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ARTICLE

Selective Determination of Volatile Sulfur Compounds in Wine by Gas Chromatography with Sulfur Chemiluminescence Detection

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Volatile sulfur compounds can be formed at various stages during wine production and storage, and some may impart unpleasant "reduced" aromas to wine when present at sensorially significant concentrations. Quantitative data are necessary to understand factors that influence the formation of volatile sulfur compounds, but their analysis is not a trivial undertaking. A rapid and selective method for determining 10 volatile sulfur-containing aroma compounds in wine that have been linked to "off-odors" has been developed. The method utilizes static headspace injection and cool-on-column gas chromatography coupled with sulfur chemiluminescence detection (GC-SCD). Validation demonstrated that the method is accurate, precise, robust, and sensitive, with limits of quantitation around 1 μg/L or better, which is below the aroma detection thresholds for the analytes. Importantly, the method does not form artifacts, such as disulfides, during sample preparation or analysis. To study the contribution of volatile sulfur compounds, the GC-SCD method was applied to 68 commercial wines that had reductive sensory evaluations. The analytes implicated as contributors to reductive characters were hydrogen sulfide, methanethiol, and dimethyl sulfide, whereas carbon disulfide played an uncertain role.

KEYWORDS: Gas chromatography; sulfur chemiluminescence detection; wine aroma; volatile sulfur compounds; headspace analysis; wine storage

INTRODUCTION

Over 700 volatile compounds are known to contribute to wine aroma and flavor (*1*−*5*), including compounds containing one or more sulfur atoms. Volatile sulfur compounds can be formed by biological and chemical mechanisms at various stages during wine production and storage and often have low aroma detection thresholds (from ng/L to μg/L range). Although there are many sulfur compounds that can potentially be of significance to wine aroma and flavor, we focused this study on volatile sulfur compounds found after fermentation that are relevant to "reduced" aromas and "off-odors". Typically, these were the more potent, low molecular weight and low boiling point sulfur compounds, along with related acetates and disulfides. There is a great deal that remains to be understood about these compounds, most notably their impact on negative perceptions of wine aroma and the effects of closure type and storage conditions on their formation and stability.

A number of low molecular weight volatile sulfur compounds are known to impart unpleasant "reduced", "onion", "asparagus", "burnt rubber", or "garlic" aromas to wine (*2*, *6*−*9*). Distinctive "off-odors" can be attributed to specific compounds, such as "rotten egg" from hydrogen sulfide (H_2S) (*2*, *7*), "putrid", "garlic", or "onion" from methanethiol (MeSH) (*7*, *10*), and "(canned) corn" or "(cooked) asparagus" from dimethyl sulfide (DMS) at higher concentrations (*6*, *7*, *11*). Despite these negative associations, at lower levels DMS can give a pleasant "black currant" aroma and has been shown to enhance fruity notes in the presence of other volatile wine components (*12*−*14*). Other volatile sulfur compounds, such as carbon disulfide (CS_2), which may be thought of as negative contributors to wine aroma, are not necessarily so at lower concentrations (*12*). Even H_2S may contribute to the bouquet of a young wine and add complexity to wine aroma at low levels at which it is not perceived as a fault (*15*, *16*). **Table 1** shows aroma descriptors and detection thresholds for 10 volatile sulfur compounds previously found in wine that are generally regarded as causing problems or faults, especially when present at high concentrations.

H_2S, the most volatile sulfur compound being considered, is used by yeasts to make sulfur-containing amino acids and small peptides (e.g., cysteine, cystine, methionine, glutathione) that are important in yeast cell metabolism and growth (*19*). It is well established that yeast strain can be a major factor in H_2S accumulation during fermentation (*19*−*21*). As such, a large amount of H_2S can be produced during grape must fermentation, along with other organic sulfur compounds (*22*). Elemental sulfur, sulfate, or sulfite can be reduced by yeast to produce H_2S, with its production also affected by factors such as juice clarity and must nutrients, particularly yeast assimilable nitrogen (YAN) (*15*, *23*−*26*). Formation of H_2S may also occur due to the

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Table 1. Aroma Descriptors and Detection Thresholds for 10 Volatile Sulfur Compounds Previously Found in Wine

compound		odor descriptor[a]	threshold (μg/L)
hydrogen sulfide	H_2S	rotten egg, sewage-like, vegetal	1.1 −1.6 (*16*)[b]
methanethiol	MeSH	rotten cabbage, burnt rubber, putrefaction	1.8 −3.1 (*17*)
ethanethiol	EtSH	onion, rubbery, burnt match, sulfidy, earthy	1.1 (*6*)
methyl thioacetate	MeSAc	sulfurous, cheesy, egg	50[c](*18*)
ethyl thioacetate	EtSAc	sulfurous, garlic, onion	10[c](*18*)
dimethyl sulfide	DMS	*black currant*,[d] cooked cabbage, canned corn, asparagus	25 (*6*)
diethyl sulfide	DES	garlic, rubbery	0.9 (*6*)
carbon disulfide	CS_2	*sweet, ethereal, slight green*,[d] rubber, sulfidy	>38 (*12*)
dimethyl disulfide	DMDS	vegetal, cabbage, intense onion-like	29 (*6*)
diethyl disulfide	DEDS	onion	4.3 (*6*)

[a] In-house and ref *8*. [b] Literature source. [c] In beer. [d] At low levels.

presence of metals and metal cations (iron, zinc, manganese, etc.) (*24*, *27*). Production of excess H_2S can potentially lead to the formation of other sulfur-containing compounds, such as MeSH and ethanethiol (EtSH) and their acetates (*15*, *25*). Although some undesirable sulfur compounds may be removed from wine through copper fining, this approach is not effective for sulfides, disulfides, and thioacetates and is limited to H_2S and thiol removal (*15*, *25*). An unwanted side effect of copper fining may be the formation of disulfides and trisulfides exhibiting additional undesirable aromas (*25*, *28*), as well as problems associated with wine instability (*29*) and additional wine-processing logistics.

Fermentation aside, debate remains in the wine industry regarding the propensity of volatile sulfur compounds to form under closures of differing oxygen transfer rates. It could be inferred that, from a finite pool of sulfur compounds at bottling, equilibria may form between thiols, disulfides, and thioacetate esters and other wine matrix components, resulting in the release of potent sulfur compounds during storage under low-oxygen permeable closures (*30*−*33*). In addition to the complexity of wine thiol chemistry, other changes to volatile sulfur compounds may be evident. During model studies of wine aging, the concentration of DMS has been shown to increase to potentially unpleasant levels depending on its release from precursor compounds (*34*) and availability of YAN during fermentation (*35*). The delayed effect on DMS accumulation and the rerelease of thiols demonstrate that fermentation and processing effects can still manifest themselves some time after bottling, when closure type also plays a critical role. Clearly, the ability to monitor volatile sulfur compounds easily and in a timely manner is therefore of great utility in undertaking such fermentation and storage studies.

