Chapter 11

Occurrence and Analysis of Sulfur Compounds in Wine

Daniela Fracassetti and Ileana Vigentini

Additional information is available at the end of the chapter

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Abstract

Sulfur compounds play an important role in the sensory characteristics of wine. These molecules can derive from the grape, in which the non-volatile forms are usually present as glycosylated molecules, the metabolic activities of yeast and bacteria, the chemical reactions taking place during the wine aging and storage, and the environment. The sulfur compounds include molecules positively correlated to the aromatic profile of wine, namely the volatile thiols, and are responsible for certain defects, imparting notes described as cabbage, onion, rotten egg, garlic, sulfur and rubber. Due to the low concentration of these molecules in wine, their high reactivity and the matrix complexity, the analytical methods which enable their detection and quantification represent a challenge. The solid phase microextraction (SPME) technique has been developed for sulfur compounds associated with off-flavors. The analysis of volatile thiols usually requires a derivatization followed by gas chromatography (GC)-MS or UPLC-MS methods. Besides the sulfur-containing aromas, another sulfur compound that deserves mention is the reduced glutathione (GSH) which has been widely studied due to its antioxidant properties. The analysis of GSH has been proposed using a liquid chromatography technique (HPLC or UPLC) coupled with fluorescence, MS and UV detectors.

Keywords: sulfur off-flavors, volatile thiols, reduced glutathione, sample preparation, analysis, wine

1. Introduction

Sulfur-containing compounds strongly affect the sensory properties of wine and must. They include aromatic molecules, off-flavors and a well-known non-volatile compound with antioxidant properties such as glutathione. The sulfur-containing compounds can be derived from the grape in which the non-volatile forms are usually present, the metabolic activities of yeast and bacteria, the chemical reactions taking place during the wine aging and storage, and the environment [1]. Their presence in wine can be the result of both enzymatic and non-enzymatic

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mechanisms. In the first case, the sulfur compounds represent the products of metabolic and fermentative pathways whose substrates are both amino acids and some sulfur-containing pesticides. When yeast and bacteria metabolize these thiols, the released sulfur compounds are generally considered off-flavors [2]. Non-enzymatic processes include photochemical, thermal and other chemical reactions of sulfur compounds during winemaking and storage [3]. The reactions involving the sulfur-containing amino acids, in particular, can bring about the light-struck taste in case the bottled white wine is exposed to light greatly affecting the sensory properties [4, 5]. The sulfur-containing off-flavors impart negative notes such as cabbage, onion, rotten egg, garlic, sulfur and rubber [6]. Among them, hydrogen sulfide is probably the best-known sulfur compound in wine. Hydrogen sulfide is a very reactive species and can trigger reactions generating compounds such as mercaptans, dimethyl sulfide and polysulfide, which also have a negative impact on the wine aroma. The long-chain polyfunctional sulfur compounds, also known as volatile thiols, are one of the most important groups of aroma compounds in wine, which confer pleasant aromatic notes at trace levels; at high concentrations, these compounds can be objectionable yet [3, 7–9]. Volatile thiols such as 4-mercapto-4-methylpentan-2-one (4MMP), 3-mercaptohexan-1-ol (3MH) and 3-mercaptohexyl acetate (3MHA) are particularly important, for example, for characterizing the typical aroma of Sauvignon Blanc wine [10]. Moreover, in some wines, 4-mercapto-4-methylpentan-2-ol (4MMPOH) is also detected but its concentration is lower than the thiol-related aromas mentioned above [11].

Besides the sulfur compounds contributing to the sensory characteristics of wine, the glutathione is of particular interest due to its antioxidant properties. This tripeptide can limit the browning of white must as it reduces back the *o*-quinones to the correspondent phenols and, consequently, the formation of the brown polymers are avoided [12].

2. Aromatic compounds containing sulfur

2.1. Sulfur off-flavors

Sulfur-containing compounds are mainly associated with off-odors due to the presence of hydrogen sulfide and mercaptans whose concentrations are typically low as well as their perception threshold [2]. The most abundant sulfur off-flavors found in wine are represented by hydrogen sulfide, methanethiol, ethanethiol, dimethylmercaptans (dimethylsulfide, dimethyldisulfide, dimethyltrisulfide) [13] and other sulfur-containing compounds responsible for off-flavors in wine (**Table 1**). Hydrogen sulfide is the most recognized sulfur compound associated with the rotten egg aroma, it is highly volatile and has a low perception threshold (10–80 μg/L). Even if hydrogen sulfide could be easily removed by copper treatment [14], it is very reactive specie participating in the generation of other mercaptans, such as dimethylsulfide and polysulfide, and ethanthiol, the latter due to its combination with ethanol or acetaldehyde [7]. *Saccharomyces cerevisiae* can release this compound through the degradation of sulfur-containing amino acids and the reduction of elemental sulfur, sulfite or sulfate [15]. However, its synthesis is limited when nitrogen derived from either amino acids (except for cysteine) or ammonium is added [13]. Variable amounts of hydrogen sulfite can be released during the alcoholic fermentation. Smith and co-authors [16] suggest that the sulfur-containing

