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Synthesis and Stable Isotope Dilution Assay of Ethanethiol and Diethyl Disulfide in Wine Using Solid Phase Microextraction. Effect of Aging on Their Levels in Wine

ANDREA BELANCIC MAJCENOVIC,[†] RÉMI SCHNEIDER,[‡,§] JEAN-PAUL LEPOUTRE,[‡] VALÉRIE LEMPEREUR,[§] AND RAYMOND BAUMES*[,‡]

Pontificia Universidad Catolica de Chile, 4860 Vicuña Mackena, Santiago, Chile; UMR Sciences pour l'Oenologie, INRA, 2 place Viala, 34060 Montpellier Cedex, France; and ITV France, B.P. 320, 210 Boulevard Vermorel, 69661 Villefranche-sur-Saône, France

Ethanethiol and diethyl disulfide (DEDS) most often occurred at levels above their olfactive threshold in wines with nauseous sulfur-linked smells. As ethanethiol is very oxidizable and chemically reactive, a stable isotopic dilution analysis of both ethanethiol and its disulfide in wines using solid phase microextraction and GC-MS was developed. The latter involved the determination of the proportion of DEDS formed by oxidation of the thiol during the analysis conditions, which was obtained by the use of two differently labeled disulfide standards. An original synthesis of labeled ethanethiol standards in conditions minimizing oxidation was developed, and the corresponding labeled diethyl disulfides were obtained from these thiols. This analytical method was used to follow the levels of these sulfur compounds during aging in a young red wine spiked with ethanethiol and added with enological tannins, with or without oxygen addition. The total levels of these two sulfur compounds were shown to decrease steadily after 60 days of aging, up to 83%. The effect of oxygen sped this decrease, but the effect of enological tannins was very slight. Residual ethanethiol was detected in its disulfide form from ~36% in the nonoxygenated wines to 69% in the oxygenated samples.

KEYWORDS: Wine; aging; aroma; stable isotope dilution assay; sulfur compounds; SPME; ethanethiol; diethyl disulfide; tannin

INTRODUCTION

Sulfur-containing compounds generally exhibit intense aroma properties due to their extremely low odor thresholds. Depending on their levels in beverages and foods they contribute favorably to the aroma or to off-flavor. Among the volatile sulfur compounds of wine, those mostly associated with nauseous sulfur-linked smells are hydrogen sulfide, carbon disulfide, methanethiol, and ethanethiol, as well as the sulfides, polysulfides, and thioacetates derived from these thiols (*1*, *2*). Hydrogen sulfide has a very nauseous smell of rotten egg; during yeast fermentation it accumulates at concentrations very superior to its olfactive perception threshold, but due to its high volatility, it is rapidly eliminated from the fermentation medium (*3*, *4*). Thus, among those sulfur-related off-odors occurring most frequently in wines, mostly dimethyl sulfide and ethanethiol and, to a lesser extent, methanethiol and diethyl disulfide (DEDS) were detected at levels above their olfactive perception threshold (*5*, *6*). Whereas the influence of dimethyl sulfide on the aroma of wine can be perceived positively (*7*, *8*), that of methanethiol, ethanethiol, and their oxidized disulfide forms is generally negatively perceived (*9*, *10*).

Primary thiols are highly reactive chemical species. On the one hand, they are very susceptible to oxidation, with the major oxidation products being disulfides; on the other hand, they are soft nucleophiles, susceptible to reaction with a lot of electrophilic carbons occurring in wines (*11*−*16*). Therefore, these sulfur compounds were susceptible to transformation during enrichment for their quantitative determination. Several analytical methods for analyzing them in wines were reported previously (*17*, *18*), but few paid enough attention to this problem (*15*, *19*). As shown by Mestres et al. (*17*), solid phase microextraction (SPME) appeared to be the most convenient sampling technique to analyze volatile sulfur compounds in wine. Indeed, this solvent-free technique, requiring minimum sample handling, should minimize their possible chemical modification during the sampling period. However, according to these authors (*17*), the method was less satisfactory for the more volatile and oxidizable sulfur compounds. Similarly, Murray (*20*) demonstrated limitations to the use of SPME for quantification of mixtures of volatile sulfur compounds, as the relative proportions of these components adsorbed onto the fiber depended on their ratio in the mixture. Furthermore, level variations in wines during aging are poorly understood (*13*, *21*).

* Author to whom correspondence should be addressed (telephone 33 0499612228; fax 33 0499612857; e-mail baumes@ensam.inra.fr).
[†] Pontificia Universidad Catolica de Chile.
[‡] INRA.
[§] ITV France.