Due to the volatile nature, reactivity, and relatively low abundance of these sulfur compounds in fermented beverages, specialized techniques are required for their determination. Volatile sulfur compounds have been quantified in wine using solid-phase microextraction (SPME) followed by gas chromatography−pulsed flame photometric detection (GC-PFPD) (*36*, *37*), GC−flame photometric detection (GC-FPD) (*38*, *39*), GC−mass spectrometry (GC-MS) (*13*, *40*), and GC−atomic emission detection (GC-AED) (*41*). Unfortunately, sampling of such compounds by SPME may suffer from matrix effects (*37*, *39*), and artifact formation or sample losses upon injection (*38*, *42*, *43*) can give potentially spurious results. Furthermore, H_2S in particular may be less reliably determined using SPME methods (*36*, *37*) and is often missing from analyses employing this technique. In contrast to wine sampling by SPME, other methods involved static headspace or purge and trap techniques along with GC-FPD (*7*, *44*, *45*), GC-AED (*35*, *46*, *47*), and GC−sulfur chemiluminescence detection (GC-SCD) (*48*). Despite the existence of methods for the analysis of volatile sulfur compounds in wine, there is still a need for a rapid, accurate, and reliable method involving minimal sample preparation so large numbers of samples can be analyzed relatively quickly.

In this paper we describe the development and validation of a novel method for volatile sulfur compound analysis using static headspace−cool-on-column (HS-COC) GC-SCD, with ethylmethyl sulfide (EMS) and propyl thioacetate (PrSAc) as internal standards. The method enables the quantitation of 10 volatile sulfur compounds in wine, with its utility demonstrated through application to studies relevant to wine fermentation and storage.

MATERIALS AND METHODS

Materials. Reference standards of ethanethiol (EtSH, 99.7%), dimethyl sulfide (DMS, 99.8%), diethyl sulfide (DES, 99.3%), dimethyl disulfide (DMDS, 99.8%), diethyl disulfide (DEDS, 99.9%), carbon disulfide (CS_2, 99.9%), and ethylmethyl sulfide (EMS, 96.0%) were of the highest purity as supplied by Sigma-Aldrich (Castle Hill, NSW, Australia). *S*-Methyl thioacetate (MeSAc, 98.8%), *S*-ethyl thioacetate (EtSAc, 99.5%), and propyl thioacetate (PrSAc, 99.7%) were of the highest purity obtainable from Lancaster Synthesis (Jomar Bioscience, Kensington, SA, Australia). The remaining chemicals listed below were of analytical reagent grade quality or better. Sodium hydrosulfide hydrate ($NaSH \cdot xH_2O$, 74.0%), sodium thiomethoxide (NaSMe, 101.8%), and potassium hydrogen tartrate (Fluka) were supplied by Sigma-Aldrich and Merck tartaric acid and sodium chloride (NaCl) were obtained from Rowe Scientific (Lonsdale, SA, Australia). BDH acetaldehyde was supplied by Thermo Fisher (Scorseby, VIC, Australia), Ajax Finechem ethylenediaminetetraacetic acid disodium salt (disodium EDTA) was supplied by Rowe Scientific, ethanol (99.5%, Rowe Scientific) was redistilled in-house prior to use, and water was obtained from a Milli-Q purification system (Millipore, North Ryde, NSW, Australia). Fermentation-related alcohols, acids, and esters were obtained from Sigma-Aldrich.

All solvents and analytical standards were verified for purity by GC-MS and GC-AED or GC-SCD prior to use. EtSH, DMS, DES, DMDS, DEDS, CS_2, EMS, MeSAc, EtSAc, and PrSAc were stored at −20 °C to prevent degradation. Containers of $NaSH \cdot xH_2O$ and NaSMe were sparged with nitrogen and stored in a desiccator at room temperature.

Wine Samples. Commercial, bottled wines of various vintages (2004−2008) and varieties (40 red wines and 28 white wines) were obtained from Australian wine producers. The wines were thought to have "reductive" characters on the basis of preliminary sensory assessments by winemakers, wine judges, and trained sensory panelists during informal evaluations.

Preparation of Standard Solutions. *Stock Standard Solutions of Volatile Sulfur Compounds.* Individual stock standard solutions of EtSH, DMS, DES, DMDS, DEDS, CS_2, EMS, MeSAc, EtSAc, and PrSAc were prepared by injecting 100 μL of neat standard into 50.0 mL of ethanol contained in a 125 mL Sure-Seal bottle (Sigma-Aldrich) that had been crimp-capped and sparged with nitrogen. The density for each reference standard was used to calculate the actual concentration (approximately 2 g/L), and the solutions were stored at −18 °C for up to 24 months except for EtSH, which was stored for only 6 months.

Global Standard Solution of Volatile Sulfur Compounds. A global standard solution of known concentration (with DMS, MeSAc, and

Table 2. Concentrations of Fermentation-Related Volatiles Added to the Model Wine Used for Calibration Purposes

compound	mg/L	compound	mg/L
ethyl esters		**acids**	
ethyl acetate	20	acetic acid	40
ethyl lactate			
		propanoic acid	
ethyl propanoate		2-methylpropanoic acid	8
ethyl 2-methylpropanoate		butanoic acid	
ethyl butanoate		octanoic acid	
ethyl 2-methylbutanoate			
ethyl 3-methylbutanoate	2	2-methylbutanoic acid	
ethyl hexanoate		3-methylbutanoic acid	
ethyl octanoate		hexanoic acid	2
ethyl decanoate		decanoic acid	
ethyl dodecanoate			
		alcohols	
acetates		2-methylpropanol	
2-methylpropyl acetate		butanol	
2-methylbutyl acetate		2-methylbutanol	
3-methylbutyl acetate	2	3-methylbutanol	20
hexyl acetate		hexanol	
2-phenylethyl acetate		2-phenylethanol	

EtSAc each at approximately 50 mg/L and CS_2, DES, DMDS, and DEDS each at approximately 12.5 mg/L) was prepared by adding, via syringe, aliquots of each stock standard solution of DMS, DES, DMDS, DEDS, CS_2, MeSAc, and EtSAc into a capped and nitrogen-sparged 125 mL Sure-Seal bottle containing 60.0 mL of ethanol. The solution was stored at −18 °C for up to 6 months.