Compound	Olfactory description	Perception threshold (μg/L)	Range in wine (μg/L)
Hydrogen sulfide	Rotten eggs, reduced taste	0.001–150	0–370
Methanethiol	Cooked cabbage, reduced taste	0.3	0–16
Ethanthiol	Onion, rubber, putrefaction	1.1	0–50
Dimethyl sulfide	Cabbage, asparagus, corn, molasses	10–160	0–910
Carbon disulfide	Cabbage, rubber	>38	0–18
Dimethyl trisulfide	Cabbage, onions, cooked vegetable	0.1	0–111
Diethyl sulfide	Garlic	0.93–18	0–10
Dimethyl disulfide	Cooked cabbage, asparagus, onions	20–45	0–160
Diethyl disulfide	Garlic, onion, burnt rubber	4.3–40	0–160
2-Mercaptoethanol	Barnyard-like, poultry, farmyard	130	0–400
Methylthioacetate	Sulfurous, rotten vegetables	300	0–115
S-Ethylthioacetate	Sulfurous	40	0–180
2-(Methylthio)-1-ethanol	Cauliflower, French bean	250	0–139
2-(Methylthio)-1-propanol	Cauliflower, cooked cabbage	1200	0–5655
2-(Methylthio)-1-butanol	Onion, garlic, earthy	100	0–180
Benzothiazole	Rubber	50–350	0–30
5-(2-Hydroxyethyl)-4-methylthiazole	Green	100–1000	5–50

Table 1. Common fermentative sulfur compounds and off-flavors found in wines [3, 22, 120].

precursors could produce hydrogen sulfite, methanthiol and dimethylsulfide during fermentation and not all these compounds are released in gaseous form. The chemistry behind the reactions and regulation mechanisms involving hydrogen sulfite and other mercaptans produced by the yeast is not well understood [17]. Methionine can be metabolized by yeast to produce fusel alcohol, methionol and 3-methylthio-1-propanol imparting cabbage and cauliflower aromas. Cysteine is a precursor of S-containing heterocycle compounds; these compounds can be metabolized by *Oenococcus oeni* and aroma descriptors such as sulfury, floral, fruity, toasted and roasted can be imparted to the wine [2, 18]. The microbial degradation of these amino acids can lead to the presence of dimethyl sulfide which has shown the property of enhancing the wine aroma due to the interactions with other volatiles, such as esters and norisoprenoids [19, 20]. Nevertheless, dimethyl sulfide imparts notes such as asparagus, corn and molasses which are undesired characters in white wine to a certain extent, although the aroma complexity is increased [21]. The levels of dimethyl sulfide, methionol, diethyl sulfide and diethyl disulfide increase as the aging time and storage temperature increase and they can influence the aroma complexity of aged wine [13, 22]. Mercaptans are responsible for reduced aroma in wine. Different strategies can be used for the removal of sulfur off-flavors. The addition of diammonium phosphate or other yeast-based nutrients leads to an increase of the readily assimilable nitrogen enabling the yeast to convert sulfur-containing amino acids into hydrogen sulfide [23]. The aeration of wine during the alcoholic fermentation as well as wine racking in aerated conditions showed a protective effect against the formation of sulfur off-flavors. The oxygen positively contributes to the fermentative capacity of the yeast [24, 25]. Thereafter, the oxygen presence maintains lower levels of sulfur off-flavors during storage [26] probably due to either the formation molecular mass sulfur compounds or the quinone ability for trapping the sulfur compounds [16]. The aging of the lees can have a positive effect since the mercaptans can link to the cysteinyl residues of the yeast cell wall to give disulfides [27]. Wine treatment with copper or silver showed to be affective for the removal of hydrogen sulfide as copper or silver sulfide precipitates were formed [28]. However, recent studies have indicated that copper addition post-bottling increases the level of sulfur off-flavors during aging [24, 29, 30]. The effect of copper still needs to be clarified as well as its dosages.

The sulfur-containing compounds are associated with the occurrence of light-struck taste, a defect affecting white wine bottled in clear bottles and exposed to light. The photochemical oxidations may affect different wine components including phenols, acids and alcohols [31, 32]. The formation of the light-struck taste is related to the presence of riboflavin, a vitamin highly sensitive to light and methionine. The pathway proposed by Maujean and Seguin [4] involves the photoxidative degradation of methionine to give methional and reduced riboflavin. Methional is unstable when exposed to light and decomposes to acrolein and methanethiol (**Figure 1**). Two molecules of the latter eventually yield dimethyl disulfide (DMDS). The light-struck taste is described as cooked cabbage mainly due to the formation of methanethiol and DMDS though hydrogen sulfide may have a role [33–35]. Light exposure can also lead to the formation of other undesirable compounds, such as furfural [36] which has been positively correlated to the "cooked vegetable" aroma of white wines stored under oxidative conditions [37, 38]. Not all wines are susceptible to developing this defect. The concentration of riboflavin plays the major role [5, 39] and when it is lower than 80–100 μg/L, the risk of the light-struck taste decreases. The riboflavin content in the grape hardly exceeds a few tens of micrograms per liter and the metabolic activities of *S. cerevisiae* affect its levels in wine [40, 41]. The levels of riboflavin can be reduced by means of wine treatment with bentonite or charcoal as well as by using a low producer yeast strain of this vitamin. Finally, nutrients commonly employed for favoring yeast growth could further increase the amount of riboflavin in wine [41]. Moreover, the amounts of methionine could influence the occurrence of light-struck taste as well as the presence of oxygen [42]. The light-struck taste can be limited by the addition of antioxidant compounds. The use of gallic tannins seems to be particularly promising in protecting wine possibly due to the binding of sulfur off-flavors and the quinones present in the polyphenol-based mixture [42].

