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Application of a Modified Method for 3-Mercaptohexan-1-ol Determination To Investigate the Relationship between Free Thiol and Related Conjugates in Grape Juice and Wine

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Supporting Information

ABSTRACT: A method has been developed for determining 3-mercaptohexan-1-ol (3-MH) in wine and grape juice using gas chromatography with conventional electron ionization (EI) mass spectrometry. The limit of quantitation of 40 ng/L was achieved with excellent precision using stable isotope dilution analysis (SIDA) combined with headspace solid-phase microextraction (SPME) of derivatized 3-MH. This method was used in combination with HPLC-MS/MS analysis of the individual diastereomers of 3-*S*-cysteinylhexan-1-ol (Cys-3-MH) and 3-*S*-glutathionylhexan-1-ol (Glut-3-MH), which are known precursors of the volatile thiol 3-MH. Commercial and small-lot winemaking trials were evaluated to determine the concentrations of precursors and free 3-MH at various stages of grape processing and winemaking. Five Sauvignon blanc clones were also assessed for precursors and free thiol during ripening, revealing the presence of 3-MH in the unfermented juices and a stark increase in precursor concentrations in the latter stage of ripening. Additionally, differences due to sample freezing and mode of juice preparation were revealed for the precursors, and a set of commercially available wines was analyzed to investigate the amounts of precursors and free 3-MH in Sauvignon blanc and other white wine varieties. There was seemingly no relationship between precursor concentrations in juice and 3-MH concentrations in wine. This was somewhat understandable, because the formation of precursors appears to be a dynamic process affected by a multitude of factors, beginning with grape ripening and continuing during vinification.

KEYWORDS: wine aroma, 3-mercaptohexan-1-ol, wine thiol precursors, SIDA, HPLC-MS/MS, GC-MS

INTRODUCTION

Thiol-containing compounds, in particular 3-mercaptohexan-1-ol (3-MH), have been shown to be important varietal aroma impact compounds of Sauvignon blanc.[1] The aroma of 3-MH is described as "grapefruit" or "passionfruit", and its potency can be demonstrated by its low aroma detection threshold of 60 ng/L determined in a hydroalcoholic solution.[1] In Sauvignon blanc the amount of 3-MH varies widely; it has been found to range from 26 ng/L[2] to almost 13000 ng/L.[3] Although the presence of 3-MH is commonly associated with Sauvignon blanc, it has also been documented for other varieties including Gewurztraminer, Riesling, Colombard, Petit Manseng, Semillon, Pinot gris, Riesling, Muscat, Pinot blanc, Sylvaner, Cabernet Sauvignon, and Merlot.[1,4−7] The alcohol functionality of 3-MH may be acylated during fermentation of grape juice to form 3-mercaptohexyl acetate (3-MHA),[8] which is also a potent aroma compound and typically amounts to up to 10% of the concentration of 3-MH.[3,7] 3-MHA is not detected in all wine varieties and does not have a direct precursor in grape berries. As such, we have focused on the more abundant 3-MH along with its known precursors, the odorless cysteine and glutathione conjugates.

The analytical determination of wine thiols such as 3-MH at near-threshold concentrations in wine is particularly difficult, because extremely low levels need to be reliably determined (low ng/L to μg/L range). Some of the difficulties experienced with 3-MH analysis are poor sensitivity and poor chromatography, where intense tailing of peaks may arise from adsorption due to the thiol functionality on stationary phases or residual active sites that can occur in gas chromatographic (GC) systems.[9] Accurate analysis of thiols can also be compromised by their instability and capacity to react with oxygen or other oxidants, and thiols may form complexes and precipitates with different metal ions.[10]

The most common methods applied to the analysis of varietal thiols use *p*-hydroxymercuribenzoate (*p*-HMB) solutions to facilitate thiol extraction.[11] Although quite specific for thiols, *p*-HMB is highly toxic, and the methods employed are expensive, use large volumes of wine, and involve tedious or complicated extractions.[1,4,11] Despite these limitations, further modifications of this method have been published.[9,12] These modified methods improved the quality of the extracts containing the thiols, but still did not solve the difficulties associated with analytical sensitivity and compound stability. Such analytical methods have also been enhanced by employing stable isotope dilution analysis.[13] Unfortunately, these methods are still lengthy, the procedures are intricate, and the use of mercury complexes has not been eliminated.

Researchers have also employed derivatizing agents such as 2,3,4,5,6-pentafluorobenzyl bromide (PFBBr)[10] with on-fiber

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derivatization[14] or in-cartridge (SPE) derivatization.[15] These methods apparently have not been routinely adopted so far, possibly due to limitations with the linear range, along with reactivity, repeatability, and sensitivity issues.[15,16] More recently, Rodriguez-Bencomo et al.[16] and Mateo-Vivaracho et al.[2] separately revisited the use of PFBBr and again optimized methods using in-cartridge (SPE) derivatization followed by solid-phase microextraction (SPME) of the derivatives to overcome some limitations of the earlier methods. These various derivatization methods use gas chromatography−mass spectrometry (GC-MS) with negative chemical ionization (NCI) mass spectrometry, which is well suited to compounds with high electron affinity such as these derivatives,[17,18] but an NCI instrument option may not be readily available. As such, a GC-MS method using electron ionization (EI) would be a desirable alternative if the sensitivity proved adequate.