Dilute Standard Solution of EtSH. A dilute standard solution containing a known concentration of EtSH (approximately 10 mg/L) was prepared by adding, via syringe, 600 μL of the EtSH stock standard solution into a capped and nitrogen-sparged 125 mL Sure-Seal bottle containing 100.0 mL of ethanol. The solution was stored at −18 °C for up to 3 months.

Stock Standard Solution of NaSH (for H_2S) and NaSMe (for MeSH). Due to the impracticality of working with gaseous H_2S and MeSH, a suitable alternative employed the sodium salts of these analytes, which were dissolved in cold water (4 °C) and used immediately. Individual stock solutions of known concentration (approximately 300 mg/L) were prepared in amber volumetric flasks. The concentrations of NaSH and NaSMe were calculated using the purity reported in their respective certificates of analysis (Sigma-Aldrich).

Dilute Standard Solution of NaSH (for H_2S) and NaSMe (for MeSH). Individual dilute standard solutions of known concentration containing NaSH or NaSMe (approximately 7.5 mg/L) were prepared in cold water (4 °C) in 200 mL amber volumetric flasks and used immediately.

Internal Standard Mix. An internal standard solution containing known concentrations of EMS (approximately 20 mg/L) and PrSAc (approximately 50 mg/L) was prepared in an amber volumetric flask by diluting the respective stock standard solutions with ethanol. The internal standard solution was stored at 4 °C for 3 months.

Preparation of Model Wine. Aqueous ethanol (12% v/v) was saturated with potassium hydrogen tartrate, and the pH was adjusted to 3.2 with tartaric acid solution (40% w/v). Fermentation-derived volatiles (ethyl esters, acetates, alcohols, and fatty acids) were added to approximate the concentrations commonly found in wine (**Table 2**).

Sample Preparation. Wine samples were cooled to 4 °C in their original containers prior to opening, and all sample handling was completed in a temperature-controlled room at 4 °C. An aliquot of wine (10 mL) was added to a 20 mL amber glass headspace vial containing 2 g of NaCl and a 3 × 8 mm magnetic stir bar. Internal standard solution (25 μL) was added to give known final concentrations of EMS (approximately 50 μg/L) and PrSAc (approximately 125 μg/L). Acetaldehyde (4 μL) was added to each white wine sample vial. The vial was tightly sealed with a white PTFE/blue silicone lined screw cap (Grace Davison Discovery Sciences, Baulkham Hills, NSW, Australia).

Instrumentation. *Gas Chromatography*. The samples were analyzed using an Agilent 6890 gas chromatograph (Forest Hill, VIC, Australia) equipped with a Gerstel multipurpose sampler (MPS 2XL, Lasersan Australasia, Robina, QLD, Australia) and coupled to either an SCD or AED. Instrument control and data analysis were performed with Agilent GC ChemStation software, rev. B.03.01 and Maestro software integrated version 1.3.3.51/3.3. The gas chromatograph was fitted with a 15 m × 0.25 mm FactorFour VFWAXms fused silica capillary column, 0.50 μm film thickness (Varian, Mulgrave, VIC, Australia) connected with a fused silica universal straight connector (Grace Davison Discovery Sciences) to a 60 m × 0.25 mm VICI ValcoBond VB-5 fused silica capillary column, 0.50 μm film thickness (Chromalytic Technology, Boronia, VIC, Australia), with a 2 m × 0.53 mm retention gap. Helium (Air Liquide ultrahigh purity), linear velocity = 37 cm/s, flow rate = 2.7 mL/min in constant flow mode, was used as the carrier gas. The initial oven temperature was held at 5 °C for 5 min, increased to 150 at 5 °C/min, and held at this temperature for 5 min. The cool-on-column (COC) inlet (Agilent G3440A) (pressurized to 252.69 kPa) was held at 30 °C for 10 min and ramped at the same rate as the oven. The oven and COC inlet were cryogenically cooled with liquid nitrogen.

Sulfur Chemiluminescence Detection. An Agilent 355 SCD sulfur chemiluminescence detector coupled to the GC was used with the default SCD parameters recommended by Agilent and sulfur trap gas purifiers on all gas lines (Agilent). The detector base temperature was held at 200 °C and the Dual Plasma Controller at 800 °C. The reagent gases were air (Air Liquide instrument grade), 60.0 sccm; hydrogen (Air Liquide ultrahigh purity), 45.0 sccm; and ozone, generated in situ from air at 41.37 kPa.

Atomic Emission Detection. An Agilent G2350A atomic emission detector coupled to the GC was used with AED parameters optimized for sulfur sensitivity. The AED cavity block and the transfer line were held at 250 °C. Helium (Air Liquid ultrahigh purity with SAES PS2GC50 heated getter) was used for the microwave-induced plasma at a flow rate of 25 mL/min, measured at the cavity vent. Oxygen, 379.21 kPa (Air Liquide ultrahigh purity), and hydrogen, 68.95 kPa (Air Liquide ultrahigh purity), were used as the reagent gases. Sulfur (181 nm) and carbon (193 nm) emission lines were monitored. The discharge tube was cooled with water at 65 °C, and the spectrometer was constantly purged with nitrogen at 400 mL/min.

Peak Identification. Analytes were identified by comparison of their retention times with those of the corresponding pure reference compounds. Due to the detectors employed, all peaks necessarily arose from sulfur-containing compounds, and analyte identification was unambiguous.

Headspace (HS) Equipment and Conditions. The refrigerated sample vials were placed into a Gerstel peltier cooled sample tray (Lasersan) at 4 °C. The vial and its contents were heated to 45 °C for 30 min with stirring at 400 rpm. A Gerstel 1.0 mL HS syringe (Lasersan) was fitted with a custom-made dual gauge cone-tip needle (0.47 mm/0.63 mm, SGE, Ringwood, VIC, Australia), and the syringe heating block was held at 60 °C. A 100 μL static HS sample was injected into the COC inlet at 10 μL/s. The syringe was purged to atmosphere with nitrogen at 10.34 kPa (BOC grade 3.5) for 3 min after injection.