2.2. Varietal thiols

The varietal thiols including 3-mercaptohexan-1-ol (3MH), 3-mercaptohexylacetate (3MHA) and 4-mercapto-4-methylpentan-2-one (4MMP) are sulfur-containing aromas associated with the typical flavor of Sauvignon Blanc wines [11, 43, 44]. Moreover, in some wine, 4-mercapto-4-methylpentan-2-ol (4MMPOH) was also detected, but its level was lower than the volatile thiols mentioned above [44] (**Table 2**). They are characterized by some fruity aromas, like cassis [45], grapefruit [46], passion fruit [47] and guava [48]. The impact of sulfur compounds on wine aroma has been updated in literature and the varietal character is affected by several of these

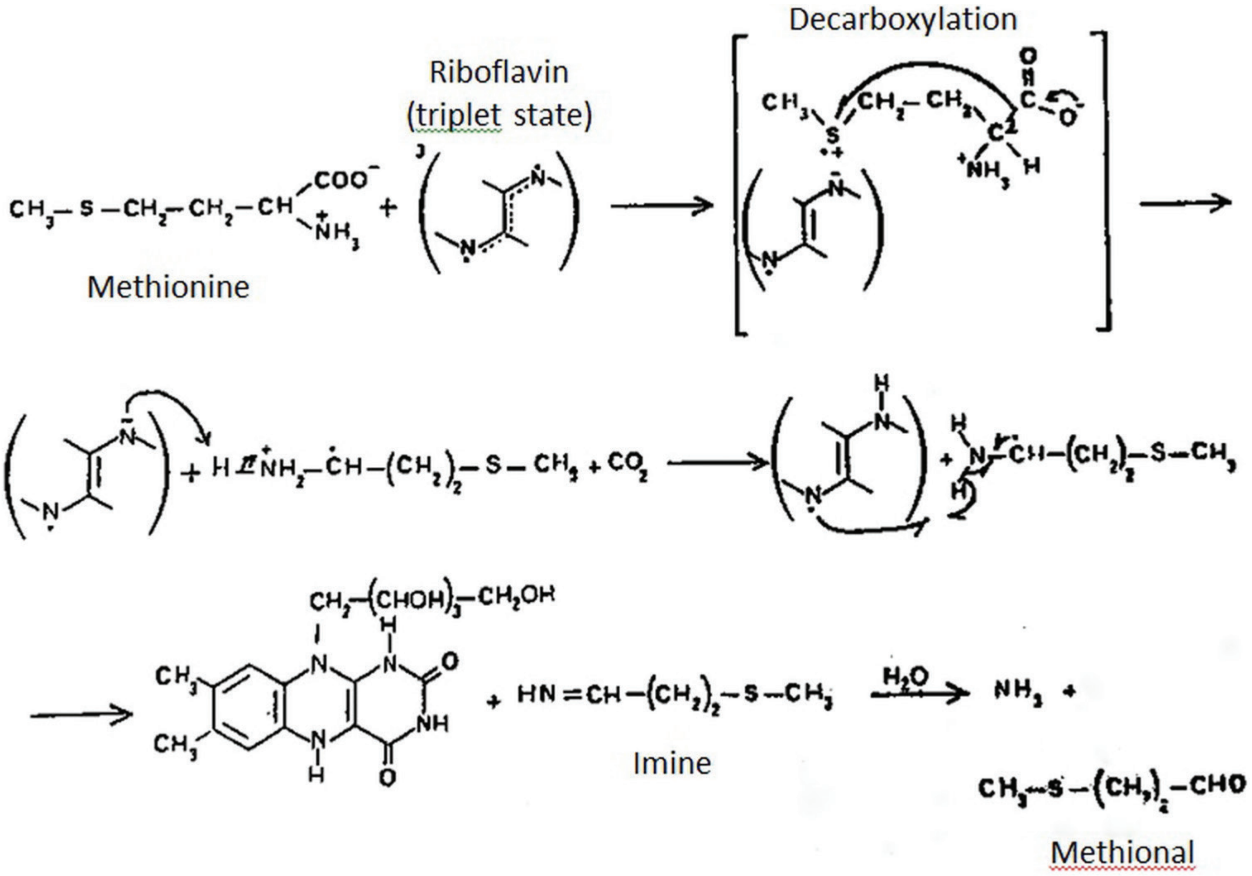
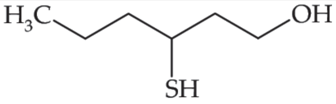
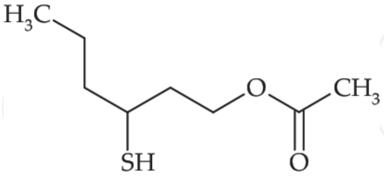
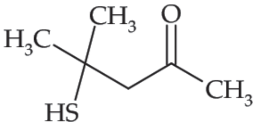
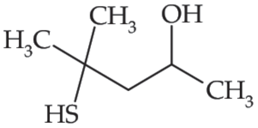


Figure 1. Reaction scheme of methional formation due to light exposure [4].

molecules if their concentration is close to the perception threshold [22, 49, 50]. 3MH and 3MHA have olfactory perception thresholds of 60 and 4 ng/L, respectively and they are responsible for passion fruit-like and grapefruit-like olfactory notes [11]. These compounds were also found in red wines [51]. The 4MMP has an olfactory perception threshold of 0.8 ng/L, and its aroma is described as box tree-like, black currant-like, or even cat urine-like when occurring at high concentration [10]. The 4MMPOH has a perception threshold of 55 ng/L in aqueous alcoholic solution and it is reminiscent of citrus zest and grapefruit [44]. 3MH is the most abundant in wine, in concentrations generally higher than its perception threshold, while 4MMPOH in wine is generally lower than its perception threshold. The level of 4MMP is dependent on the grape cultivar and it changes between different samples in the same cultivar [52]. The concentrations of 3MH and 3MHA in Sauvignon Blanc wines from different countries have been reported to be in the range 688–18,681 and 10–2507 ng/L, respectively [53–55]. Besides Sauvignon Blanc wines, the volatile thiols were detected in white wine made from different *Vitis vinifera* grape varieties, such as Gewürtztraminer, Muscat, Riesling, Sylvaner, Pinot Gris, Pinot Blanc, Colombard, Petit Manseng, botrytised Semillon and Grenache [44, 56]. Moreover, these compounds were also found in certain Italian autochthonous varieties including Verdicchio Bianco (also known as Trebbiano di Lugana) [57], Arneis [58], Grillo and Catarratto Bianco Comune [59].