To continue increasing our understanding of the varietal thiol 3-MH and its precursors, a GC-EI-MS method for analyzing 3-MH was developed and applied in conjunction with a high-performance liquid chromatorgraphy−tandem mass spectrometry (HPLC-MS/MS) precursor method.[19] Together these methods were used to evaluate the relationship between 3-MH and its glutathionyl and cysteinyl precursors in a range of commercial and research scale juices and wines.

MATERIALS AND METHODS

Materials. Samples used for analysis were obtained from retail outlets (dry white wine) or supplied by Australian producers (wine and juice). Juices from commercial producers were stored at −20 °C until required. Model wine consisted of 10% aqueous ethanol saturated with potassium hydrogen tartrate and pH adjusted to 3.2 with tartaric acid. All isotopically labeled and unlabeled standards were previously synthesized according to the procedures of Pardon et al.[20] and Grant-Preece et al.[21] The standards used were 3-mercaptohexan-1-ol (3-MH), d_{10}-3-mercaptohexan-1-ol (d_{10}-3-MH), 3-mercaptohexyl acetate (3-MHA), d_5-3-mercaptohexyl acetate (d_5-3-MHA), *S*-[(1*R*/*S*)-1-(2-hydroxyethyl)butyl]-L-cysteine (Cys-3-MH), *S*-[(1*R*/*S*)-1-(2-hydroxyethyl)butyl-1,2,2,3,3,4,4,4-d_8]-L-cysteine (d_8-Cys-3-MH), γ-L-glutamyl-*S*-[(1*R*/*S*)-1-(2-hydroxyethyl)butyl]-L-cysteinylglycine (Glut-3-MH), γ-L-glutamyl-*S*-[(1*R*/*S*)-1-(2-hydroxyethyl-2-d_1)butyl-1,2,2,3,3,4,4,4-d_8]-L-cysteinylglycine (d_9-Glut-3-MH). Stock solutions of standards were prepared volumetrically in Milli-Q water (for precursors) or redistilled ethanol (for free thiol) and stored at −20 °C, and working solutions were stored at 4 °C until required. Solvents were of Mallinckrodt nanopure grade for GC-MS analysis and Merck HPLC grade for HPLC-MS/MS analysis (Rowe Scientific, Lonsdale, SA, Australia). All chemicals were of analytical reagent grade unless otherwise stated, and water was obtained from a Milli-Q purification system (Millipore, North Ryde, NSW, Australia). Merck solvents and Merck or BDH reagents were purchased from Rowe Scientific, and other chemicals were obtained from Sigma-Aldrich (Castle Hill, NSW, Australia). The SPME fibers used were polydimethylsiloxane/divinylbenzene (PDMS/DVB) 65 μm, Carboxen/polydimethylsiloxane (CAR/PDMS) 85 μm, polyacrylate coating (PA) 85 μm, polydimethylsiloxane (PDMS) 100 μm, and divinylbenzene/Carboxen/polydimethylsiloxane (DVB/CAR/PDMS-2 cm) 50/30 μm (Supelco, Bellefonte, PA).

Assessment of Method Parameters for Analysis of 3-MH. *Liquid−Liquid Extraction from Wine.* Different solvent compositions were assessed to determine the best solvent for the extraction of 3-MH from white wine. Successive extractions (3 × 6 mL) were used to extract 3-MH from 200 mL of wine. The different solvents included pentane and mixtures of pentane/dichloromethane (4:1 and 2:1) and pentane/ethyl acetate (4:1 and 2:1). The combined organic extracts (18 mL) were then extracted with different aqueous NaOH solutions (0.01, 0.5, 1, and 2 M). When the base strength was optimized, the volume of base was evaluated and included a 1 × 6 mL extraction, 2 × 3 mL extractions, 2 × 5 mL extractions, and 2 × 6 mL extractions. The addition of 1 g of ethylenediaminetetraacetic acid disodium salt (EDTA 2Na) and 2 g of NaCl to the wine prior to the solvent extraction was also investigated.

Derivatization of Analyte. A stream of nitrogen was applied for 7 min to the basic extract to remove residual organic solvent, and 100 μL of 2,3,4,5,6-pentafluorobenzyl bromide (PFBBr) solution (20 μL of PFBBr in 50 mL of redistilled ethanol, 740 mg/L) was added to the sample. After 20 min, the extract was submitted for analysis or adjusted to pH 4−5 with either dropwise addition of concentrated HCl or addition of solid tartaric acid before analysis. The time taken between derivatization and pH adjustment (0−60 min) was also included.

GC-MS Instrumentation. The quantitation of derivatized 3-MH was carried out using headspace solid-phase microextraction/stable isotope dilution analysis coupled with gas chromatography−mass spectrometry (HS/SPME/SIDA/GC-MS). Samples were analyzed with an Agilent 6890N gas chromatograph (Santa Clara, CA) fitted with a Gerstel MPS2 autosampler and a Gerstel Cooled Injection System (CIS 4) and coupled to an Agilent 5973N mass spectrometer. Positive ion electron impact spectra at 70 eV were recorded in the range m/z 35−350 for scan runs.

Evaluation of SPME Fibers. White wine or model wine (10 mL) was spiked with 3-MH and d_{10}-3-MH at 250 ng/L and added to a 20 mL amber screw-cap GC-MS vial, along with approximately 0.1 g of EDTA 2Na. The sample was derivatized using 100 μL of 1,8-diazabicyclo-[5.4.0]undec-7-ene (DBU) solution (407 mg/L in redistilled ethanol) and 100 μL of PFBBr solution (740 mg/L in redistilled ethanol),[10] and 2 g of NaCl was added to the vial. Various SPME fibers were investigated to determine the fiber that was most efficient at absorption of the pentafluorobenzyl (PFBn) derivatives. The fibers investigated were PDMS/DVB, CAR/PDMS, PA, PDMS, and DVB/CAR/PDMS at the recommended operating temperature for each fiber.