Validation. Method precision and calibration linearity were validated by a series of standard addition experiments to model, white, and red wine matrices. Method linearity was determined for nine calibration levels, in duplicate, over the concentration ranges of 0.2−100 μg/L for H_2S, MeSH, EtSH, CS_2, DES, DMDS, and DEDS, and 1.0−400 μg/L for DMS, MeSAc, and EtSAc. Method precision was determined in all matrices using seven replicate samples spiked at low and high concentrations (5 and 20 μg/L for all analytes except DMS, MeSAc, and EtSAc, which were 50 and 200 μg/L). For quantifying the analytes in batches of unknown samples, duplicate standards (0 and 50 μg/L for all analytes except DMS, MeSAc, and EtSAc, which were 0 and 200 μg/L) were prepared using model wine and analyzed with every set of samples. To check the accuracy of the analysis, duplicate control samples, spiked with 10 μg/L for all analytes except DMS, MeSAc, and EtSAc, which were spiked at 50 μg/L, were included with every set of samples to be quantified, along with blanks. Control samples were prepared in red or white wine with known low levels of the analytes. For red and white wine matrices, 10 mg of disodium EDTA was added to all standard addition, precision sample, and control sample vials.

Statistical Analysis. The results reported for validation of the method were derived from the average of duplicate measurements for each

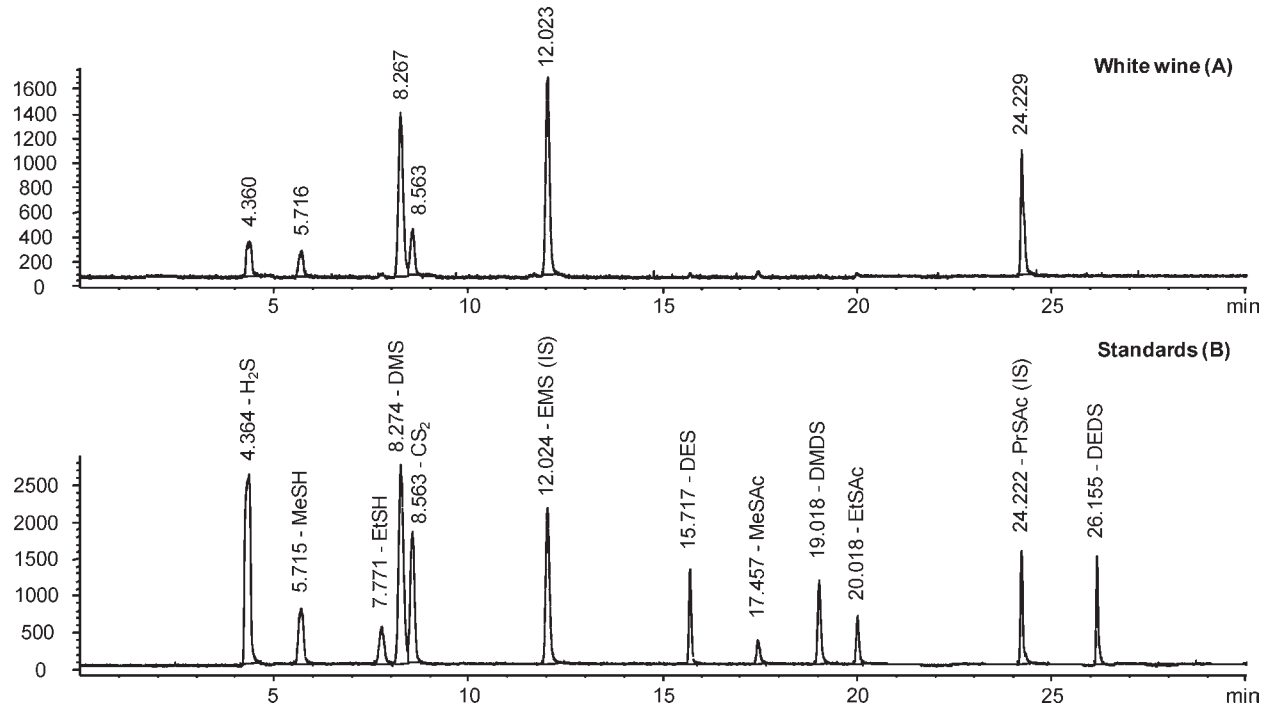

Figure 1. Typical GC-SCD chromatograms of (**A**) a white wine and (**B**) reference standards in a model wine. Analyte abbreviations appear in **Table 1**, and IS refers to internal standards ethylmethyl sulfide (EMS) and propyl thioacetate (PrSAc).

concentration of the analyte (seven replicates for repeatability samples). The limit of detection (LOD) was determined by establishing the minimum level at which the analytes could be reliably detected from the analysis of samples with known analyte concentrations. The limit of quantitation (LOQ) was determined by establishing the minimum level at which the analytes could be quantified with acceptable accuracy from the analysis of samples with known analyte concentrations.

RESULTS AND DISCUSSION

Method Development. The initial instrumentation setup consisted of HS-COC-GC-AED that selectively monitored sulfur (181 nm) and carbon (193 nm) (*47*). The COC inlet and cryogenically cooled GC oven were chosen to overcome the challenges faced in the analysis of inherently reactive and thermally labile sulfur compounds. The analytical method for the determination of fermentative sulfur compounds was then optimized over a period of time as new technologies and instrumentation were acquired. This continual improvement has increased sample throughput to cope with demand because of the widespread application of this analysis to many facets of wine research, particularly winemaking and wine shelf life studies. Method validation was completed using the SCD after incorporation of all optimized parameters.

Optimization of Headspace Sampling. Static HS injection into a COC inlet was chosen because it overcomes some of the limitations of SPME techniques and hot injector temperatures. A wide-bore retention gap, long enough to easily accommodate the injected HS volume, was used, and care was taken to optimize the speed of injection. If the injection rate is too fast, then analytes will be lost backward through the septum purge vent. Therefore, the HS must be injected at a speed slower than the column flow (i.e., < 2.7 mL/min). With manual injection, we found 10 μL/s was a convenient speed, and various injection volumes (10, 25, 50, 60, 70, 75, and 100 μL) were investigated. It was found that a volume of 70 μL was the most reproducible without loss of peak shape (data not shown). However, when using the MPS 2 (autosampler) in HS mode, the smallest volume syringe available was 1 mL, with a minimum injection volume of 100 μL. Fortuitously, an automated HS injection of 100 μL showed chromatography similar to that of a 70 μL manual injection. Higher automatic injection volumes (150, 200, and 250 μL) were investigated, but the chromatography deteriorated for the early eluting compounds (data not shown). Peak broadening was evident for H_2S, MeSH, EtSH, CS_2, DMS, and EMS, which worsened with each increase in injection volume, resulting in complete loss of resolution between CS_2 and DMS.