The content of volatile thiols decreases during wine aging, according to the oxidative conditions. Glutathione, sulfur dioxide and anthocyanin content exert a protective effect. In contrast,

Compound	Structure	Olfactory description	Perception threshold (ng/L)[a]	Range in wine (ng/L)[b]
3-mercaptohexan-1-ol (3MH)		Passion fruit, grape fruit, guava[c]	60	26–18,000
3-mercaptohexylacetate (3MHA)		Passion fruit, grape fruit, box tree, guava[d]	4	0–2500
4-mercapto-4-methylpentan-2-one (4MMP)		Box tree, black currant, passion fruit[e]	0.8	0–40
4-mercapto-4-methylpentan-2-ol (4MMPOH)		Citrus zest[f]		0–90

[a] In model wine solution [8, 10, 11, 147].

[b] Range *Vitis vinifera* wines [11, 28, 53–55, 92, 148].

[c] [43].

[d] [8, 11].

[e] [8, 147].

Table 2. Contribution of volatile thiols in *Vitis vinifera* wines.

increased contact with oxygen, particularly in the presence of catechin derivatives, promotes their degradation [52, 60, 61]. Three different mechanisms proposed lead to their decay with a different degradation kinetics. The volatile thiols can be easily oxidized when oxygen and iron are present, forming disulfides [62, 63]. Since the volatile thiols act as electrophiles, they can react with phenolic compounds [64]. Moreover, the presence of oxygen and catalyst metals, namely iron and copper, lead the oxidation of phenols into the corresponding *o*-quinones which can bind the volatile thiols [65, 66]. A rapid decline of 3MHA is observed after 3 months of bottle storage, while a much slower decline in 3MH can be noticed. After 1 year of storage, the ester has completely disappeared, while 3MH is still present but its content is halved. The chemical structure of the volatile thiols affects their degradation: 4MMP, a tertiary thiol, results less able to react with *o*-quinones and decreases slower in comparison to 3MH, a secondary thiol, maybe due to its steric hindrance [63]. The wine composition and oxygen exposure are the major reasons for loss of volatile thiols both during winemaking and post-bottling [29, 67]. The presence of antioxidants, such as sulfur dioxide, ascorbic acid and reduced glutathione, limit the polyphenol oxidation either by removing oxygen from wine or by reversing and altering the oxidation process. In particular, the addition of glutathione, up to 20 mg/L before bottling, led to higher 3MH level after 6 months of storage in bottle [29].

2.2.1. Varietal thiol precursors

The varietal thiols occur in grape berry as non-volatile cysteinyl- and glutathionyl-conjugated precursors [29, 68]. 3MH bound as cysteinyl-conjugate (Cys-3MH), glutathionyl-conjugate (GSH-3MH) and also cysteinylglycin-conjugate (CysGly-3MH) have been reported [70, 71]. 4MMP occurs in the grape and must as cysteine conjugate (Cys-4MMP) and glutathione conjugate (GSH-4MMP) [72, 73]. Moreover, 3MH can be derived from either (E)-2-hexen-1-ol or (E)-2-hexenal [74, 75]. Through a lipoxygenase/lyase sequence active in the presence of oxygen, (E)-2-hexenal can be obtained from linolenic acid and converted to GSH-3MH by coupling with glutathione [75–77]. (E)-2-hexenal may act as a precursor when hydrogen sulfide is released in the early part of the fermentation [73, 74, 78]. Under aerobic conditions, *S. cerevisiae* is able to oxidize (E)-2-hexen-1-ol into (E)-2-hexenal, and the reverse process can occur under anaerobic conditions [77]. Recently, S-3-(hexan-1-al)-glutathione (GSH-3MHAl) was identified in Sauvignon Blanc juice [79] and it can be considered a precursor of thiol aromas. These compounds are split by the *S. cerevisiae* through the enzymatic activity of carbon-sulfur lyase [10, 80], the volatile thiols are liberated. 3MHA is obtained by yeast activity from 3MH esterified with acetic acid as precursor [7]. This finding correlated the ester and volatile thiol metabolism in yeast for the first time [2]. The capability of *S. cerevisiae* to liberate volatile thiols from their precursors is genetically determined; the yeast selection can represent a useful tool to favor the 3MH and 4MMP release [8, 50]. The fermentation temperature affects the varietal thiol concentrations in the resulting wines: in the range of alcoholic fermentation for white wines, at higher fermentation temperature and higher concentrations of varietal thiols is released. Among the *S. cerevisiae* strains commonly used, VL3 and EG8 release more volatile thiols, in comparison to VL1 and 522 [80]. *S. bayanus* var. *uvarum* strains and hybrids of the latter yeast and *S. cerevisiae* showed a higher ability to release both 3MH and 4MMP when compared to *S. cerevisiae* strain [81]. Furthermore, non-*Saccharomyces* yeasts, like *Torulaspora delbrueckii* and *Pichia kluyveri*, have been proposed as innovative tools to improve wine quality, being able to release varietal thiols [82, 83]. The 3MHA formation from 3MH is also dependent on the strain genetic characteristics [84]. *S. cerevisiae* strains showed the enhanced ability to hydrolyze the S-cysteinyl link rather than the ester synthetic activity. The combined use of different yeast strains, one of them having hydrolyzing ability, with stronger esterification ability, can represent a useful tool to affect the volatile thiol composition [50]. However, several researches have indicated that no clear correlation between precursor concentrations in must and free thiol concentration in wine exist [8, 16, 59, 73, 80, 85–89].

