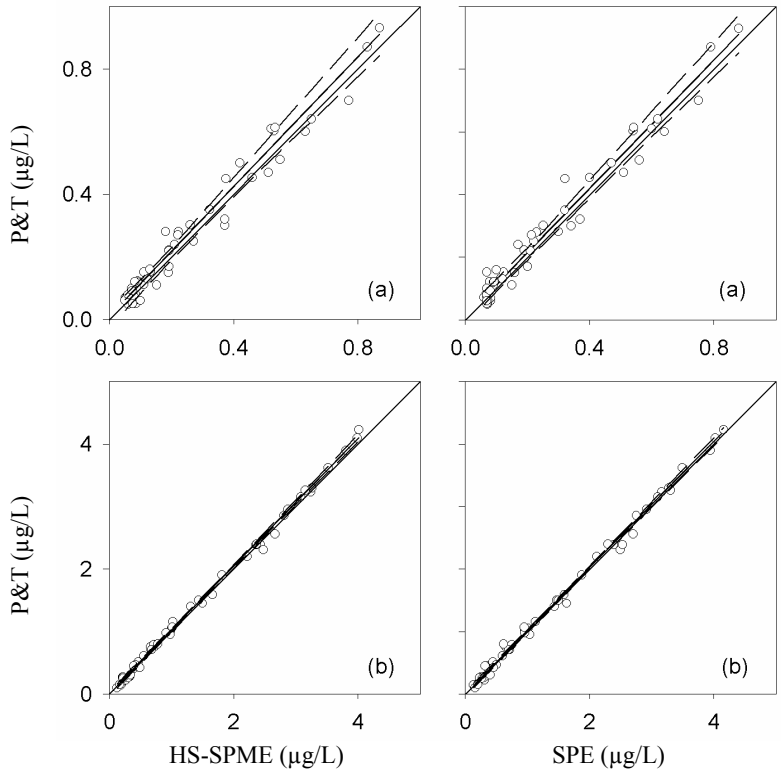


Figure 38. Comparison of the three considered sampling techniques. **a)** 3-MHA; **b)** 3-MH

The procedure of Theil, suitable to non normal *x,y* data both affected by uncertainty, fits the plotted data with a straight line with intercept *a* and slope *b* statistically equal to zero and unit, respectively [161].

3-MHA (**Figure 38a**) data are wider spread in the graph owing to their low concentration in the wine samples, near to the detection limit of the method; 3-MH data (**Figure 38b**), well above the detection limit, appear more aligned along the "reference line". The quite higher concentrations ever found in the Purge and Trap measurements in comparison with those from the other two sampling techniques are most probably due to a better extraction of the analytes by the Purge and Trap than by the HS-SPME and SPE approaches.

Nevertheless the methods can be considered equivalent since the reference straight line ($\alpha = 0$ and $\beta = 1$) belongs to the 95% confidence bands of the regression line.

5.3.8 Thiol precursors SPE method

We first analysed a Sauvignon Blanc juice without a pre-concentration step. This approach did not give any interesting result as the concentration level in the juice was too low to be detected by direct injection. Consequently a preliminary enrichment step using an SPE cartridge was introduced. Different cartridges (**Table 24**) were tested.

Table 24. Different SPE cartridges tested. Bed packing of 100 mg for all the cartridges

Cartridge	Brand	Functional Group	Pore Size
Oasis MAX	Waters	dimethyl butylamine	80 Å
Bond Elut C18	Varian	C18	60 Å
Bond Elut LMS	Varian	crossed-linked styrene divinyl benzene	300 Å
Bond Elut PPL	Varian	functionalized styrene divinyl benzene	150 Å

The best results were achieved by using the strong anionic exchange SPE cartridge Oasis® MAX as shown in **Figure 39.**