The optimal incubation time was investigated by comparing identical spiked cask white wine samples, nominally containing only trace amounts of DMS and CS_2, after varying the length of time that samples were incubated (10, 20, 30, 40, 50, and 60 min) with and without stirring. Only two incubation temperatures were investigated (35 and 45 °C) because of the thermal lability of the analytes. We found that equilibrium of the analytes between the headspace and wine was reached relatively quickly and reproducibly by stirring for 30 min at 45 °C. The optimization of the headspace parameters was undertaken using the original GC-AED system, and no major changes were made to sample preparation or sample introduction when the analytical method was further developed for use with GC-SCD.

Chromatographic Resolution. To achieve the separation of the 10 volatile sulfur compounds listed in **Table 1** by GC, a combination column similar to that used by Hill and Smith (*49*) was chosen with 15 m of a polar phase column connected to 60 m of a nonpolar phase column (VB-5). Originally, a SGE SolGel wax column was used as the polar phase, but using the FactorFour wax column enabled the cryogenic oven function to be utilized more effectively, allowing the GC oven temperature program to begin at 5 °C compared to 30 °C for the SolGel wax phase. The cooler oven start provided much better peak shape for MeSH and adequate resolution between EtSH, DMS, and CS_2 (**Figure 1**).

The COC inlet was held at 30 °C during headspace injection and maintained at that temperature until the oven temperature program also reached 30 °C. The COC inlet temperature ramp program then matched the oven program. The inlet could not be

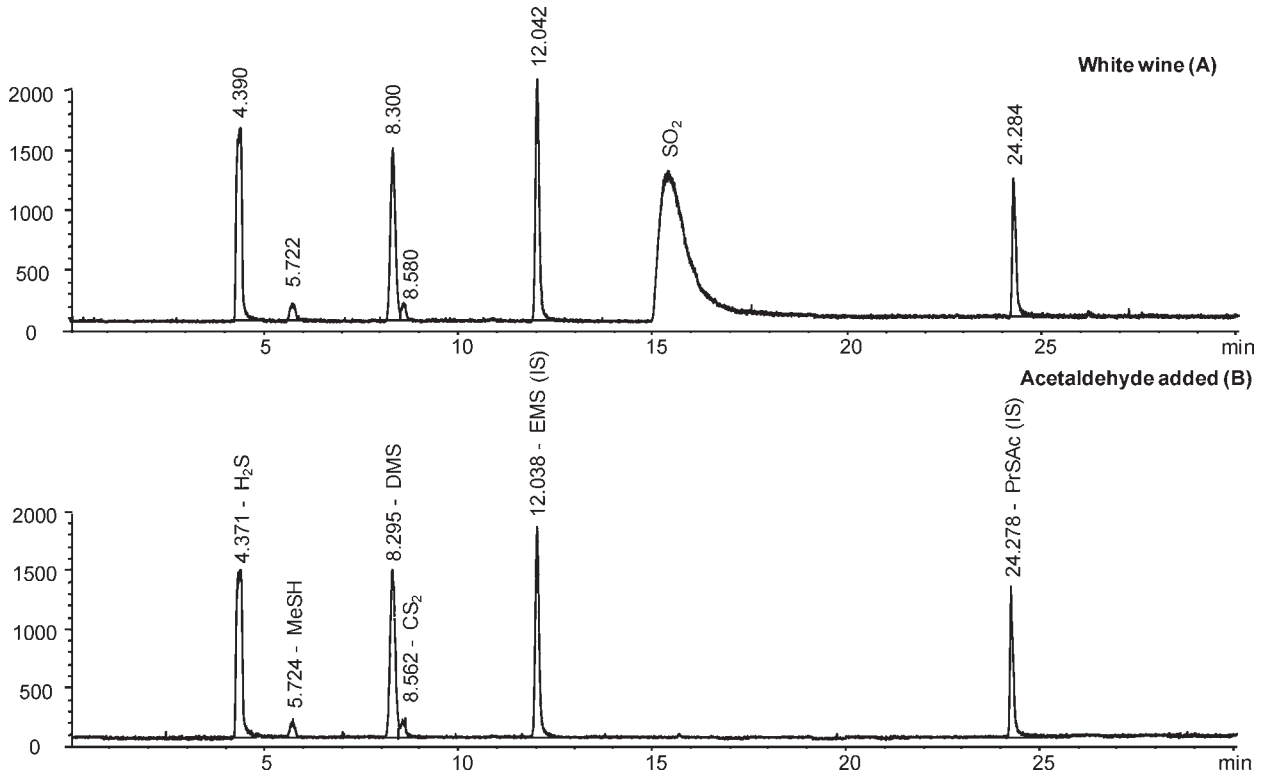

Figure 2. GC-SCD chromatograms of a white wine (**A**) without acetaldehyde addition and (**B**) with addition of acetaldehyde to bind free SO_2.

held at a lower temperature, for example, 5 °C, because the analytes were cold trapped in the syringe needle during the injection process and only partial transfer of the analytes into the inlet ensued.

Checking Artifact Formation. High incubation, injector, or oven program temperatures used in other methods may contribute to the formation of disulfides or the breakdown of thioacetates (*38*, *42*, *43*). Although our method enabled the use of cooler injector and oven temperatures, we checked that no artifacts (such as disulfides) were produced in the GC injector block or the GC column using our new approach. When individual reference standards were sampled and injected under the method conditions, only the single peaks expected for the analytes were observed. Furthermore, we employed a cooled sample tray that maintained at 4 °C the samples awaiting incubation, to prevent analyte degradation in the sample vial.

Analyte Detection. Although the AED was a very selective and sensitive detector for analyzing sulfur compounds, in our experience it was not very cost-effective and suffered from reliability issues. With the novel combination of sample introduction to the GC and column choice providing acceptable resolution and peak height, another sulfur-specific detector was sought. FPD has been utilized by others (*38*, *39*), but drawbacks of this system include nonlinear detector response and hydrocarbon quenching. Alternative forms of detection were PFPD and SCD, which have been shown to be more sensitive than FPD (*36*, *48*, *49*). Perhaps of most relevance to our situation was the finding that SCD was equivalent to AED for sulfur detection sensitivity, although the AED had a linear dynamic range 1 order of magnitude higher (*50*). On the basis of an evaluation of the available options, an Agilent 355 SCD was identified as the most suitable replacement for the AED. We have since found the SCD to be sensitive and selective, more economical, and easier to operate and maintain than the AED. In contrast to Hill and Smith, who reported a loss in sensitivity after analysis of one or two samples using an SCD (*49*), our SCD has proven to be very stable throughout hundreds of injections, with only minor routine maintenance required for optimum performance. In addition, the interference from air that affected H_2S resolution with the AED does not occur with the SCD (data not shown).