The harvest has a significant effect on the content of thiol precursors in juice. Capone and Jeffery [90] showed that machine harvest and transportation for 12 h of the grapes led to higher levels of thiol precursors in comparison to hand harvest grapes immediately processed. The authors suggested the minimal berry damage results in less formation of the precursors. More than half of the total cysteine conjugates is located in the grape skin; as a consequence, an increased skin contact time augments Cys-3MH in grape musts, while it has little effect on Cys-4MMP and Cys-4MMPOH concentrations [91–93]. Stronger pressing conditions allow a major extraction of thiol precursors in comparison to free run juice. Roland and coauthors [94] demonstrated an easier extraction of Cys-3MH in comparison to GSH-3MH due to their distribution in different parts of the skin. The produced wines contained 3MH at higher concentrations when the juice was collected at the end of the pressing cycle. On the contrary, Patel et al. [95] found lower amounts of 3MH and 3MHA in wine produced from juice obtained from stronger pressing conditions in which the thiol precursors were present in higher amounts. This can be due to the higher extraction of oxidizable phenols inducing the rapid decrease of glutathione and the oxidation of varietal thiols. The pressing performed on an industrial scale caused a strong decrease of thiol precursors. The factors affecting the GSH-portion degradation of the precursors are not completely clear and mechanisms, other than oxidation or proteolysis, could induce the loss of the thiol precursors [59].

3. The reduced glutathione

The reduced glutathione (GSH) is a tripeptide constituted by ʟ-cysteine, γ-glutamic acid and glycine exerting antioxidant and detoxifying activities in the cell [96, 97]. This compound exerts several activities in must and wine. The antioxidant property of GSH is well known: it can reduce the *o*-quinone deriving from the enzymatic oxidation carried out by the polyphenoloxidase enzymes (PPO) on the tartaric esters of hydroxycinnamic acids. This hinders the formation polymers causing the browning of must [12]. During aging, the *o*-quinones are produced as a result of the non-enzymatic oxidation (also known as chemical oxidation) of *o*-diphenols [98]. The level of *o*-diphenols in wine is correlated to the browning of white wines [28, 98, 99]. Caffeoyl-tartaric acid (caftaric acid) and coumaric-tartaric acid (coutaric acid) are some of the most abundant hydroxycinnamic acids in must representing the substrate mainly oxidized by the enzymatic action. The GSH can reduce the oxidized caftaric acid, generating 2-*S*-glutathionyl caftaric acid, also known as Grape Reaction Product (GRP) [100]. The GRP is not a substrate of the PPO and it can trap the *o*-quinone, limiting the formation of brown polymers which are responsible for color changes of white must and wine. GRP can oxide enzymatically by the *Botrytis cinerea* laccase and chemically by the caffeoyl-tartaric acid quinone. In this way, the GRP quinone is originated; it can be a substrate of condensation reaction with the phenols. This molecule is responsible for brown compound formation. The high GSH concentration allows a second nucleophile attack, in position 5 of the benzyl ring. The 2,5-diglutathionyl caftaric acid (GRP2) is formed and is a substrate of the laccase action [12]. With low GSH concentration, the GRP can be oxidized by the excess caftaric acid quinones which can cause an intense browning (**Figure 2**) [101, 102].

GSH is able to limit the loss of the flavoring volatile thiols acting as a competitor for the reduction of the quinones [103]. In fact, GSH concentration is about a thousand times higher than that of volatile thiols which are protected against oxidation. Lavigne and Dubourdieu [103] reported that when the GSH concentration ranged from 6 to 10 mg/L it slowed down the decrease of volatile thiols. Additionally, other aromatic compounds, such as isoamyl acetate (3-methyl-1-butyl acetate), ethyl hexanoate and linalool (3,7-dimethylocta-1,6-dien-3-ol), are better protected during bottle storage [104]. GSH can limit the formation of sotolon (3-hydroxy-4,5-dimethyl-2(5H)-furanone), a compound responsible for the atypical aging character of white wine [103]. It confers aroma descriptors such as dried fig and rancid and its perception threshold is 7 μg/L [105]. Besides sotolon, 2-aminoacetophenone (1-(2-aminophenyl)-ethanone) is also responsible for the atypical aging and it has a lower perception threshold than sotolon, corresponding to 1 μg/L. Both sotolon and 2-aminoacetophenone concentrations increase due to the exposition of wine to oxygen during bottling [106]. GSH can have a protective action on the wine aroma during the oxidative aging. GSH reduces the formation of both these off-flavors during storage. Moreover, GSH can have a positive effect on white wine color which appears to be more stable during aging [103, 107]. The first source of GSH is the grape in which it can exceed 200 mg/L of grape juice according to grape cultivar, environmental conditions and viticultural practices [108]