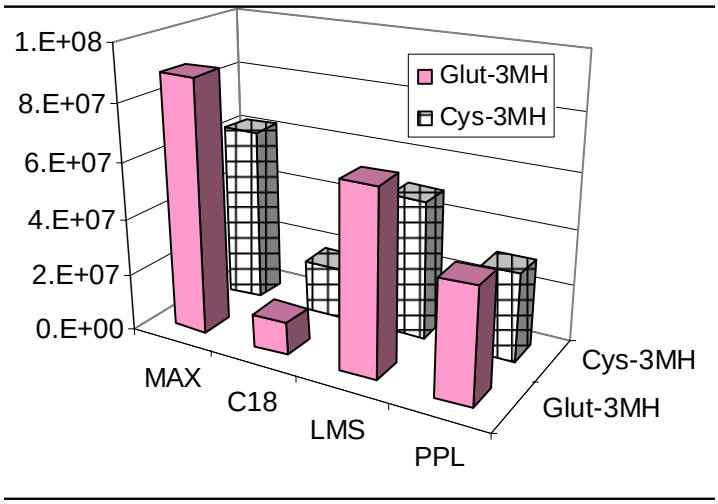


Figure 39. Analytical response using different SPE cartridges

The extraction was carried out according to the protocol reported in **Table 25** and changing the juice pH to 9 by means of an NaOH (1M) solution before the percolation of the sample through the cartridge.

Table 25. SPE protocol to analyse 3-MH precursors

Step	Solvent	Volume
Activation	Methanol	6 mL
Activation	Water	6 mL
Loading	Grape juice	50 mL
Washing	Methanol/Sodium Acetate (95:5)	6 mL
Elution	Methanol with 2% formic acid	10 mL

5.3.9 HPLC-MS/MS analysis of thiol precursors

An aqueous solution of synthetic Glut-4MMP, Cys-3MH and Glut-3MH (2 mg/L each) was firstly infused into the mass spectrometer in positive ionisation mode to obtain and optimise the fragmentation pattern of the analyte by collision induced dissociation (CID). This resulted in the choice of mass transitions, for multiple reaction monitoring (MRM) experiments, m/z 406.4→331.3, m/z 406.4→259.3 and m/z 406.4→174.2, 408.4 → 261.1, 408.4 → 171.1, 408.4 → 161.9, 222.1 → 205.1, 222.1 → 101.1, 222.1 → 83.0, for Glut-4MMP, Glut-3MH and Cys-3MH, respectively.

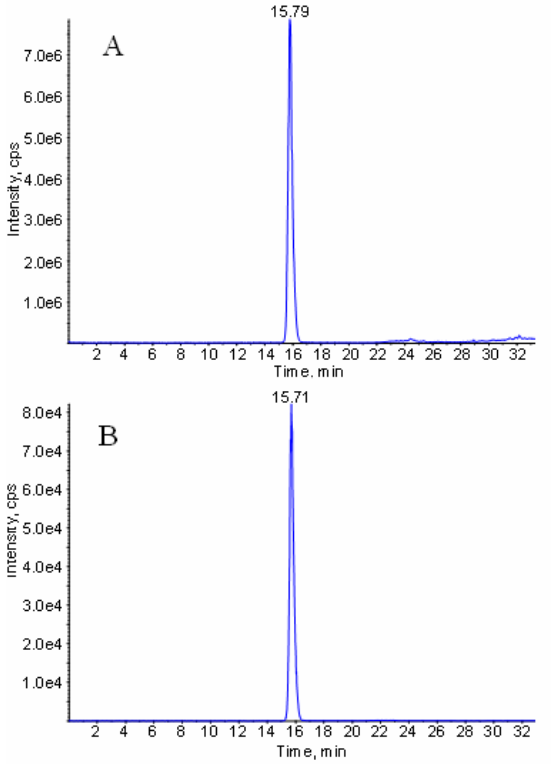


Figure 40. Example of EPI (**A**) and MRM (**B**) chromatogram of Glut-4MMP

Next we recorded the total ion EPI and MRM chromatograms (**Figure 40**) of the Glut-4MMP, Glut-3MH and Cys-3MH reference compounds.

The spectrum taken from the EPI chromatogram displayed the fragmentation of the parent ions m/z 406, m/z 408 and m/z 222 for Glut-4MMP, Glut-3MH and Cys-3MH, respectively. The peaks at m/z 331 and m/z 259 correspond to the loss of glycine and glutamic acid residues, respectively. Accordingly for Glut-3MH, the peaks at m/z 261, m/z 171 and m/z 161 represent the loss of glutamidyl, water + glutamidyl + glycyl and glutamidyl + alkyl chain fragments, respectively. The Cys-3MH peaks at m/z 205 and m/z 101 represent the loss of water and cysteinyl fragments, respectively. Retention time of the studied analytes are reported in **Table 26**.