Internal Standards, Acetaldehyde Addition, and Stock Solutions. For accurate quantitation labeled internal standards, which are ideal for GC coupled with MS, were an obvious choice to investigate. However, preliminary work showed there was insufficient resolution of labeled and unlabeled compounds, preventing quantitative determination in this manner. With rigorous validation of the analytical method using SCD, we confirmed that EMS and PrSAc were suitable internal standards for the determination of sulfur compounds in wine, in accord with previous studies employing these internal standards (*36*, *37*, *48*, *49*).

Storage and repeated analysis of the stock standard and global standard solutions over many months verified the stability of the sulfur compounds prepared in Sure-Seal bottles and stored at −18 °C under nitrogen. Details of the maximum storage times adopted for the various solutions are provided under Materials and Methods. Furthermore, the Sure-Seal system minimized disagreeable odors emanating from the storage bottles.

Occasionally, interference from sulfur dioxide (SO_2) was found when some white wines were analyzed according to the described method. Most often they were young white wines with higher free SO_2 levels and, when present, the SO_2 peak coeluted with DES and MeSAc (**Figure 2**). To prevent any potential interferences, acetaldehyde was routinely added to white wine sample vials to bind free SO_2 without affecting the other compounds, in accord with other findings reported in the literature (*36*, *38*).

Validation. Typical coefficients of determination (R^2), repeatability values, limits of detection and quantitation, and linear ranges are summarized in **Table 3**. The model wine used for the standard addition calibration had characteristic fermentation volatiles added (see **Table 2**) to better approximate the headspace of a true wine matrix as opposed to a simple buffered water/ethanol mixture. This resulted in calibration functions with

similar slopes and R^2 values for model wine, white wine, and red wine for individual compounds (data not shown), justifying the use of spiked model wine for analyte quantitation in batches of samples. The precision of the analysis, determined at low and high analyte concentrations, gave < 10% relative standard deviation (RSD) for all compounds investigated. Calibration functions were linear throughout the ranges tested and gave R^2 values generally > 0.99 (**Table 3**). The method sensitivity was based on assessment of known concentrations of the analytes to establish the minimum levels required for reliable peak identification (LOD) and precise integration (LOQ). The low noise of the SCD (10 μV) meant the usual estimation of 3 and 10 times the signal-to-noise for LOD and LOQ, respectively, provided unrealistic limits. The LOQs shown in **Table 3** were entirely appropriate for the analytes and comparable to those of other methods. Blank runs, recoveries, and negative controls were checked regularly to evaluate method performance, and duplicate calibrants were run with each batch of samples for quantitation purposes.

Application of the Method to Sensory Studies Involving Commercial Wines. The optimized GC-SCD method was used to determine the concentration of 10 volatile sulfur compounds in 68 commercial wines selected on the basis of sensory descriptors such as "reduced", "struck flint", or "off-odor" that typically indicate the presence of volatile sulfur compounds. The samples comprised seven white wine varieties, one white wine blend, six red wine varieties, and four different red wine blends from numerous wine regions of Australia with vintages ranging from 2004 through 2008. **Table 4** shows the concentration ranges of sulfur compounds determined for each variety that was studied.

Table 3. Validation Data for the Analysis of 10 Volatile Sulfur Compounds in Wines by GC-SCD

		RSD[b]				
analyte[a]	R^2	5 μg/L	50 μg/L	LOD[c]	LOQ[d]	range (μg/L)
H_2S	0.9975	3.3	4.2	0.2	0.5	0.2−100
MeSH	0.9882	6.6	5.6	0.2	0.5	0.2−100
EtSH	0.9952	5.9	4.6	0.2	0.5	0.2−100
DMS[e]	0.9973	3.9	2.6	1.0	2.0	1.0−400
CS_2	0.9915	9.4	4.0	0.2	0.5	0.2−100
DES	0.9974	5.4	3.8	0.2	0.5	0.2−100
MeSAc[e]	0.9983	4.2	4.1	1.0	2.0	1.0−400
DMDS	0.9983	2.8	5.0	0.2	0.5	0.2−100
EtSAc[e]	0.9993	5.6	3.7	1.0	2.0	1.0−400
DEDS	0.9972	3.6	6.2	0.2	0.5	0.2−100

[a] Abbreviations are the same as in **Table 1**. [b] RSD, % relative standard deviation for repeatability ($N = 7$). [c] LOD, limit of detection (μg/L). [d] LOQ, limit of quantitation (μg/L). [e] Repeatability at 20 μg/L and 200 μg/L.

Hydrogen Sulfide. H_2S was detected in every white wine, with the highest level of 35.0 μg/L for a Riesling wine (**Table 4**). For the red wines, H_2S was detected in 33 of 40 samples, with the highest level being 8.7 μg/L in a Shiraz wine. At low levels, H_2S may add complexity to wine aroma but higher levels remaining after fermentation may lead to undesirable traits, such as "rotten egg" or "sewage-like" odors. However, there was a lack of consensus in the literature about the actual aroma threshold for H_2S in wine. Articles and books quote vastly different aroma thresholds, anywhere from 1 ng/L to 150 μg/L in wine (*8*) with a commonly used range of 10−80 μg/L (*15*). In contrast, we found that in the set of 68 "reduced" wines, only 5 wines (4 Riesling and 1 Sauvignon blanc/Semillon) contained > 10 μg/L of H_2S. As a result, the aroma detection threshold of H_2S in wine was revisited and found to be 1.1 and 1.6 μg/L in red and white wine, respectively (*16*). This means in most of the 68 wines assessed as "reduced", H_2S could be contributing to the reductive aromas, although more investigation is required. To highlight this point, H_2S was dismissed as the cause of an "off-odor" by Rauhut et al. for wines presenting "sulfurous off-flavor" (*48*), and Fang and Qian analyzed commercial wines with no apparent "off-flavor" (*36*), although H_2S was found in both studies at concentrations similar to those in our work. Additionally, Lopez et al. analyzed commercial wines with up to 30 μg/L of H_2S yet none had discernible "off-flavors" (*37*).