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Some constituents of wine, such as tannins, could possibly fix these compounds, which will result in a lower negative olfactive impact; this effect could be increased by the addition of enological tannins with or without oxygen addition (*22*, *23*).

In this study, ethanethiol and its disulfide were chosen as models to develop a stable isotope dilution assay (SIDA) for the quantitative determination of these thiols and their disulfides, taking into account their interconversion. This method was used to follow during aging the levels of these compounds spiked in a young red wine, with or without addition of enological tannins and oxygen.

MATERIALS AND METHODS

Chemicals and Other Materials. Dichloromethane (ultrapure grade) and the ethanethiol used in the aging experiments were obtained from Merck, Darmstadt, Germany, and absolute ethanol (99.8%) was from Carlo Erba, Val de Reuil, France. Carbon disulfide, triphenylsilanethiol (98%), triethylamine (99.5%), 2-fluoro-1-methylpyridinium *p*-toluenesulfonate (95%), ethylenediaminetetraacetic acid (EDTA, 99.5%), sodium chloride (98%), [2,2,2-$^2H_3$]ethanol (99+ atom % D), [$^2H_6$]-ethanol (99+ atom % D), the Carboxen−polydimethylsiloxane (CAR−PDMS, 75 μm) fibers, and the SPME manual holder (Supelco, Bellefonte, PA) were purchased from Sigma-Aldrich (St Quentin Fallavier, France). All glassware was cleaned by washing with acetone, alcohol, and dichloromethane, followed by oven baking at 100 °C prior to use.

Natural and Model Base Wine. To obtain the model base wine, 3.5 g of tartaric acid was added to 120 mL of ethanol and 800 mL of water, and the pH of the mixture was adjusted to 3.5 with 1 M aqueous sodium hydroxide. The volume of the solution was then adjusted to 1 L.

An experimental Syrah red wine from Languedoc, made at the pilot scale in the "Institut Cooperatif du Vin" (ICV, Lattes, France) according to standard wine-making procedure, was used for the aging experiments. At the end of malolactic fermentation, 10 μg/L of ethanethiol (purchased from Merck) was added to this wine. Then it was stored in 1 L polyethylene flasks with or without treatment with six different enological tannins T1−T6 (200 mg of tannin added to 1 L of wine). The botanical origins of tannins T1−T6 were, respectively, grape seeds, grape skins, oak apples, quebracho wood, oak wood, and chestnut wood. The flasks were securely stoppered and kept at 18 °C during the aging in the ICV. The air treatment was carried out every other week by removing from the flask one-third of the wine, which was then put back in the flask. After aging, the flasks were transferred to INRA (Montpellier), where they were kept at 4 °C until analysis.

Gas Chromatography Coupled with Mass Spectrometry (GC-MS). GC-MS analysis was carried out using a Hewlett-Packard gas chromatograph 5890 series II fitted with a 60 m fused-silica column (0.32 mm i.d. and 1.0 μm film thickness), coated with DB5 (J&W Scientific). The injector temperature was held at 300 °C throughout the analysis. Transfer of the sample to the GC-MS column was accomplished by keeping the SPME fiber for 1 min in the heated chromatograph injector.

The carrier gas was helium 6.0 (Linde gaz, Marseille), with a flow rate of 1.5 mL/min. The oven temperature program was 30 °C (for 3 min), increased at 1 °C/min to 45 °C, held at this temperature for a further 3 min, then increased at 20 °C/min to 250 °C, and held at this temperature for 5 min. The GC instrument was coupled to a Hewlett-Packard 5989A mass selective detector and an MS chemstation. The transfer line was heated at 250 °C. The electron impact (EI) energy was 70 eV, and the MS source and quadrupole temperatures were set at 250 and 120 °C, respectively. EIMS spectra were recorded in the range of 30−350 amu at 0.5 s intervals.

The following ions in the full-scan mass spectra of the sulfur compounds were used for quantification: for ethanethiol, *m*/*z* 62; for [$^2H_5$]ethanethiol, *m*/*z* 67; for DEDS, *m*/*z* 122; for [$^2H_6$]diethyl disulfide, *m*/*z* 128; for [$^2H_{10}$]diethyl disulfide, *m*/*z* 132.