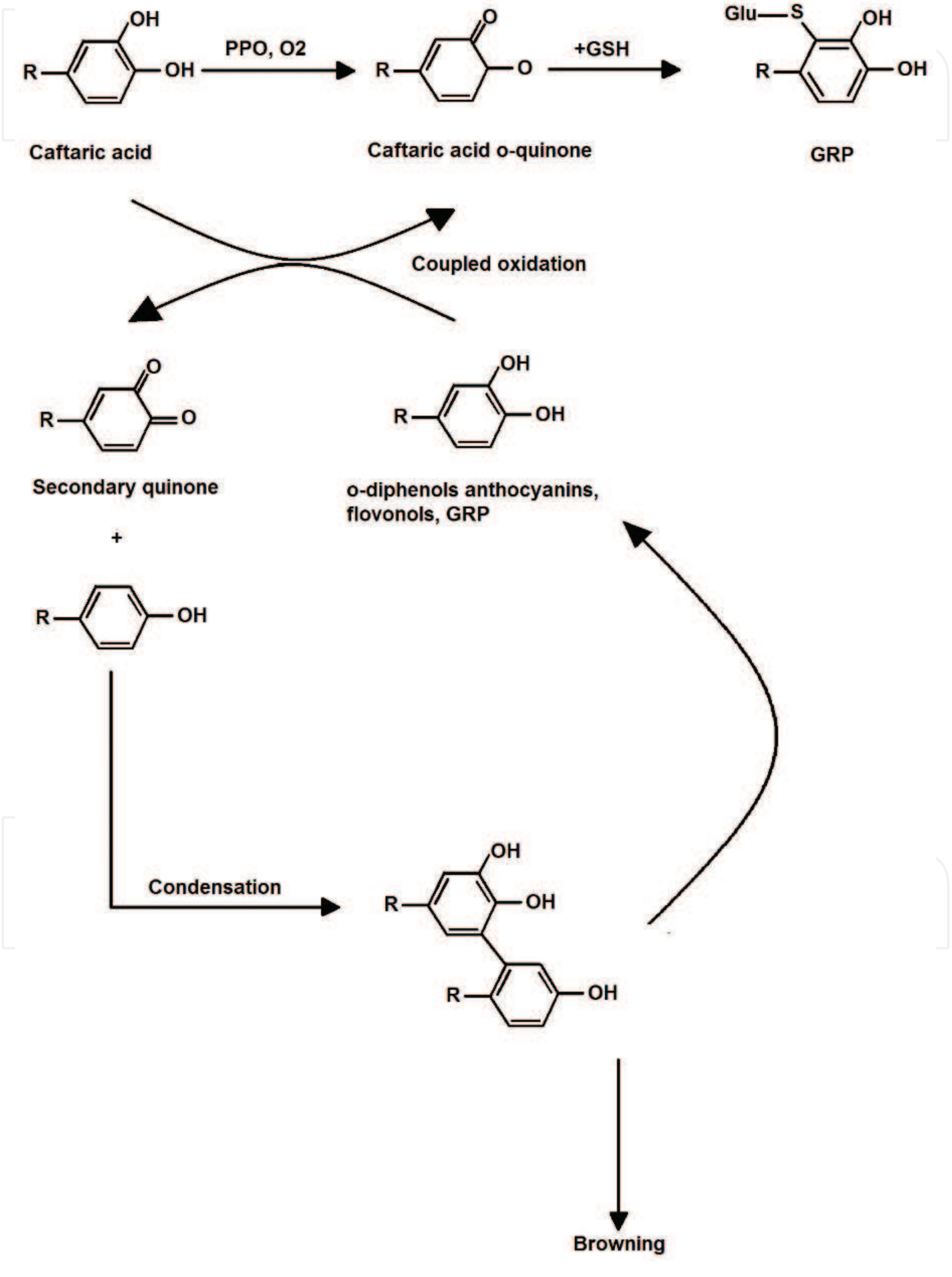


Figure 2. Scheme of GRP and browning compound formation [100–102].

and the amounts of readily assimilable nitrogen in the soil [103]. Lower levels were found in must up to 100 mg/L [108] and it can be affected by exposure to oxygen, tyrosinase activity and pre-fermentative grape skin maceration [93, 109]. The loss of GSH in must production can negatively affect the formation of precursors of the varietal thiol compounds [73] as well as the residual content of GSH during wine aging. Glutathione was reported to be consumed by *S. cerevisiae* at the beginning of the alcoholic fermentation and then to be released by the yeast cell lysis [103, 110]. At the beginning of the alcoholic fermentation GSH almost disappears and then its concentration increases as an effect of the yeast cell synthesis and lysis. However, winemaking on an industrial scale did not preserve GSH in must and the level of this antioxidant increased during the alcoholic fermentation due to the yeast metabolism [111]. Lavigne and Dubourdieu [103] reported the GSH level becomes stable 1 month after the alcoholic fermentation is started. On the contrary, in recent researches, the highest levels of GSH were observed after the racking [111, 112]. The concentration of GSH in wine is lower than in the juice and grapes and it ranges from 3 to 35.5 mg/L [109, 111–113] and can be increased through the choice of an adequate yeast strain [15]. No GSH is released from yeast under nitrogen starvation during the alcoholic fermentation [103, 111]. The GSH concentration decreased after the racking as well as during wine aging on lees [57, 95, 111] maybe due to the adsorption of GSH on the yeast lees as occurs for other low-molecular weight thiols during wine aging [114].

4. Analytical methods

The analytical methods developed for the analysis of sulfur compounds in wine need to overcome the low concentrations and the high reactivity of these molecules as well as the complexity of the matrix. Sulfur compounds in wine are frequently divided into “light” (boiling point < 90°C) and “heavy” (boiling point > 90°C) compounds [3] indicating difficulty in using a relevant common sampling/enrichment technique.