Methanethiol. MeSH was detected in all but 1 white wine and in 30 of the 40 red wine samples. The highest level of MeSH was 8.0 μg/L in a Chardonnay wine, whereas the highest level in the red wines was 5.0 μg/L in a Shiraz wine (**Table 4**). MeSH was attributed to "off-odor" in wines with levels > 4 μg/L in one study (*7*), whereas almost 5 μg/L in a wine from another study posed no sulfur-related issues (*36*). Levels of MeSH around 1.5 μg/L have been associated with "off-odor" in wines (*48*), but it was apparent that no wine aroma detection threshold was

Table 4. Concentration Ranges of Volatile Sulfur Compounds in Commercial Australian Wines from 2004 to 2008 with Noted "Reductive" Characters (Analyzed May 2009)

		concentration (μg/L)									
variety	no. of wines	H_2S	MeSH	EtSH	DMS	CS_2	DES	MeSAc	DMDS	EtSAc	DEDS
Chardonnay	4	1.5−5.0	3.0−8.0	nd[a] −0.5	20.0−185.0	0.5−5.0	nd	nd−7.0	nd	nd	nd
Pinot gris	1	2.0	3.0	nd	11.0	0.5	nd	nd	nd	nd	nd
Riesling	10	0.5−35.0	nd−3.0	nd	11.0 −37.1	nd−21.1	nd−0.4	nd	nd	nd	nd
Sauvignon blanc	6	0.8−4.0	1.7−6.0	nd	25.0−118.2	1.0−13.5	nd−0.4	nd	nd	nd	nd
Sauvignon blanc/Semillon	4	2.0−13.0	1.0−4.0	nd−1.0	25.0−76.0	0.5−14.8	nd−0.4	nd−2.1	nd	nd	nd
Semillon	1	2.0	3.0	nd	13.5	2.0	nd	nd	nd	nd	nd
Verdelho	1	1.0	1.6	nd	47.7	18.6	0.4	nd	nd	nd	nd
Viognier	1	0.5	3.0	nd	78.0	6.0	nd	nd	nd	nd	nd
Cabernet Merlot	2	0.5−0.8	0.4−1.0	nd	102.5−106.0	3.5−15.6	nd−0.4	nd	nd−1.5	nd	nd
Cabernet Sauvignon	5	nd−1.6	nd−1.5	nd	88.0−379.5	3.0−20.0	nd−0.4	nd−10.0	nd	nd	nd
Durif	1	2.0	2.0	nd	61.0	1.0	nd	18.0	nd	nd	nd
Grenache/Shiraz/Merlot	1	0.7	0.7	nd	111.0	18.0	0.4	nd	nd	nd	nd
Merlot	3	0.5−1.2	nd−1.6	nd	48.0−235.0	8.0−17.0	nd−0.4	3.0−8.0	nd	nd	nd
red wine blend	1	1.0	0.2	nd	195.0	14.5	0.4	4.7	nd	nd	nd
Sangiovese	1	nd	nd	nd	68.0	4.0	nd	nd	nd	nd	nd
Shiraz	22	nd−8.7	nd−5.0	nd−0.7	28.0−765.0[b]	2.0−45.1	nd−0.5	nd−12.5	nd−1.5	nd	nd
Shiraz/Cabernet Sauvignon	2	0.5−1.0	1.0−1.2	nd	85.0−228.4	4.0−17.4	nd−0.4	4.1 −7.5	nd	nd	nd
Shiraz/Viognier	2	nd−1.0	1.0	nd	57.0−112.0	2.0−6.0	nd	nd−6.0	nd	nd	nd

[a] Not detected. [b] Extrapolated value, outside the calibration range.

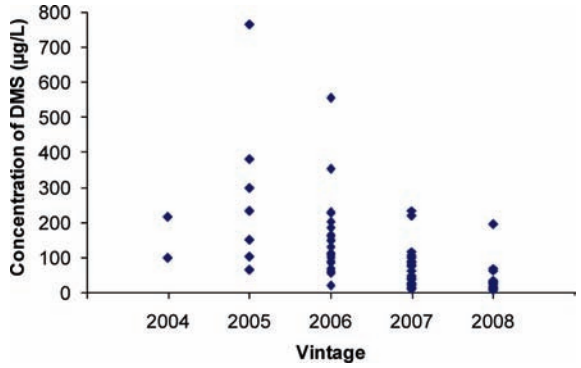

Figure 3. Concentration of DMS in 68 commercial wines (2004−2008) analyzed in May 2009 by GC-SCD.

available in the literature. Given that MeSH was measured in 57 of the 68 wines shown in **Table 4**, quite often at levels 3−4 times above which it might pose a problem, it was prudent to accurately determine the aroma detection threshold in wine. We have recently shown that the aroma detection thresholds for MeSH in white and red wines were 3.1 and 1.8 μg/L, respectively (*17*). This threshold study highlighted the matrix-dependent nature of volatile sulfur compounds, as suggested previously (*36*), and indicated that MeSH could have contributed to the "reductive" characters of quite a number of red and white wines listed in **Table 4**.

Ethanethiol. EtSH was detected in only one red wine and two white wines ($\leq$1.0 μg/L, **Table 4**), with all being below the reported white wine aroma threshold of 1.1 μg/L (*6*). In contrast, Park et al. regularly detected EtSH in a set of California wines considered to have "sulfide off-odors" (*7*), whereas Rauhut et al. barely detected EtSH in a number of wines from various countries displaying "off-flavor", except for one which had 5.5 μg/L (*48*). In other studies involving commercial wines without sulfur "off-odor", EtSH was detected on average 2−3 times above its threshold concentration in numerous Spanish wines (*37*, *44*), whereas no EtSH was present in a number of wines from California, Oregon, and Canada (*36*). It is doubtful that method sensitivity has affected the various results, so in the absence of elevated must sulfur levels during winemaking (*15*), the impact on sulfide "off-odor" from EtSH appears to be negligible.