Table 26. Retention times of the considered thiols precursors

Analyte	Retention Time (min)
Glut-4MMP	15.75
Glut-3MH	22.55
Cys-3MH	15.31
d_8-Glut-3MH (I.S.)	21.91

5.3.10 Validation HPLC-MS/MS method for Glut-3MH and Cys-3MH analysis

Since no information about the level of Glut-3MH is reported in the literature we decide to adopt the same concentration range for both Cys-3MH and Glut-3MH. In particular linearity was studied with 7 calibration levels between 0.05 and 0.75 mg/L (calibration points: 0.05, 0.10, 0.20, 0.35, 0.50 0.60, 0.75), with three replicates for each level. Detection limits were calculated, according to the Hubaux-Vos method [34], using the following concentrations: 0.00, 0.005, 0.01, 0.15, 0.25 mg/L.

The regression parameters of the straight line model used and the value of the detection limits are given in **Table 27**.

Table 27. Regression parameters and detection limit for Glut-3MH and Cys-3MH

Analyte	Intercept	Intercept SD	Slope	Slope SD	R^2	$S_{y/x}$	LD (ppm)
Glut-3MH	0.013	0.008	1.53	0.02	0.983	0.081	0.01
Cys-3MH	0.024	0.015	1.12	0.05	0.991	0.065	0.02

5.3.11 Identification of 4-S-glutathionyl-4-methylpentan-2-one in a Sauvignon Blanc juice extract

While the identification of the glutathione conjugate of 3-MH was described in 2002 as a grape juice component [117], no evidence has been reported for the presence of the glutathione conjugates of 4-MMP (Glut-4MMP) in grape juice. The apparent absence of these glutathione conjugates was of interest to us, as these compounds are likely to be related to the biosynthesis of their 4-*S*-cysteinyl congeners and thus to the corresponding free odorants.

Analysis of the Sauvignon Blanc juice extract by HPLC-MS/MS revealed the presence of Glut-4MMP after comparing the mass spectrometry data obtained from the juice with data obtained for the synthetic reference compound. Initially, a total ion chromatogram in scan mode was obtained, and the extracted ion chromatogram for m/z 406 displayed a peak at the correct retention time of 16 min (data not shown).

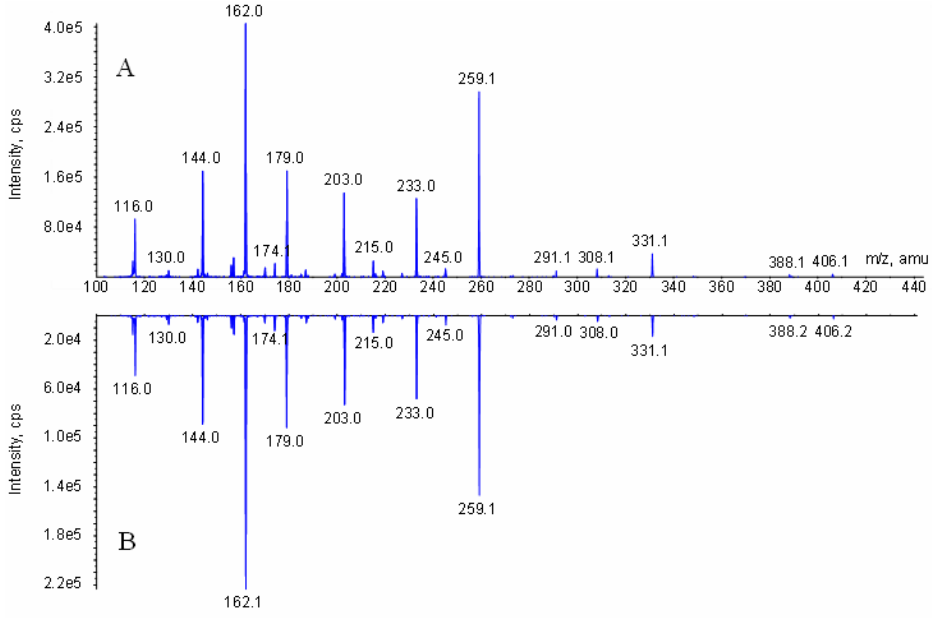


Figure 41. Overlap of EPI mass spectrum for synthetic (**a**) and extracted (**b**) Glut-4MMP

Further HPLC-MS/MS analyses in EPI and MRM mode, showed how retention times, and more significantly the EPI mass spectrum of parent ion m/z 406 (**Figure 41**) were in perfect agreement with those of the synthetic reference compound.

5.3.12 First identification of thiols precursors in leaf blades

The presence of aroma compound precursors in several plant tissues, other than berries, has been reported. So far only glycosides were found in leaf blades and petioles [168, 169]. This evidence prompted our investigation in evaluating the possible presence of thiol precursors (either cysteinylated or glutathionylated ones) in leaf blades.