Gas Chromatography Coupled with Atomic Emission Detection (AED). After synthesis, for each dichloromethane solution of ethanethiol, [$^2H_3$]ethanethiol, [$^2H_5$]ethanethiol, DEDS, [$^2H_6$]diethyl disulfide, and [$^2H_{10}$]diethyl disulfide, an aliquot was diluted with ethanol in a flask under nitrogen and the levels of each compound were quantified by GC-AED monitored on sulfur-selective acquisition using a solution of carbon disulfide (10 μL at 2.5 g/L) in dichloromethane as internal standard. The system consisted of an HP 5890 series II GC equipped with an HP 7673A automatic sampler and coupled to an HP 5921A atomic emission detector. The GC conditions were the same as above for GC-MS, with the difference that the extracts were injected on-column. The injector temperature was held at 30 °C for 1 min, then increased at 180 °C/min to 200 °C, and held at this temperature throughout the analysis. Two microliters of the extracts was injected in the GC. The temperatures of the AED were as follows: inlet temperature, 250 °C; transfer line, 250 °C; and cavity block, 290 °C. Element-selective chromatograms were obtained for carbon- and sulfur-containing compounds (emission wavelength at 193.03 and 181.40 nm, respectively). Helium was used for the plasma at 4.16 bar. The reagent gas was oxygen at 1.73 bar and hydrogen at 4.85 bar. The spectrometer was purged using ultrapure nitrogen 5.0 Norme Aga at 1.4 bar. The discharge tube was water cooled to 65 °C.

Calibration Curves. A model base wine was used to obtain the calibration curves. They were plotted for the target compounds, ethanethiol and DEDS, as well as [$^2H_{10}$]diethyl disulfide. Serial dilutions of aliquots of the ethanol solutions of these analytes in 15 mL of a model base wine were made separately under nitrogen in a 22 mL septum-sealed glass vial used for SPME. Then the corresponding labeled internal standard was added to each diluted solution. The calibration curves were obtained from these solutions by SPME analysis (see below) coupled to GC-MS.

Ethanethiol. Peak area ratios (peak area of the ion *m*/*z* 62/peak area of the ion *m*/*z* 67) were plotted against the concentration ratios (ng of ethanethiol/390 ng of [$^2H_5$]ethanethiol) for the following ethanethiol concentrations: 19.5, 48.8, 195, 390, and 780 ng. The resultant curve was linear [response ratio = (1.066 × concentration ratio); $R^2 = 0.987$].

DEDS. Integrated peak area ratios (peak area of ion *m*/*z* 122/peak area of *m*/*z* 128) were calculated and plotted against the concentration ratios (ng of DEDS/90 ng of [$^2H_6$]diethyl disulfide) for the following DEDS concentrations: 31.8, 63.75, 127.5, 255, and 510 ng. The resultant curve was linear [response ratio = (0.9355 × concentration ratio); $R^2 = 0.988$].

[$^2H_{10}$]Diethyl Disulfide. Integrated peak area ratios were calculated and plotted against the concentration ratios (ng of [$^2H_{10}$]diethyl disulfide/90 ng of [$^2H_6$]diethyl disulfide) for the following concentrations: 22.5, 45, 90, 180, and 360 ng. The resultant curve was linear [response ratio = (0.9798 × concentration ratio); $R^2 = 0.992$].

Isolation of Volatile Sulfur Compounds from Wines Using SPME. A 75 μm CAR−PDMS fiber was used as reported previously by Mestres et al. (*17*). Before each extraction, the SPME fiber was conditioned at 280 °C for 30 min as prescribed by the supplier. Sodium chloride (1.75 g) and 0.08 g of EDTA were put under nitrogen into a 22 mL septum-sealed glass vial used for SPME, and then 15 mL of wine was transferred into the vial using a syringe. The wine was spiked with [$^2H_5$]ethanethiol and [$^2H_6$]diethyl disulfide (see levels in **Tables 1**−**3**) as internal standards, and it was stirred at 500 rpm. Then the SPME fiber was introduced through the septum into the vial and exposed to the headspace at room temperature for 30 min. The desorption was carried out immediately after in the heated chromatograph injector of the GC-MS system.

Reproducibility Study. One hundred milliliters of the Syrah red wine was spiked with 250 μL of a stock solution containing either 6.5 μg/mL of ethanethiol or 0.4 μg/mL of DEDS, respectively, and a series of five analysis was carried out using the method described above.