Dimethyl Sulfide. Of the volatile sulfur compounds examined, the largest overall concentration range was noted for DMS, with 11.0−185.0 μg/L in the white wines and 28.0−765.0 μg/L in the red wines (**Table 4**). DMS is an interesting sulfur compound that can be beneficial to wine aroma at low levels (perhaps up to 100 μg/L), increasing the perceived fruitiness (*13*, *14*). At high levels it may mask fruity aromas and impart unpleasant "canned corn", "cooked cabbage", or "vegetal" type aromas (*6*, *7*, *11*). Certainly the levels encountered in some of the wines we analyzed can be expected to negatively affect the aromas of those wines. Higher levels of DMS are often found in older wines because DMS tends to increase in concentration as wine ages (*34*, *35*). Although the wines were not analyzed at bottling, this trend may be evident within the 68 wine samples we analyzed, in accord with Segurel et al. (*13*). **Figure 3** shows that all of the older wines from 2004 and 2005, except one, contained $\geq$100 μg/L DMS, whereas all of the younger wines from 2008, except one, contained $<$100 μg/L. Wines from 2006 and 2007 were between these extremes, although more wines from 2006 had $>$100 μg/L of DMS and most wines from 2007 had less than this amount. It can also be seen from **Figure 3** that in general as the wines aged, the levels of DMS encountered for a particular vintage diverged to a greater extent, which may relate to the amounts of DMS precursors remaining in the wine (*13*, *34*).

Carbon Disulfide. CS_2 was detected in all samples except two Riesling wines, with concentrations up to 21.1 and 45.1 μg/L for white and red wines, respectively (**Table 4**). CS_2 was first identified in wines by Leppänen et al. in amounts up to 10 μg/L (*45*), although it may be present at $<$5 μg/L, even in wines bearing an "off-odor" (*48*). CS_2 appears to be a ubiquitous sulfur compound in wine, previously found in concentrations up to 2.3 μg/L in a white wine and 17.8 μg/L in a red wine (*44*). Higher concentrations may be associated with reduced wines (*8*), but spiking of CS_2 into a white wine at almost 38 μg/L appeared to have no effect on the aroma (*12*). The impact of CS_2 on wine aroma is not well understood, and an aroma threshold study in wine appears to be absent from the literature. Negative descriptors relating to CS_2 may be due to impurities in commercially available material (*25*), but a greater understanding of its role in "reductive" characters is required.

Diethyl Sulfide. DES was detected in 24 wines with concentrations of $\leq$0.5 μg/L (**Table 4**), which were below its white wine aroma threshold of 0.93 μg/L (*6*). The values we encountered were in accord with fault-free wines from California, Oregon, and Canada (*36*) and below those identified in Spanish wines, which contained up to 1.9 and 2.6 μg/L of DES in white and red wines, respectively (*37*). Another study of wines from Tarragona revealed above-threshold levels of DES up to 7.8 μg/L in a white wine and 5.4 μg/L in a red wine (*44*). The levels of DES encountered in the reports on Spanish wines may be expected to yield wines with "rubbery" or "garlic" descriptors, yet there was no mention of this effect. It could be hypothesized that above-threshold levels of DES may be tolerated in some wines as a result of matrix interactions or simply that the detection threshold is higher in certain wine varieties. In our situation it appeared unlikely that "reductive" characters resulted from the amounts of DES present.

Methyl and Ethyl Thioacetates. MeSAc was detected at low levels in 29 of the red wines ($\leq$18 μg/L) and only 2 of the white wines ($\leq$7.0 μg/L, **Table 4**), in all cases below the aroma detection threshold of 50 μg/L determined in beer (*18*). The results for MeSAc were broadly similar to fault-free wines from North America (*36*), Spain (*44*), and Europe (*45*), and a variety of wines with and without sulfur "off-odor" from various regions (*48*). EtSAc was not detected in any of the 68 wines we analyzed. This contrasted with other work, in which EtSAc was found either in trace amounts for wines with and without sulfur "off-odor" (*48*) or, in the case of sound wines, at several micrograms per liter (*44*, *45*) and up to 22 μg/L in white wine and 13 μg/L in red wine (*36*). In one reported case, a Pinot noir with "off-odor" had very high levels of both MeSAc (115 μg/L) and EtSAc (56 μg/L), but this seemed to be an extreme example (*48*). The presence or absence of MeSAc and EtSAc appeared to be somewhat related to the amounts of their associated thiols, MeSH and EtSH. In particular, MeSAc could serve as a precursor for MeSH release during storage, potentially elevating the "reductive" characters. For example, 10 μg/L of MeSAc could liberate 5.34 μg/L of MeSH depending on closure oxygen transmission rate and other factors. The nature of the conditions that lead to any changes between thioacetates and thiols upon storage needs further investigation, however.

Dimethyl and Diethyl Disulfides. DMDS was present in only five red wines ($\leq$1.5 μg/L, **Table 4**) at levels well below its white wine aroma detection threshold of 29 μg/L (*6*). DEDS, with a reported detection threshold in white wine of 4.3 μg/L (*6*), was not detected in any of the wines we analyzed. These results are generally in agreement with concentrations reported from other studies (traces up to several μg/L) of wines either presenting sulfur "off-odor"(*48*) or with no sulfur-related issues (*36*, *45*). As with

other volatile sulfur compounds, Spanish red and white wine varieties tended to have greater amounts of DMDS or DEDS (up to 5.2 μg/L) (*37*, *44*), even though no sensory faults were apparent. A larger study of California wines with "sulfide off-odors" found 14 wines with concentrations of DEDS ranging from 5.0 to 86.6 μg/L, at which DEDS probably caused strong "rubbery" characters when present in concentrations of at least 5 times its detection threshold value (*7*). Although it is a worthy exercise to determine these disulfides in wine when the dynamics of interrelated fermentation-derived sulfur compounds are evaluated, in the case of the 68 wines we analyzed these disulfides are unlikely to have contributed to any "reductive" character.

In summary, the advent of this method for the quantitation of 10 volatile sulfur compounds in wine allows the expeditious analysis of samples from studies relating to optimization of fermentation and storage. The HS sampling arrangement and COC introduction into the GC eliminates tedious sample preparation or the possibility of artifact formation, and the SCD provides excellent selectivity and sensitivity. The method has been validated and applied to an extensive set of wines ascribed with "reductive" sensory attributes. On the basis of aroma detection thresholds where known, the analytes implicated as contributors to "reductive" characters were H_2S, MeSH, and DMS, whereas CS_2 played an undetermined role. MeSAc, although not present above its aroma detection threshold at the time of analysis, could act as a source of perceivable MeSH over time depending on a range of factors including closure oxygen transmission rate.

ABBREVIATIONS USED

HS, headspace; COC, cool-on-column; SCD, sulfur chemiluminescence detection; YAN, yeast assimilable nitrogen; PFPD, pulsed flame photometric detection; FPD, flame photometric detection; AED, atomic emission detection; LOD, limit of detection; LOQ, limit of quantitation.

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