Using the chromatographic method developed to analyse thiol precursors in grape products it has been possible to identify 3-*S*-glutathionylhexenal and 3-*S*-glutathionylhexanol in Sauvignon Blanc grape leaves (**Figure 42**).

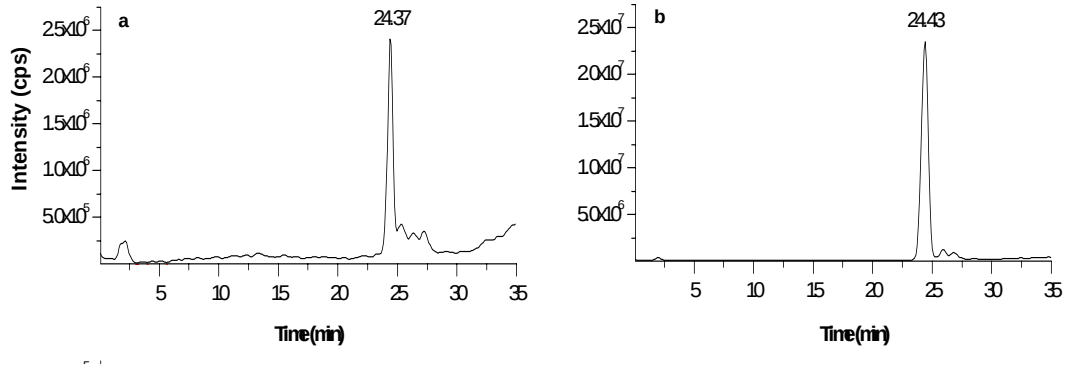


Figure 42. Chromatogram showing the presence of thiols precursors in grape vine leaves

To the best of our knowledge this is also the first time varietal sulfur compound precursors have been identified in grape vine leaves. The evidence generated from these experiments leads to more questions, e.g. to what extent are these glutathionylated precursors transported into the berry?

Due to their known role as flavour precursors, any transport of thiol conjugates from grapevine leaves to the berries must be considered as it might have a strong influence on the final product. In particular what has been found it has also important consequences on wine physiology and viticulture as it can influence vineyard practices and lead to future changes in vine cultivation.

5.4 Conclusions

On the basis of the several studies performed during my PhD about the varietal sulfur compounds a good knowledge on this topic has been achieved from our group and some interesting remarks can be proposed.

All the three analytical approaches developed to analyse VSCs showed interesting potentiality for a further application in wine science.

In particular, the SPE procedure is a further successful application in the quantification of the two important varietal thiols in the context of analysis of volatiles in the wine matrix.

The proposed HS-SPME sampling method coupled with a GC-MS procedure, permitted an easier quantification of two tropical fruit scenting thiols of high oenological interest in the context of sensorial relevance.

The new Purge and Trap method represents a further improvement in the development of analytical procedure useful for quantifying important molecules which are able to condition the wine aroma. Additionally the Purge and Trap method represents a simple tool, more sensitive and more robust, to control and quantify the two considered species.

The internal standard chosen confirmed its effectiveness in overcoming the possible matrix effects observed during sampling optimisation and in dedicated experiments with red and white wines, deprived of all sulphur volatiles and suitably enriched with thiols.

A robust statistic approach, namely the Theil's regression procedure, showed its suitability in the comparison of data drawn from different analytical methods and affected by similar uncertainties. HS-SPME method, giving results comparable to those obtained with the SPE and Purge and Trap methods, appeared to be easier and cheaper to apply to the wine process control laboratory.

The SPE, HS-SPME and Purge and Trap procedures, both precise and accurate, satisfy oenological requirements, giving detection limits near or even lower than the sensory threshold for each analyte. Moreover these methods do not require toxic or pollutant reagents allowing a safer application in laboratory dedicated to routine analysis.

The investigation on thiols precursors improved our knowledge on VSCs, furthers our understanding of the presence of volatile thiol precursors and complements previous studies that identified other conjugated precursors in grape juices. This information will aid future studies aimed at determining the inter-relationships between the thiol precursors found in grapes and juice, and the resulting aroma-active compounds in wine. Moreover, as the *S*-cysteinyl conjugates are always found along the metabolic pathway of the degradation of the relevant *S*-glutathionyl conjugates, these glutathione derivatives might be the real precursors to a range of compounds important to wine sensory properties.