Headspace Extraction Conditions. Investigations included extraction temperatures of 30, 50, 60, 80, and 100 °C for various times using the PDMS/DVB fiber, along with the effect of agitating the sample during extraction. The agitation conditions were 700 rpm (agitation on-time of 10 s and off-time of 1 s) with an extraction time of 30 min.

Chromatography. A 30 m × 0.25 mm i.d., 0.25 μm film thickness, J&W DB-5MS column (Biolab Australia Limited, Scoresby, VIC, Australia) was initially trialed, followed by a 60 m column of the same type. The chromatographic conditions were 50 °C for 2 min, then a ramp of 3 or 5 °C/min to 215 °C, followed by 50 °C/min to 300 °C and holding at this temperature for 15 min. The transfer line was 300 °C, and the injection temperatures were 250 or 270 °C for 15 min followed by ramping the injector to 300 °C at 12 °C/s and holding at this temperature for 10 min.

Effect of PFBBr Storage Time. Solutions of PFBBr were prepared by adding 20 μL of PFBBr to a 50 mL volumetric flask containing redistilled ethanol and topping up to the mark with redistilled ethanol. Fresh derivatization solutions were repeatedly prepared over an 8 month period using an original bottle of PFBBr for comparison with solutions prepared from a new reagent purchased after 8 months. Comparisons were undertaken within the same batch of GC-MS samples.

Checks for Interferences from 3-MHA Hydrolysis During Incubation. Headspace extraction temperatures of 50 and 80 °C were assessed for 250 ng/L each of d_5-3-MHA and 3-MHA spiked into 6 mL of 1 M NaOH and derivatized with 100 μL of PFBBr solution. Samples were reacted for 20 min at room temperature, adjusted to pH 4−5 with tartaric acid, and the headspace was extracted for 30 min and analyzed using the optimized GC-MS method.

Optimized Method for Analysis of 3-MH. *Preparation of Juice and Wine Extracts.* An aliquot (200 μL) of an ethanol solution

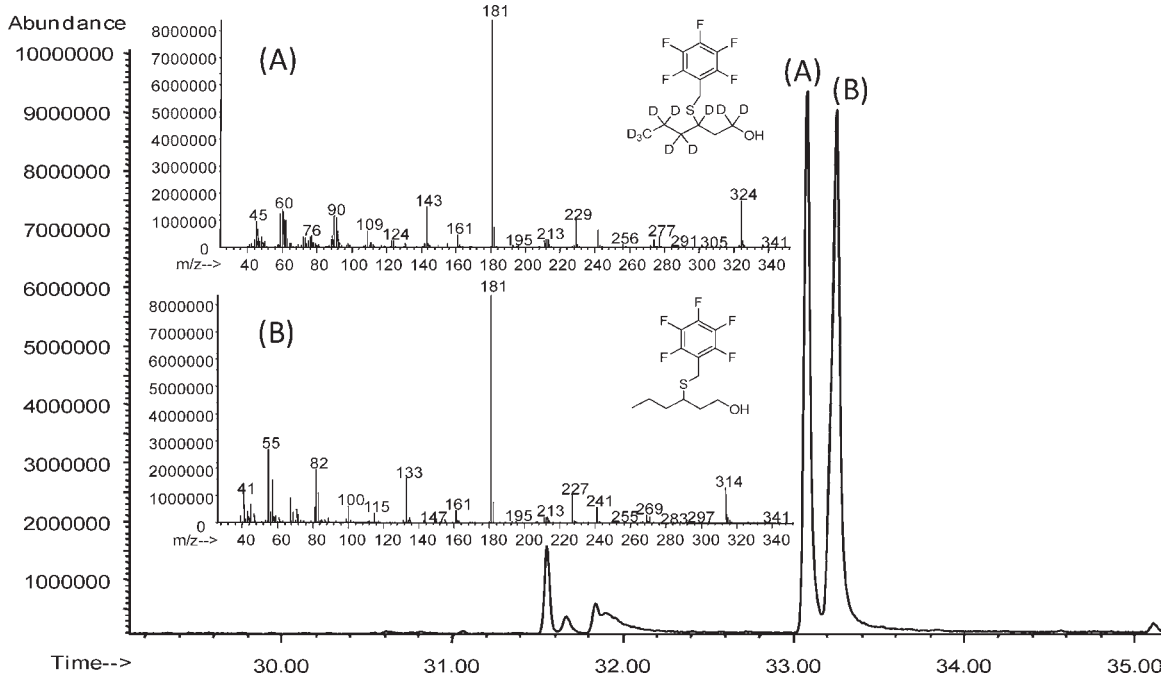

Figure 1. Total ion chromatogram of the pentafluorobenzyl derivatives of 3-MH for a standard mixture and mass spectra of (A) d_{10}-3-MH PFBn derivative and (B) 3-MH PFBn derivative.