4.1. Sulfur off-flavors

The most common technique employed for the analysis of sulfur compounds associated with off-flavors in wine is static headspace analysis by means of solid phase micro extraction (SPME) combined with gas chromatography (GC) coupled with different detectors (**Table 3**). Methods in dynamic headspace by using Purge and Trap equipment was also described as an alternative to the static headspace. In static headspace technique, the analytes reach the equilibrium state between the liquid and gas phases and then are adsorbed in a fiber. In dynamic space, the analytes in the gas phase into the headspace and the atmosphere around the sample is constantly swept away by a flow of carrier gas, taking volatile analytes with it. Through this technique, the equilibrium state is not reached and, thus, more of the volatile dispersed in the matrix will pass into the headspace whose size results in increased sampling phase. As a consequence, the trapping stage of the analysis offers good sensitivity [43, 115]. The proper combination of sorbent and temperature may permit collection and concentration of specific analytes while venting others. Despite this, the instrumentation requires more complexity and it is more expensive than other sampling techniques, such as the static headspace. Moreover,

Analytical technique		Advantages	Disadvantages
Dynamic headspace	Purge and trap—gas chromatography	Increased sampling phase; good sensitivity	Many sources of errors
Static headspace	Solid phase micro extraction (SPME)—gas chromatography	Cheaper and simpler instrumentation	Compromise between sensitivity and extraction/temperature conditions needs to be properly set
SPME fiber			
	Carboxen-polydimethylsiloxane (CAR-PDMS)[a]	Suggested for sulfur compounds with low boiling point	Less versatile than CAR-PDMS-DVB fiber
	Carboxen-polydimethylsiloxane-divinylbenzene (CAR-PDMS-DVB)[b]	Best extraction yield for the analytical conditions applied	Suitable analytical method setting for increasing the extraction yield (sample temperature, ionic strength, extraction time)

[a] [117, 118].

[b] [22, 119, 120].

Table 3. Analytical methods for the determination of sulfur off-flavors.

many sources of error in Purge and Trap instruments have been reviewed [116]. As for the dynamic headspace, the choices of the proper fiber as well as sample temperature and the presence of salt increasing the ionic strength can improve extraction yield in static headspace. In particular, the use of carboxen-polydimethylsiloxane fiber (CAR-PDMS) [117, 118] or carboxen-polydimethylsiloxane-divinylbenzene (CAR-PDMS-DVB) [22, 119] has been proposed for the analysis of fermentative sulfur compounds. The use of the latter fiber has been shown to produce good results in terms of repeatability and reproducibility [22]. Moreover, the best modified ionic strength has been obtained by using magnesium sulfate. Due to the different boiling temperature of the fermentative sulfur compounds, a good compromise needs to be set to allow for the adsorption of these compounds through the SPME technique. As reported by Nguyen and co-authors [120], the incubation of the sample was carried out at 45°C for 5 min and the extraction at 45°C for 30 min under agitation. The GC equipment was coupled with MS detector and the compounds of interest were detected in single ion monitoring (SIM) mode and quantified by means of different internal standards properly chosen. The analytical methods described above are suitable for the analysis of the volatiles characterized by low boiling point (lower than 90°C) that is not the case for 3MH, 3MHA and 4MMP.

4.2. Varietal thiols

Among headspace sampling techniques, only purge and trap has been used to analyze 3MH and 3MHA in wines, reaching detection limits closer to their perception threshold [121]. Despite this, the other heavy volatile sulfur aromas were not identified at perception threshold, thus leaving derivatization procedures as the most promising technique for the extraction and analysis of volatile thiols. Due to the low concentration of these compounds, in the order of magnitude of ng/L, the sample preparation has provided a liquid/liquid extraction followed by the evaporation of the organic solvent. As the varietal thiols are present in wine in low concentration as well as being highly reactive, the deuterated analogues are commonly used as internal standards compensating for the eventual loss taking place during the sample preparation [122].

Among the derivatizing compounds, the use of *p*-hydroxymercuribenzoate (pHMB) was first proposed by Tominaga and co-authors [10]. The volatile thiols were derivatized with pHMB, isolated with the use of strong basic anion exchange column followed by the liquid/liquid extraction with dichloromethane. The sample was concentrated and analyzed by GC-MS. As a result of the analytical method improvement, varietal thiols were quantified in several wines produced with *Vitis vinifera* grape varieties [44], suggesting these sulfur compounds play a key role in the aroma of different white wines. Besides 3MH, 3MHA and 4MMP, the method allows the identification and quantification of two other sulfur-containing aromas, such as 2-furanmethanethiol [11] and benzenemethanethiol [123] in wines. The identification of these two latter molecules, together with the identification of ethyl 3-mercaptopropionate, established the role of certain volatile thiols in the bouquet of aged champagne wines [124]. Although very efficient, this procedure is time consuming. In an effort to reduce the time required for the sample preparation, a covalent chromatography was employed for the enrichment of the thiols from the wine extract. Specifically, a cross-linked agarose gel containing phenylmercuric chloride was used where the volatile thiols were trapped after their liquid/liquid extraction with dichlomethane [122]. The ability of some common solid phase extraction sorbents to retain organomercuric salts for selective concentration of thiols in wines was also proposed using styrene-divinylbenzene copolymer sorbent. Nevertheless, the organomercury salt that is formed for the detection of varietal thiols by the analytical methods described (considered as hazardous poison) still remains the key point of this method.

Other analytical approaches employ pentafluorobenzyl bromide as a derivatizing agent, which transforms thiols into their corresponding pentafluorobenzyl derivatives [125–128]. The derivatizing reaction is usually carried out in a purified extract (i.e. water) [128], organic solvent [126], in-cartridge [127], or in-fiber [125]. Another very promising derivatizing agent in the gas chromatography analysis of thiols is ethyl propiolate, which is able to derivatize thiols directly in wine matrix and is a suitable derivatizing reagent for the electron impact mass spectrometry detection system [129].