Synthesis of Ethanethiol, [$^2H_3$]Ethanethiol, and [$^2H_5$]Ethanethiol. 2-Fluoro-1-methylpyridinium tosylate (567 mg; 98%, 1.9 mmol), 0.28 mL of triethylamine, and 4 mL of dichloromethane were placed at room temperature and under nitrogen atmosphere into the Claisen flask of a distillation apparatus. To this suspension was added dropwise with a syringe a solution of 0.12 mL of ethanol (2.1 mmol) in 1 mL of dichloromethane, and the mixture was stirred for 1 h. Then a solution of 585 mg of triphenylsilanethiol (95%, 1.9 mmol) in 2 mL of dichloromethane was added with a syringe into the Claisen flask, and

Table 1. Precision Assay for Ethanethiol and Diethyl Disulfide Spiked in a Syrah Red Wine[a]

analysis no., $n = 5$	ethanethiol level (μg/L) measured[b]	DEDS level (μg/L) measured[b]	DEDS level (μg/L) corrected[c]	analytical oxidation percentage[d]
mean	15.5	1.15	1.10	0.24
SD[e]	1.6	0.07	0.05	0.12
%CV[e]	10.4	5.9	4.7	51.0

[a] 16.2 μg/L ethanethiol and 1 μg/L diethyl disulfide. [b] 15 μL of a stock solution containing 32.2 μg/mL [$^2H_5$]ethanethiol and 1 μg/mL [$^2H_{10}$]diethyl disulfide and 15 μL of a stock solution containing 6 μg/mL [$^2H_6$]diethyl disulfide were added as standards to 15 mL of wine. [c] Level of DEDS measured minus the level of DEDS formed from ethanethiol by oxidation during the analysis (see text). [d] Molar percentage calculated from [$^2H_{10}$]diethyl disulfide formed from [$^2H_5$]ethanethiol by oxidation during the analysis (see text). [e] SD, standard deviation; %CV, variation coefficient.

the stirring at room temperature under nitrogen atmosphere was continued overnight. Then the receiver was placed in an ice−water bath and the Claisen flask in an oil bath heated to ∼50−60 °C for distilling the ethanethiol formed together with dichloromethane. The distillation was stopped when ∼4.5 mL of liquid was obtained in the receiver, which was then closed under nitrogen with a septum and kept at −20 °C. [$^2H_3$]Ethanethiol and [$^2H_5$]ethanethiol solutions in dichloromethane were obtained using the same procedure starting from [$^2H_3$]-ethanol and [$^2H_6$]ethanol, respectively. The yields, obtained by quantitation of these solutions using GC-AED, ranged from 5 to 15%. The corresponding diethyl disulfides were detected as secondary products from 3.6 to 6.1% w/w relative to the thiols: EI-MS (70 eV), *m*/*z* (%) 62 (100), 61 (20), 47 (55), 29 (97) for ethanethiol; *m*/*z* (%) 65 (100), 64 (16), 47 (54), 32 (76) for [$^2H_3$]ethanethiol; *m*/*z* (%) 67 (100), 66 (6), 49 (52), 34 (80) for [$^2H_5$]ethanethiol.

Synthesis of DEDS, [$^2H_6$]Diethyl Disulfide, and [$^2H_{10}$]Diethyl Disulfide. Two milliliters of the ethanethiol solution in dichloromethane obtained as described above was diluted with 2 mL of dichloromethane and added with 4 mL of aqueous sodium hydroxide (32% solution). A fine stream of oxygen was passed through the mixture stirred at room temperature for 24 h. The flow of oxygen introduced was low, so that the stripping of the organic phase was low. Then the organic layer containing the DEDS formed was added with dichloromethane to have a concluding volume of 4 mL, separated, and kept at −20 °C. [$^2H_6$]-Diethyl disulfide and [$^2H_{10}$]diethyl disulfide solutions in dichloromethane were obtained using the same procedure starting from [$^2H_3$]ethanethiol and [$^2H_6$]ethanethiol, respectively. The yields, obtained by quantitation of these solutions using GC-AED, ranged from 80 to 94%. EI-MS (70 eV), *m*/*z* (%) 122 (100), 94 (52), 66 (68) for DEDS; *m*/*z* (%) 128 (100), 98 (41), 97 (13), 68 (34), 67 (33) for [$^2H_6$]diethyl disulfide; *m*/*z* (%) 132 (100), 100 (57), 68 (71) for [$^2H_{10}$]diethyl disulfide. No thiol was detected in these solutions.

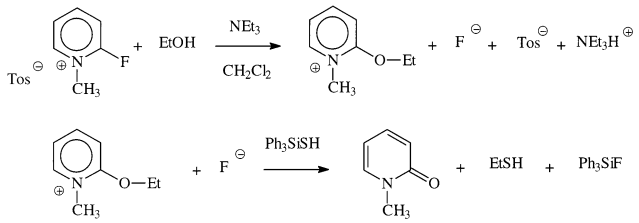

Figure 1. Synthesis of ethanethiol from ethanol and triphenylsilanethiol.