containing d_{10}-3-MH (300 ng/L) was added to the sample to be analyzed in a 200 mL glass volumetric flask, and the flask was topped up to the mark with the sample. The flask was shaken, 1 g of EDTA 2Na and 2 g of NaCl were added, and the flask was shaken again. The sample was extracted from the volumetric flask by thorough mixing with pentane (3 × 6 mL). The combined pentane extracts were stored in a spark-proof freezer at −20 °C for up to 3 days without any noticeable degradation. The pentane extracts were washed with $NaHCO_3$ solution (5 mL, 0.3% w/v, pH 6) and extracted with ice-cold 1 M NaOH (6 mL), and the aqueous NaOH extract was transferred into a 20 mL glass SPME vial. A stream of nitrogen was applied to the basic extract for 7 min to remove any traces of pentane, and the extract was derivatized with 100 μL of PFBBr solution for 20 min at room temperature. The sample was then adjusted to pH 4−5 with approximately 0.5 g of tartaric acid, 2 g of NaCl was added, and the vial was sealed for GC-MS analysis. One operator could potentially complete 25 samples per day using this method.

Quantitative GC-MS of 3-MH Derivatives. The GC-MS system described above was employed. The autosampler was fitted with an automated 65 μm PDMS/DVB SPME fiber and a Gerstel peltier cooling tray set at 4 °C. The gas chromatograph described above was fitted with a J&W DB-5MS fused silica capillary column (60 m × 0.25 mm i.d., 0.25 μm film thickness). The carrier gas was helium (BOC gases, ultrahigh purity), and the flow rate was 1.6 mL/min. The oven temperature started at 50 °C, was held at this temperature for 2 min, then increased at 3 °C/min to 215 °C, then increased at 50 °C/min to 300 °C, and held at this temperature for 15 min. The CIS 4 injector was held at 270 °C for 15 min, then heated at 12 °C/s to 300 °C, and held for 10 min. The transfer line was maintained at 300 °C. The sample was extracted at 80 °C for 30 min while agitating at 700 rpm (10 s on, 1 s off) and desorbed in the inlet for 15 min. The splitter, at 50:1, was opened after 180 s. Injection was done in splitless mode with an inlet pressure of 45.0 psi maintained until splitting. The SPME injection sleeve (Gerstel, 1 mm i.d.) was borosilicate glass. For quantitation, mass spectra were recorded in selected ion monitoring (SIM) mode (Figure 1). The ions monitored in SIM runs were m/z 143, 181, and 324 for d_{10}-3-MH and m/z 133, 181, and 314 for 3-MH. Selected fragment ions were monitored for 20 ms each. The underlined ion for each compound was the ion typically used for quantitation, having the best signal/noise ratio and the least interference from other matrix components. The other ions were used as qualifiers.

Analytical Method Validation. The analytical method was validated by a series of duplicate standard additions of unlabeled 3-MH (0, 1, 10, 25, 50, 100, 250, 500, 1000, 2000, and 5000 ng/L) to a model wine, and a 2008 commercial unwooded Chardonnay (13.5% v/v ethanol, pH 3.56, titratable acidity = 6.2 g/L, SO_2 (free) = 26 mg/L, SO_2 (total) = 127 mg/L), which was subsequently found to contain 376 ng/L of 3-MH. To determine the precision of the analysis, seven replicate samples were spiked at two different 3-MH concentrations, 25 and 500 ng/L. The limit of detection (LOD) and limit of quantitation (LOQ) were determined using the visual evaluation method. LOD was determined by establishing the minimum level at which the analyte could be reliably detected from the analysis of samples with known concentrations of analyte (signal/noise ratio of about 3). LOQ was determined by establishing the minimum level at which the analyte could be quantified with acceptable accuracy and precision (<2% relative standard deviation) from the analysis of samples with known analyte concentrations. For quantifying the analytes in batches of unknown samples, duplicate standards in model wine were prepared at the same time as the juice and wine samples with 3-MH at concentrations of 0, 20, 1000, and 5000 ng/L. To ensure that the accuracy of the analysis was maintained, duplicate control samples, spiked with 100 ng/L 3-MH, were included with every set of samples to be quantified. All validation and calibration samples were extracted, derivatized, and analyzed according to the optimized method.

Commercial Juice and Wine Samples. Bottled Australian wines of various vintages from different regions were purchased from local retailers and included five each of Riesling, Sauvignon blanc, Pinot gris, Gewurztraminer, and Muscat varieties. Juices and the corresponding wines were provided by a local winery and included one Chardonnay, one Pinot Grigio, two Sauvignon blanc, and five Riesling sample sets.

Juice Samples During Ripening. Five Sauvignon blanc clones (H5V10, F7V7, F4V6, Q9720, and 5385) grown in a single vineyard within consecutive rows in the Adelaide Hills region of South Australia were sampled at different stages of maturity. The sampling time points

were spaced at approximately 2 week intervals, beginning at veraison (February 1) and ending with harvest (March 11). Samples were hand-harvested, and 400 plucked and randomized berries containing 20 mg/kg SO_2 added as potassium metabisulfite (PMS) were homogenized with a household stick mixer (Breville Wizz Stick). The homogenate was centrifuged (Beckman J2-21M/E, Beckman Coulter, Gladesville, NSW, Australia) at 4000 rpm (2830*g*) for 10 min at 15 °C, and the supernatant was used for analyses. The time taken between steps such as homogenizing and centrifuging or centrifuging and extract preparation was never more than 30 min. Basic compositional details of the samples appear in Supplementary Figure 1 in the Supporting Information.