Moreover, Piano and co-authors [55] proposed an analytical method in which the varietal thiols are detected by liquid chromatography (UPLC) coupled with MS/MS. The sample preparation required several steps in order to protect the thiol aromas from oxidation and the liquid/liquid extraction in order to achieve the concentration of the analytes. The *o*-phthaldialdehyde (OPA) is used as derivatizing agent. The method allows the quantification of 3MH and 3MHA, but the derivatization of 4MMP does not occur. The formation of the OPA derivative of 4MMP was probably prevented by the hydrogen bonding between the thiol group and the carbonyl moiety within the compound itself, and its steric hindrance. Derivatization of 4MMP was an issue when other derivatizing reagents were used [127].

4.2.1. Varietal thiol precursors

The analytical methods proposed for the determination of volatile thiol precursors include both indirect and direct methods [71]. In the first case, the precursors are transformed in volatile compounds and determined by both GC and HPLC coupled with MS detector. In the second case, the precursors are quantified after their proper purification.

In the first method developed, the Cys-4MMP was detected by GC coupled with flame photometric detectors (FDP) by synthetizing it and 4MMP was then detected [69]. Tominaga and co-authors [130] quantified the cysteinylated precursors by GC-MS after their derivatization. The addition of labeled thiol precursors allowed the quantification of those contained in the must by GC-FDP and GC-MS [131]. The GC coupled either with atomic emission detection (AED) [132] or detection-capture mass spectrometry (DCMS) detector [86] were also proposed employing propyl thioacetate as internal standard and derivatizing the released volatile thiols with ethyl chloroformate, respectively. The precursors of 3MH were quantified using the labeled isotopic standard d10-3MH by GC-MS.

Both GC-MS and liquid chromatography techniques were used for the direct determination of thiol precursors. In the case of GC-MS, their derivatization is necessary and different derivatizing agents were proposed [133–135]. In the case of liquid chromatography, both HPLC-MS and HPLC-MS/MS were applied. The use of these types of equipment did not require a derivatization of thiol precursors, but a solid phase extraction (SPE) was applied achieving the sample purification. The quantification was carried out by means of the patterns of labeled compounds [73, 87, 136–139] as well as without them [59, 72]. The quantification by liquid secondary ionization mass spectrometry (LSIMS) was also reported [140].

4.3. The reduced glutathione

Several analytical methods have been proposed for GSH quantification in the grape, must and wine using different analytical techniques [141]. GSH was quantified after a treatment with glutathione reductase enzyme in white wine [113]. In grapevine tissues, the GSH was derivatized with 5,5′-dithiobis-2-nitrobenzoic acid (DTNB) after the enzymatic treatment and the detection was performed spectrophotometrically at 412 nm [142]. The determination of total GSH could be performed after the enzymatic treatment which allowed the breakdown of disulfide bridges. The GSH could be derivatized pre-column with *o*-phthalaldehyde (OPA) and quantified by HPLC; the detection was than conducted by fluorescence [143, 144]. The use of the fluorescence detector allowed the GSH quantification after derivatization with 2,3-naphthalenedialdehyde (NDA), as described by Marchand and de Revel [145]. The technique involved the pre-column derivatization and the separation by HPLC. The determination of oxidized glutathione was also allowed by enzymatic treatment with glutathione reductase. The fluorescence detector was employed for the GSH determination after its derivatization with monobromobimane (mBB); the reaction adduct was identified by capillary electrophoresis [110]. As described above, the mBB is photodegradable and a GSH content underestimation could occur [146]. The method proposed by Du Toit et al. [109] was based on liquid chromatography coupled with a mass spectrometry detector (LC-MS/MS). The determination of both reduced and oxidized glutathione was allowed in the same run. The ethanol present in wine had to be removed prior to the analysis and this step could cause an underestimation of the GSH content. Another analytical method for the GSH quantification was developed using the atomic adsorption spectrometry [147]. The method is based on the reactivity of the mercury toward the thiols. The liquid chromatography (both HPLC and UPLC) coupled with

UV detector allows the quantification of GSH after its derivatization with *p*-benzoquinone [148]. The sample preparation is not required for must and wine, while the juice needs to be obtained from grape under reductive condition prior to the derivatization [111]. Suitable preparative conditions are mandatory for monitoring GSH concentration during must extraction and clarification since such wine making steps are responsible for the chemical and enzymatic oxidations.

5. Conclusions

Several sulfur compounds occur in wine with a strong influence on its aromatic profile. On one hand, some of these molecules impart negative notes and their presence in wine should be counteracted and limited. The formation mechanisms are not completely clear and further researches are needed also to better understand the treatments and/or the winemaking practices potentially decreasing the appearance of these undesired compounds. On the other hand, the varietal thiols are responsible for positive characteristics of wine aroma up to a certain extent. Their protection is essential for the maintenance of the aromatic profile throughout both the winemaking and storage of wine by means of antioxidants of which reduce glutathione can represent a good natural candidate. Further studies will be necessary to investigate the fate of their precursors during winemaking on an industrial scale as well as to increase the aromatic potential of the produced wine. In the last decades, improvements of the analytical methods have been carried out in terms of sensitivity and identification of new odorants has been achieved. The goal for the researches has being the set up more sensitive and less-time consuming methods with a reduced impact on the environment in order to minimize defects and optimize the aromatic profile during winemaking.

Author details

Daniela Fracassetti* and Ileana Vigentini

*Address all correspondence to: daniela.fracassetti@unimi.it

Department of Food, Environmental and Nutritional Sciences (DeFENS), Università degli Studi di Milano, Milan, Italy

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