RESULTS AND DISCUSSION

Preparation of standard solutions of thiol compounds is a very difficult task due to their high volatility and oxidizability (*24*). Increased problems were foreseable when labeled analogues of these compounds were desired, which needed further elaboration. To solve these problems, we developed a method to prepare directly from labeled and unlabeled ethanol standard solutions of the corresponding ethanethiols in an indifferent atmosphere.

Synthetic Standard Solutions. For the preparation of thiols from alcohols, the utilization of suitable combinations of onium salts of azaarenes and sulfur-containing nucleophiles has been reported (*25*). To synthesize ethanethiol and its labeled analogues, we chose the same onium salt, but we used triphenylsilanethiol as sulfur source. This reagent was reported previously as a solid hydrogen sulfide equivalent in the ring opening of epoxides (*26*). This combination proved to be suitable for the development of a two-step synthesis and purification (distillation), carried out one-pot in an inert atmosphere to avoid any contact with air. The first step consisted of the activation of the starting material, ethanol, with 2-fluoro-1-methylpyridinium tosylate to give the 2-ethoxy-1-methylpyridinium salt and the fluoride anion (**Figure 1**). The key step of the synthesis consisted of the S_N2 type reaction of the pyridinium salt with the sulfhydryl anion generated in situ from triphenylsilanethiol by reaction with the fluoride anion liberated in the first step (**Figure 1**). Using this procedure, the deuterated ethanethiols were obtained from the corresponding deuterated ethanols. As the labeling of the synthesized ethanethiols was obtained from the starting material, this synthesis could be used to obtain ^{13}C-labeled ethanethiol from more expensive ^{13}C-labeled ethanols. The concentrations of the distilled ethanethiol solutions were determined by GC-AED (sulfur detection) using carbon disulfide

Table 2. Statistical Treatment (Student Test) of the Effects of Time Aging, Aeration, and Addition of Enological Tannins on the Levels of Ethanethiol and Diethyl Disulfide[a] in a Red Wine Initially Spiked with 10 μg/L Ethanethiol

		ethanethiol			DEDS		
aging time (days)	aeration treatment	without tannins[b]	with tannins[c]	tannins effect	without tannins[b]	with tannins[c]	tannins effect
30	without	5.6 (0.2)[d]	6 (1.4)	ns[e]	3.2 (0.2)	3.4 (0.5)	ns
	with	2.6 (0.6)	2.2 (0.5)	ns	2.2 (0.8)	2.7 (0.2)	ns
	aeration effect	$p < 0.1\%$[e]	$p < 1\%$		$p < 5\%$	$p < 5\%$	
60	without	1.6 (0.4)	1.0 (0.2)	ns	1.1 (0.1)	1.2 (0.2)	ns
	with	0.9 (0.2)	0.6 (0.3)	ns	0.9 (0.1)	1.1 (0.1)	ns
	aeration effect	ns	ns		$p < 1\%$	ns	
aging effect	without	$p < 0.1\%$%	$p < 0.1\%$		$p < 0.1\%$	$p < 0.1\%$	
	with	$p < 1\%$	$p < 1\%$		ns	$p < 0.1\%$	

[a] 15 μL of a stock solution containing 26 μg/mL of [$^2H_5$]ethanethiol and 1.2 μg/mL of [$^2H_{10}$]diethyl disulfide and 15 μL of a stock solution containing 4.4 μg/mL of [$^2H_6$]diethyldisulfide were added to 15 mL of wine as standards for SPME. [b] Three samples for each aging time and each aeration treatment were analyzed ($n = 3$). [c] Six samples treated with 200 mg/L of enological tannins Ti (each one added with only one enological tannin) for each aging time and each aeration treatment were analyzed ($n = 6$). [d] Mean levels (μg/L) and 95% confidence intervals (in parentheses) of the sulfur compounds in the sampes analyzed ($n = 3^b$ or $n = 6^c$). [e] Level of significance of the Student test; ns = not significant.