Winemaking. Sauvignon blanc wines were prepared from the five Adelaide Hills clones by a contracted research winemaker. Hand-harvested fruit in good condition (between 57 and 66 kg of each clone) was collected and delivered to the winemaking facility and stored at 0 °C in a coldroom for 24 h. Fruit from each clone was pressed individually in two 30 kg lots using a 50 kg stainless steel membrane press, juice was collected into 30 L fermentation vessels (see Supplementary Table 1 in the Supporting Information for basic juice compositional data), and 4 mL/hL Ultrazyme CPL and 50 mg/L SO_2 (added as PMS) were added. The juice was cold settled at 0 °C in a coldroom for 72 h, racked off lees, and moved to a 15 °C fermentation room. The juice was inoculated with Maurivin PDM yeast at 0.25 g/L and fermented to dryness, and 60 mg/L SO_2 (added as PMS) and 0.1 mg/L $CuSO_4$ were added. The wine was racked off gross lees and cold stabilized at 0 °C in a coldroom for 72 h, and the stable wine was passed through a Z6 grade filter (polishing, nonsterile) and then a 0.45 μm sterile membrane and bottled under ROTE closures in 375 mL bottles. Basic compositional data for the finished wines appear in Supplementary Table 2 in the Supporting Information.

Effects of Juice Preparation and Sample Freezing. For preparation of fresh juices at harvest, 400 plucked and randomized berries (approximately 500 g) containing 20 mg/kg of SO_2 added as PMS were homogenized with a household stick mixer (Breville Wizz Stick). The homogenate was centrifuged (Beckman J2-21M/E) at 4000 rpm (2830*g*) for 10 min at 15 °C, and a 10 mL aliquot of supernatant was prepared for precursor analysis. The remaining grapes and juices were carefully packaged and frozen (−20 °C). After 2 months, the frozen samples were thawed for precursor analysis; the thawed grapes were processed as described for the fresh samples to obtain juice aliquots for analysis.

Preparation of Juice and Wine Extracts for Precursor Analysis. Extracts were prepared according to the procedure in Capone et al.,[19] with a slight modification to the drying and reconstitution procedure. An aliquot (100 μL) of an aqueous solution containing both diastereomers of d_8-Cys-3-MH and d_9-Glut-3-MH (50 μg/L of each diastereomer) was added to 9.9 mL of grape juice or wine. The sample was passed through a 6 mL, 500 mg Strata SDB-L cartridge (Phenomenex, Lane Cove, NSW, Australia), previously conditioned with 6 mL of methanol followed by 6 mL of water. The cartridge was dried under air for 5 min and eluted with 2 mL of methanol. The eluate was collected and concentrated to dryness using nitrogen at 25 °C on a Zymark TurboVap LV evaporator (John Morris Scientific, Chatswood, NSW, Australia). The sample was then reconstituted with 500 μL of methanol, followed by the addition of 200 μL of Milli-Q water, and the sample was vortexed (Vortex IKA MS1 minishaker, Crown Scientific, Wingfield, SA, Australia) for 10 s. The sample was filtered through an Acrodisc syringe filter (0.45 μm, 13 mm, Pall Gelman Life Sciences, Cheltenham, VIC, Australia) in readiness for HPLC-MS/MS analysis.

HPLC-MS Instrumentation. All HPLC-MS/MS analyses were carried with an Agilent 1200 instrument (Agilent, Forest Hill, VIC, Australia) equipped with a binary pump and connected in series to a 4000 Q Trap hybrid tandem mass spectrometer with TurboV source and TurboIon-Spray probe (Applied Biosystems/MDS Sciex, Concord, ON, Canada) as previously described.[19]

Statistical Analysis. The results reported for the calibration of the method were derived from the average of two replicate measurements for each concentration of analyte (and seven replicates for repeatability samples). The effects of the various treatments were analyzed using one-way or two-way analysis of variance (ANOVA) (GenStat 11.0, VSN International Ltd., Hemel Hempstead, U.K.). Other statistical data were obtained using Microsoft Excel 2007.

RESULTS AND DISCUSSION

Method Optimization. For maximum accuracy and precision, stable isotope dilution analysis (SIDA) was used for quantitative GC-MS determination of 3-MH in juice and wine. We chose to analyze 3-MH as the PFBn derivative to overcome problems associated with thiol reactivity and adsorption on surfaces. To maximize method sensitivity with a conventional electron ionization GC-MS while avoiding mercury complexes, we developed a liquid−liquid extraction and in situ derivatization technique. All aspects of the method were optimized as outlined below, from liquid−liquid extraction of the analyte and derivatization to headspace sampling and chromatographic parameters.

Extraction of 3-MH from Wine Samples. The objective at this step was to use liquid−liquid extraction of samples to obtain maximum transference of 3-MH into aqueous base for derivatization. Preliminary investigations of extraction solvent composition were assessed, including pentane and mixtures of pentane/dichloromethane and pentane/ethyl acetate, with back extraction using different base concentrations. Whereas the different solvent combinations afforded various extraction efficiencies, we also observed that higher base concentrations improved back extraction of 3-MH. With a base concentration above 2 M, however, a decrease in extraction was observed (data not shown). This is in accord with Yabroff's findings for other mercaptans and explained in terms of the salting-out of neutralized mercaptans from the aqueous phase by higher NaOH concentrations.[22] In all cases 1 M NaOH was chosen for optimal analyte extraction into the aqueous phase, although 0.5 M NaOH gave similar results (data not shown).