The development of dedicated HPLC-MS/MS method to quantify thiols precursors allows a further step in the knowledge of the relationship between free and conjugated forms.

Finally, evidence is presented for the first time for the presence of glutathionylated precursors on grape vine leaves, which corroborates the function of enzymes in the biogenesis of thiol precursors in other vine tissues and lead towards new question about the formation, accumulation and transport of these molecules.

Currently we are carrying out the quantification of varietals sulfur compound precursors during ripening and during fermentation. We took into consideration different zones with diverse maturation rate.

To the best of our knowledge these informations are missing in the literature, so being available all the analytical tools and owing the knowledge to study and understand possible behaviours, we are trying to fill this gap.

6. CONCLUSIONS

This Ph.D. allowed to highlight the potentiality and the contribute of Analytical Chemistry in the study of complex matrices, as oenological products. In particular Mass Spectrometry and statistical procedures resulted to play a vital role in the evaluation of such matrices.

Mass spectrometry is the most effective technique in unravelling the complexity of foodstuff, especially in the multicomponent analyses. Statistical procedures are necessary to gain a deeper knowledge of analytical responses, to define the best way to optimise methods and to overcome analytical artefact.

In this thesis several interesting results were achieved; in particular it has been possible to investigate an important class of molecules influencing wine aroma. In this case the contribution of analytical chemistry has been crucial in defining analytical methods and statistical procedure to evaluate instrumental response.

The topics explored in these 3 years and the outcomes obtained can be summarised as follows:

- definition of a statistical procedure to overcome matrix effects [29]:
- development of HS-SPME/GC-MS method to analyse fermentative sulfur compounds and to investigate how ageing, variety and oenological practices can affect their level [8, 21, 70];
- development of HS-SPME, SPE and P&T GC/MS and LC-MS/MS methods to analyse varietal sulfur compounds and their precursors, respectively.
- identification of a new putative precursor, synthesis of the reference standard and definition of a new robust procedure to compare responses from different analytical procedures [42, 118, 145].

Concerning the first topic faced in this thesis (i.e. matrix effect), the variance component model (VCM) appeared to be satisfactory in dealing with matrix effects. In

particular the definition of an overall calibration curve for all the matrices considered permitted to handle this artefact.

The shorter analysis time and the chance of having one calibration curve for all the matrices studied outweigh the higher detection limit and the slightly higher uncertainty.

Fermentative sulfur compounds represent an interesting topic in wine science. Up to now this class of molecules has been always considered as depreciating wine aroma and many efforts have been put in to understand how to limit their presence and to control their evolution. Recently they have been revaluated because of their potential positive sensory contribution when present at low concentration. Owing to their reactivity and low level in grape products, no thorough methods were present in the literature.

In this thesis we defined the best HS-SPME/GC-MS conditions to analyse, to the best of our knowledge, the widest range of sulfur compounds proposed in the literature. We optimised and validated this method and we have been able to draw important considerations about the effects of ageing, variety and common oenological practices on the level of some important fermentative sulfur compounds.

The last topic investigated in these three years and disclosed in this thesis has been the study of varietal sulfur compounds. These molecule are present as conjugated forms in specific varieties and are released during fermentation by means of a β-lyasic activity present in some *Saccharomyces Cerevisiae* strains. The sensory effect of these compound is highly positive and extremely appreciated in wines. The micro-trace level (cf. ng/L) of varietal sulfur compounds in final wine made their study and quantification particularly difficult.

In these thesis we proposed three different GC-MS approaches exploiting different pre-concentration procedures (i.e. Solid Phase Extraction, Head Space Solid Phase Microextraction and Purge & Trap) to analyse these molecules. We also proposed a robust statistical procedure to compare the analytical responses obtained with these three approaches, namely the Theil's method.

Afterwards we focussed our attention on the precursors of varietal thiols. We synthesised the reference compounds, we defined LC-MS/MS methods to quantify them and also we identified a new putative precursor.

All the results achieved by this thesis further our knowledge on analytical chemistry applied on wine science and permit to draw important information on sulfur compounds in oenological matrices.

In particular it has been possible to investigate the potentiality of mass spectrometry in analysing complex matrices, it has been possible to exploit the most advance analytical techniques and important statistical and analytical tools are provided to continue in the study of oenological products and other foodstuff.

This information can be also correlated according to a metabolomic point of view with microbiological, technological and genetical informations to further improve our comprehension on grape products.

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