Table 3. Statistical Treatment (Student Test) of the Effects of Time Aging, Aeration, and Tannins Addition on the Oxidation Percentage of Ethanethiol[a]

aging time (days)	aeration treatment	without tannins[b]	with tannins[c]	tannins effect
30	without	36 (2)	37 (4)	ns
	with	46 (1)[d]	55 (4)	$p < 1\%$
	aeration effect	$p < 0.1\%$[e]	$p < 0.1\%$	
60	without	42 (5)	55 (8)	ns
	with	49 (7)	69 (15)	ns
	aeration effect	ns[e]	ns	
aging effect	without	ns	$p < 1\%$	
	with	ns	ns	

[a] The oxidation percentage of ethanethiol was defined as the weight percentage of DEDS level relative to the total level of ethanethiol and DEDS calculated from the levels shown in **Table 2**. [b,c] See the corresponding footnotes in **Table 2**. [d] Mean level (%) and 95% confidence intervals (in parentheses) of the oxidation percentage of ethanethiol in the samples analyzed ($n = 3$[b] or $n = 6$[c]). [e] See the corresponding footnote in **Table 2**.

as internal standard. The interest of this detection system was that the response of a compound was proportional to the number of sulfur atoms giving rise to the signal (*27*). Despite the care being taken to prevent oxidation, the corresponding diethyl disulfides were obtained as secondary products (from 3.6 to 6.1% w/w relative to ethanethiol as assessed by GC-AED), showing the high oxidizability of ethanethiol. The labeling of the prepared [$^2H_3$]ethanethiol and [$^2H_5$]ethanethiol was consistent with the molecular ions at *m*/*z* 65 and 67, respectively, and the fragment ions at *m*/*z* 32 ($CD_3CH_2^+$) and 34 ($C_2D_5^+$), respectively, in their mass spectrum, showing the occurrence of three and five deuterium atoms in their molecule.

The corresponding labeled and unlabeled diethyl disulfides were easily obtained by oxidation with oxygen in two-phase basic conditions of the ethanethiol solutions obtained as described above. The concentrations of the DEDS solutions were determined by GC-AED using carbon disulfide as internal standard. As the residual thiols were trapped in the basic aqueous phase, no trace of ethanethiol was detected in the DEDS solutions. The labeling of the prepared [$^2H_6$]diethyl disulfide and [$^2H_{10}$]diethyl disulfide was consistent with the molecular ions at *m*/*z* 128 and 132, respectively, and the fragment ions at *m*/*z* 98 ($CD_3CH_2SSD^+$) and 100 ($C_2D_5SSD^+$), respectively, in their mass spectrum, showing the occurrence of 6 and 10 deuterium atoms in their molecules.

Analytical Method. Mestres et al. (*28*, *29*) showed that SPME with CAR−PDMS fibers was a satisfactory sampling technique of thiols and disulfides in wine. We used this technique with the parameters optimized by these authors to sample ethanethiol and DEDS. The conditions of the chromatographic analyses used were not much different from those reported by these authors, the major difference being that no cryogenic trap was used. However, to circumvent the problems reported by Murray (*20*) and those related to the volatility and chemical reactivity of thiols, a SIDA was used. Indeed, labeled internal standards have physicochemical properties very similar to those of their natural analogues, which should correct the fluctuations related to the method.

SIDA Assay Using GC-MS. The deuterated analogues of the target sulfur compounds, [$^2H_5$]ethanethiol and [$^2H_6$]diethyl disulfide, were obtained as described above and used to determine separately the calibration curves of natural ethanethiol and DEDS, respectively. It has to be noted that [$^2H_5$]ethanethiol is not the reduced form of the deuterated DEDS standard used. It was chosen as it was partly oxidized during its synthesis and quite likely during the analysis, as well as natural ethanethiol.

Indeed, the basic principle of SIDA is that the labeled standard and the analyte have very similar behaviors toward all factors that influence their analysis. However, when the analyte can be generated during the analytical process from precursors occurring in the same matrix, its quantitative determination will be excessive, irrelevant to its natural level, as the labeled standard cannot have any precursor but the ones with natural isotopic abundances, which are negligible (especially with more than three deuteriums). In our experiments, ethanethiol can be considered a precursor of DEDS, as it could be oxidized to this compound during the analytical process. Therefore, [$^2H_5$]-ethanethiol was used not only as the labeled standard for ethanethiol but also as the labeled analogue of the precursor of DEDS to monitor its oxidation. To quantitate [$^2H_{10}$]diethyl disulfide formed from [$^2H_5$]ethanethiol oxidation, it was necessary to synthesize it to determine its calibration line versus [$^2H_6$]-diethyl disulfide used as labeled standard.