The combination of pentane and 1 M NaOH was identified as being the most ideal for 3-MH extraction. This was due to a number of factors: pentane separated readily from the wine and from the hydroxide solution; pentane was more compatible with hydroxide solution (ethyl acetate can hydrolyze, dichloromethane tended to form an emulsion); the effectiveness of back extraction from pentane was superior due to its low polarity. EDTA 2Na was also added to wine and juice samples to prevent analyte oxidation during extraction. Different volumes of 1 M NaOH were investigated for back extraction of the analyte from pentane, along with a bicarbonate wash of the organic phase prior to extraction with aqueous NaOH. Using a greater volume of base for back extraction simply resulted in a more dilute sample, leading to a loss of sensitivity upon analysis (data not shown). For maximum back extraction the base solution was used cold, leading to a slight improvement in extraction efficiency, as observed by Yabroff for other mercaptan extractions.[22] The bicarbonate wash greatly improved sensitivity of the analysis by affording a cleaner chromatogram, most likely due to the removal of other wine components, particularly interfering fatty acid compounds.[9] When method optimization was virtually complete, an assessment of salt addition to the wine prior to pentane extraction led to further improvement in sensitivity (data not shown).

Derivatization of 3-MH. After extraction into base and spiking with PFBBr solution, the effect of pH adjustment following derivatization was evaluated, using either tartaric acid or hydrochloric acid (HCl) to lower the pH or no pH adjustment at all. Adjusting with tartaric acid led to a doubling of the sensitivity compared to HCl, whereas a marginal reduction in sensitivity was noted without any acid adjustment compared to using HCl (data not shown). It was difficult to reproducibly adjust the pH using HCl, whereas a measured amount of tartaric acid regularly afforded a pH of 4−5, with an occasional minor adjustment for some samples. The slight decrease in sensitivity observed without pH adjustment may be explained by sample decomposition after derivatization. Reaction times between derivatization and pH adjustment were also investigated. Under our conditions the reaction was very rapid at room temperature, and there was minimal difference between reaction times of 0 and 20 min. For practical purposes when large numbers of samples were handled, a reaction time of 20 min was adopted. This reaction time and temperature have also been employed in other methods using derivatization with PFBBr.[2,15,16]

Headspace Sampling of Derivatives. Various fibers were investigated using derivatizing conditions similar to those of Mateo-Vivaracho et al. [10] to determine which fiber would be the most suitable for the extraction of the PFBn derivative. The fibers were PDMS/DVB, CAR/PDMS, PA, PDMS, and DVB/CAR/PDMS. The PDMS/DVB and DVB/CAR/PDMS fibers were the best performing, and PDMS/DVB was chosen as it gave the most symmetrical peak shape (data not shown). Both of these fibers have been selected previously for analysis of PFBn thiol derivatives,[14,16] although fiber optimization was not reported in those studies. We found that CAR/PDMS, PA, and PDMS were inferior fibers for extraction of derivatized 3-MH, giving peak areas around 90% lower than the best performing fibers. As such, they were all deemed to be unsuitable alternatives.

Headspace Extraction and Chromatography. Extraction temperatures of 30, 50, 60, 80, and 100 °C were assessed to determine the optimum temperature for derivative extraction. On the basis of this experimentation, 80 °C was chosen as it afforded an enhancement of sensitivity over the other temperatures investigated by 85, 59, and 80% for 50, 60, and 100 °C, respectively. Agitation during extraction was investigated, yielding a 63% improvement for extraction of the PFBn derivative relative to no agitation. Furthermore, storing the samples at 4 °C in an autosampler cooling tray, to minimize analyte degradation while awaiting extraction, improved the sensitivity of the method by 20% (data not shown). Originally, a 30 m DB-5MS column was trialed, but there was a coeluting peak under the compound of interest. A 60 m column of the same phase and a slower temperature ramp eliminated interference from any coeluters (Figure 1). The injector temperature was also increased from 250 to 270 °C to eliminate any possibility of carry-over to the next sample.

Effect of Age of PFBBr solutions. To examine the stability of solutions of PFBBr used for derivatization, numerous solutions were evaluated over an 8 month period using an original bottle of PFBBr and a newly purchased one. At the end of this time period we found a 25% reduction in sensitivity for the 3-MH PFBn derivative when using the original solution prepared from the original bottle as compared to a fresh solution from a new bottle. A freshly prepared solution from the original reagent bottle was not as detrimental to sensitivity, with an 18% reduction relative to a fresh solution prepared from a new bottle of reagent (data not shown). From these results we decided that maintaining the sensitivity of the method ideally required solutions of PFBBr to be freshly prepared, and certainly not left for periods longer than 3 months.

Investigation of 3-MHA Hydrolysis. Because the sensitivity for derivatized 3-MH greatly increased at an 80 °C incubation temperature, we wanted to confirm that this did not result from hydrolysis of coextracted 3-MHA. We first ascertained that the 3-MHA standard contained only traces of 3-MH. The 3-MHA standard was then spiked into base and derivatized, and the headspace was extracted at two different temperatures (50 and 80 °C) using a PDMS/DVB fiber. There did not appear to be any significant quantities of 3-MH generated using this procedure, with at most a 1% conversion (data not shown). Considering that the amount of 3-MHA in wine is usually 10% of the 3-MH content or less, any hydrolysis at this level would be inconsequential.

Method Validation. The standard addition function obtained for 3-MH in wine using the optimized method was linear throughout the concentration range (0−5000 ng/L), with a coefficient of determination (R^2) of 0.9976. The respective standard deviations for repeatability at 25 and 500 ng/L of added 3-MH (for a wine containing 376 ng/L of 3-MH) were 2.5 and 1.4%, highlighting the precision of the method. The LOQ and LOD, determined to be 40 and 30 ng/L, respectively, were both below the aroma detection threshold of 3-MH in wine. White and model wines both gave almost identical calibration function slopes, highlighting that quantitative analysis was not dependent on the matrix (data not shown).

3-MH and Precursors in a Survey of White Wines. After optimization and validation were completed, the quantitative GC-MS method was applied to a limited survey of commercial white wines to determine the concentration of free 3-MH. Additionally, precursors were assessed by HPLC-MS/MS to ascertain the concentrations remaining in commercially bottled wine (Table 1). Almost every wine sample we analyzed contained both diastereomers of Cys-3-MH and Glut 3-MH. Diastereomers of Cys-3-MH ranged in concentration from undetected to 106 μg/L, and diastereomers of Glut-3-MH ranged from 30 to 503 μg/L. In accordance with Capone et al.,[19] we identified the same general trends as previously noted, namely, that Glut 3-MH dominates over Cys-3-MH and (*S*)-diastereomers are more abundant than (*R*)-diastereomers. The greatest amounts of precursors were found in Sauvignon blanc, but the other varieties, including Muscat, Gewurztraminer, and Riesling, contained considerable amounts of both precursor and free 3-MH. Quantitative analysis revealed concentrations of 3-MH ranging from 47 to 3200 ng/L (Table 1), highlighting the wide dynamic range of the method, with samples found below the aroma detection threshold of the analyte to concentrations around 50 times above it. These values indicated that 3-MH has the potential to be a relevant odorant in wine varieties other than Sauvignon blanc. The amounts of precursors remaining in bottled commercial wine may be of interest as they could potentially be affected by in-mouth release, leading to enhanced retronasal perception of 3-MH aroma, as described by Starkenmann et al.[23] Furthermore, any remaining thiol precursors might potentially affect the concentration of free thiol during wine aging and storage.

Evaluation of Commercial Juice and Wine Samples. Precursor concentrations were determined for nine commercial samples prior to fermentation (juice) and after fermentation (wine), along with 3-MH, to examine if there was any obvious relationship between precursor and free thiol concentrations on a

Table 1. Concentrations of the Diastereomers of Cys-3-MH and Glut-3-MH and Free Thiol 3-MH Found in a Survey of Commercial Australian White Wine Samples[a]

	precursor diastereomers (μg/L)								free thiol (ng/L)	
	(*S*)-Cys-3-MH		(*R*)-Cys-3-MH		(*S*)-Glut-3-MH		(*R*)-Glut-3-MH		3-MH	
sample	av (SD)[b]	range	av (SD)	range	av (SD)	range	av (SD)	range	av (SD)	range
Sauvignon blanc	12 (9)	nd[c]− 24	5 (4)	nd−11	231 (96)	108−339	63 (23)	29−82	1031 (1313)	77− 3200
Riesling	8 (5)	4−16	5 (4)	1−12	90 (59)	32−167	36 (24)	15−77	415 (367)	172−1060
Muscat	37 (30)	3−77	15 (13)	2−30	200 (160)	19−405	54 (37)	12−98	241 (375)	47−911
Pinot gris	18 (22)	nd−53	8 (11)	1−26	114 (55)	57−194	34 (15)	21−55	353 (380)	108−1021
Gewurztraminer	19 (16)	nd−43	7 (6)	nd−17	149 (115)	18−315	43 (35)	13−99	435 (463)	96−1237

[a] No significant differences ($p > 0.05$) were observed between the varietals for any of the measured attributes. [b] Av, average concentration for five samples; SD, standard deviation of average for five samples. [c] nd, not detected.

Table 2. Concentrations of Cys-3-MH and Glut-3-MH in Nine Commercial Juices, along with Those of Precursors and 3-MH in the Corresponding Wines

	precursors (μg/L)				free thiol (ng/L)
	total Cys-3-MH		total Glut-3-MH		3-MH
sample	juice	wine	juice	wine	wine
Chardonnay	13.2	15.4	175.7	89.6	551
Pinot gris	23.1	30.0	466.2	322.3	398
Sauvignon blanc	54.7	38.3	642.9	374.3	410
	27.7	49.6	416.4	485.8	452
Riesling	13.7	11.9	157.0	147.4	219
	17.9	14.0	275.2	90.3	234
	13.7	11.3	88.5	97.6	162
	11.1	9.0	148.2	156.9	445
	15.4	8.5	149.3	105.9	330

commercial scale (Table 2). On the whole there were lower precursor concentrations for both types after fermentation, yet there was some variability, with higher amounts found in the wine on several occasions. Although the inter-relationships are obviously somewhat complex (i.e., precursors may form and degrade, with intermediates unaccounted for), it would be useful to predict wine 3-MH concentrations based on juice precursor amounts. Kobayashi et al. have previously shown positive correlations between precursors and free 3-MH/3-MHA for microvinifications involving Koshu grapes.[24] We assessed 3-MH, ignoring any small amount of conversion to 3-MHA that may have occurred during fermentation, along with the precursor diastereomers. The related constituents were compared on a molar basis to examine whether total juice precursors related to wine 3-MH concentrations, after allowing for the remaining precursors in the wine. In this case there was no relationship between precursors and free thiol for the matched commercial samples investigated (data not shown). This was not overly surprising, considering the commercial origin of the samples and the use of different yeasts, and the data do not account for any losses of 3-MH during the winemaking process. Nonetheless, this was our first attempt to investigate precursor and free thiol relationships in commercial samples, and it was interesting to see the high concentrations of precursors and free 3-MH in varieties other than Sauvignon blanc once again.