Thus, it was possible to correct the SIDA quantitative determination of natural DEDS by taking into account the oxidation of ethanethiol during the analysis process. Hence, assuming equal oxidizability of the different isotopomers of ethanethiol and considering negligible other reactions generating ethanethiol or DEDS during the analytical process, the oxidation reaction in the samples can be written

$$3\text{EtSH} + 3[^2H_5]\text{EtSH} \rightarrow \text{DEDS} + [^2H_5]\text{DEDS} + [^2H_{10}]\text{DEDS} \quad (1)$$

Thus, the molar analytical oxidation percentage %OX of the ethanethiol isotopomers was

$$\%\text{OX} = (\text{mol of oxidized EtSH})/(\text{mol of initial EtSH}) \quad (2)$$

$$\%\text{OX} = (3[\text{DEDS}]_{ox}/122)/(2[\text{EtSH}]/62) \quad (3)$$

$$\text{or } \%\text{OX} = (\text{mol of oxidized } [^2H_5]\text{EtSH})/(\text{mol of initial } [^2H_5]\text{EtSH}) \quad (4)$$

$$\%\text{OX} = (3[[^2H_{10}]\text{DEDS}]_{ox}/132)/(2[[^2H_5]\text{EtSH}]/67) \quad (5)$$

where $[\text{DEDS}]_{ox}$ is the concentration of DEDS formed from ethanethiol in reaction 1, [EtSH] is the initial concentration of ethanethiol occurring in the sample and measured using [$^2H_5$]-EtSH as labeled standard, and $[[^2H_{10}]\text{DEDS}]_{ox}$ is the concentration of [$^2H_{10}$]diethyl disulfide formed from [$^2H_5$]EtSH in reaction 1. It was equal to the amount measured using [$^2H_6$]diethyl disulfide as labeled standard minus the level of this compound occurring as impurity in the standard solution of [$^2H_5$]ethanethiol used; [[$^2H_5$]EtSH] is the initial concentration of [$^2H_5$]ethanethiol added to the sample as labeled standard, and 122, 62, 132, and 67 are the molecular weights of DEDS, EtSH, [$^2H_{10}$]DEDS, and [$^2H_5$]EtSH, respectively.

Hence, %OX was calculated from eq 5, and this value was used in eq 3 to calculate $[\text{DEDS}]_{ox}$. Thus, the corrected DEDS level, $[\text{DEDS}]_{corr}$, is

$$[\text{DEDS}]_{corr} = [\text{DEDS}]_{meas} - [\text{DEDS}]_{ox} = [\text{DEDS}]_{meas} - 2/3 \times 122 \times \%\text{OX} \times [\text{EtSH}]/62$$

where $[\text{DEDS}]_{meas}$ is the concentration of DEDS measured using [$^2H_6$]diethyl disulfide as labeled standard.

GC-MS in full-scan mode was used to detect the compounds desorbed from the SPME fiber used. It was sensitive enough to detect the target compounds and their labeled analogues in the concentration ranges of our study, which were above the natural levels normally occurring in wines without off-flavor. Thus, the identity of the sulfur compounds detected was easily confirmed, whereas the reconstructed ion chromatograms using one characteristic ion for each one were used for their quantitative determination. The detection limits for the ions chosen in this method were, respectively, 0.3 μg/L for ethanethiol and 0.05 μg/L for DEDS with an estimated signal-to-noise ratio of 3:1 for the Syrah red wine analyzed in this study. These values showed that the response of DEDS to the SPME fiber was much higher than that of ethanethiol, which was consistent with the results reported by Mestres (*29*) and those reported for the methyl analogues by Murray (*20*). They were in the range of those reported by Mestres using a flame photometric detection and lower than the olfactive perception thresholds of these compounds. Thus, this method seems to be adequate for analyzing these sulfur compounds in wines thought to have sulfide-like odor problems (*5*). The repeatability and recovery were determined by analyzing with the same SPME fiber five identical samples of the same red wine spiked with 16.2 μg/L ethanethiol and 1 μg/L DEDS (**Table 1**). As the extent of ethanethiol oxidation during the analysis was low, the correction of the DEDS level for this source of error was very slight, but it improved the coefficient of variation from 5.9 to 4.7%. Although relatively high for ethanethiol, these coefficients of variation were lower than those reported for the previous method (*29*) and showed the interest of coupling SPME sampling with SIDA. As observed in these repeatability experiments, the extent of ethanethiol oxidation during the analysis of the samples of the aging experiments (see below) was low, ranging from 0.1 to 1.5% and from 0.4 to 3.4% in the 30- and 60-day samples, respectively. These results showed that SPME minimized chemical modification of thiols during the sampling and supported the findings of the previous study by Mestres et al. (*29*), which emphasized the interest in using SPME to analyze sulfur compounds in wine.

Analysis of Ethanethiol and Its Disulfide in Wines during Aging: Effect of the Addition of Enological Tannins with or without Aeration. A young Syrah red wine spiked with 10 μg/L ethanethiol was chosen as a model of wine exhibiting sulfur-linked off-flavors. This wine was treated with or without addition of enological tannins from six different botanical origins and aged with or without aeration. The composition of the enological tannins used was not analyzed, but their general composition and properties were reviewed by Vivas (22). The analytical method described above was used to measure the levels of ethanethiol and DEDS during aging. That could provide an assessment of the possible extent of the removal of volatile thiols and disulfides by tannins and other wine components under different oxidative storage.

The means of the levels of ethanethiol and DEDS measured in these samples after 30 and 60 days of storage at 18 °C are shown in **Table 2**, in which the single samples treated with the different enological tannins but with the same aeration and aging treatments were pooled. Statistical treatments were performed to investigate the cause for the differences observed. On the whole, the effect of aging between 30 and 60 days was to decrease significantly the levels of these two sulfur compounds, the only aging effect not significant being observed between the DEDS levels in the aerated samples without tannins (2.2 vs 0.9 in **Table 2**). The effect of aeration appeared to speed this decrease, as the levels observed in the aerated samples were significantly lower than those in the nonaerated samples at 30 days of aging, whereas they were in the same range as those observed in the aerated samples after 60 days of storage (**Table 2**). These results were consistent with those reported previously on solutions of enological tannins in a model wine (*22*). That was typical for an induction period, longer in the nonaerated samples than in the aerated ones, which could be related to nonenzymatic, radical chain mechanisms for the removal of ethanethiol, involving problably oxidation products such as quinones (*30*−*32*). The six enological tannins used had different compositions (*22*), but their individual effects on the levels of ethanethiol and DEDS in the tannin-treated samples could not be statistically tested as only single samples were available. However, when all of the tannin-treated samples were pooled (**Table 2**), statistical treatments showed that the effect of tannins addition on the sulfur compounds levels was not significant, contrary to the aeration and aging effects. That showed that the native polyphenols in wine (or other wine components) were as efficient as the enological tannins added to remove ethanethiol and DEDS under aging conditions.

On the other hand, the oxidation ratios of residual ethanethiol (**Table 3**) were higher in the aerated samples than in the nonaerated ones, which was consistent with its oxidizability, but the differences observed were statistically significant only at 30 days of aging. Furthermore, these oxidation ratios were higher in the 60-days-aged samples than in the corresponding 30-days-aged ones, but the increase was significant only between the tannin-treated samples without aeration (37 vs 55% in **Table 3**). Indeed, between 30 and 60 days the levels of DEDS decreased regularly while those of ethanethiol decreased sharply (**Table 2**). Contrary to its very weak effect on the levels of both sulfur compounds, tannins addition increased the oxidation ratios of residual ethanethiol, but this effect was statistically significant only at 30 days in the aerated samples.

As reported previously (*11*−*13*, *22*, *30*−*32*), these results could be rationalized by assuming that when oxygen was present, the oxidation of ethanethiol to its disulfide was fast as well as that of polyphenols to quinones, accelerating the ethanethiol removal. When wine has consumed the dissolved oxygen, the oxidation reaction of ethanethiol could be reversed by reducing agents of wine (sulfite ions, glutathion, etc.), but this process would be much slower, as demonstrated for sulfite ions (*13*). Due to its high reactivity, ethanethiol would be trapped by wine electrophiles such as quinones much more quickly, resulting in apparent oxidation ratio increase. However, as discussed previously (*13*−*16*), the removal of ethanethiol and its disulfide could involve the formation of other sulfur compounds, such as trisulfides in the presence of metals, mixed disulfides of ethanethiol with other wine thiols, or ethanethiol adducts involving other wine components with soft electrophilic carbons. These reactions could generate sulfur-linked smells under aging, despite volatile thiols and disulfides elimination. Thus, the best way to get rid of these off-flavors would be to limit their formation during wine-making.

ABBREVIATIONS USED

GC, gas chromatography; MS, mass spectrometry; EI, electronic impact; SIDA, stable isotope dilution assay; AED, atomic emission detection; SPME, solid phase microextraction; EDTA, ethylenediaminetetraacetic acid; CAR−PDMS, carboxen−polydimethylsiloxane; S_N, nucleophilic substitution; DEDS, diethyl disulfide.

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