Evaluation of Precursors during Ripening. Five Sauvignon blanc clones planted in the same Adelaide Hills location were analyzed for 3-MH precursors at a number of maturity stages. There were some differences between the basic chemical parameters of the clones at each sampling time point (Supplementary Figure 1 in the Supporting Information). The precursor concentrations varied significantly throughout ripening, with low μg/kg levels of glutathione conjugates and cysteine conjugates being virtually undetectable until preharvest, although slight increases were observed as ripening progressed (Figure 2). Roland et al. observed a similar trend with greater Cys-3-MH and Glut-3-MH levels with increased ripening,[25] although their Glut-3-MH levels were much lower than reported here. It was very interesting to note the difference between preharvest and commercial harvest for the Sauvignon blanc clones, with up to a 10-fold increase in cysteine and glutathione conjugates over this 14 day period, depending on the clone (Figure 2). This coincides with the results of Kobayashi et al.,[24] who found up to 4-fold increases in precursor concentrations around a similar stage of ripeness for Koshu grapes. Additionally, the maximum concentrations we found were similar in magnitude to those reported by Kobayashi et al.[24]

Identifying the greatest amounts of precursors at commercial harvest was not surprising. Around this maturity level there would be the greatest potential for loss of membrane integrity, leading to decompartmentalization and mixing of enzymes and/or precursor constituents.[26,27] Furthermore, increases in (*E*)-2-hexenal[28] and glutathione[29] concentrations with ripening have been shown for white grape varieties previously, and these appear to be the prime constituents for glutathione conjugate formation. Somewhat related to this, from veraison to preharvest there may have been a link between Brix, titratable acidity (TA) (Supplementary Figure 1 in the Supporting Information), and thiol precursor levels for the clone samples (Figure 2). Higher Brix and lower TA values, both indicators of increased ripeness, were associated with higher precursor concentrations. The Brix and TA values were all very similar at harvest, however, so any trend related to higher precursor concentrations no longer seemed apparent. In any case, the results signify the importance of proper harvest timing decisions to maximize precursor levels.

Evaluation of 3-MH during Ripening. The five Sauvignon blanc clones were also analyzed for free thiol 3-MH at the various

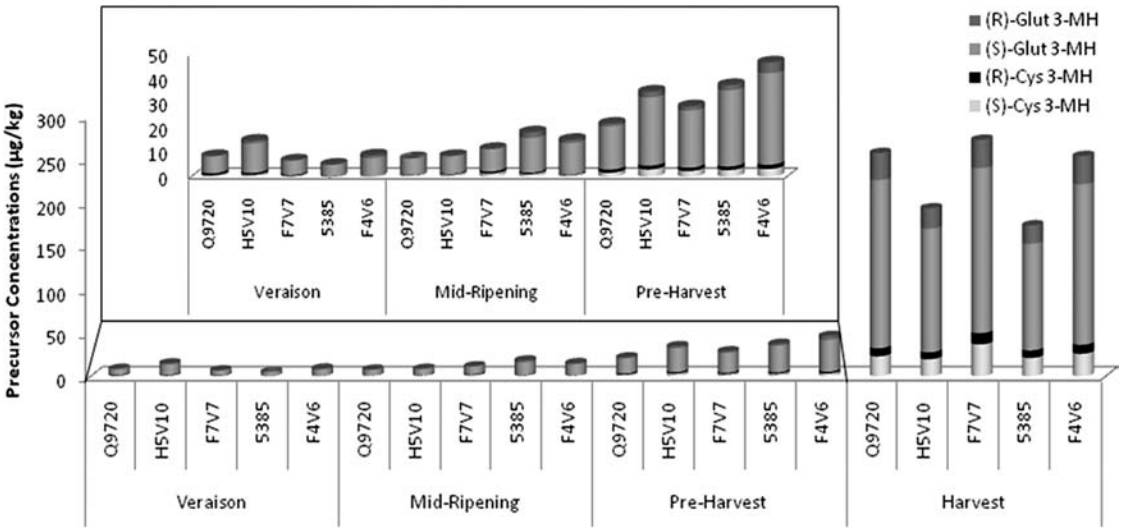

Figure 2. Concentrations of 3-MH precursor diastereomers (μg/kg) during ripening for five Sauvignon blanc clones colocated in an Adelaide Hills vineyard. The stacked bars represent the mean of each precursor diastereomer derived from three replicates. The relative standard deviations for the averages were <15%. (Inset) Expansion of the first three time points.

Table 3. Concentrations of 3-MH Determined during Ripening and after Fermentation for Five Sauvignon blanc Clones Co-located in an Adelaide Hills Vineyard

	3-MH (ng/L)				
	veraison	mid-ripe	preharvest	harvest	after fermentation
min	nd[a]	90	94	66	313
max	nd	99	110	121	419
av		95.2	98.8	82	362.4
SD		4.8	6.5	23.3	50.7

[a] nd, not detected.

maturity stages. To the best of our knowledge this was the first time that 3-MH was directly measured during grape ripening. In addition, this appeared to constitute the first study in which actual 3-MH concentrations have been determined in grape juice without any exogenous enzymatic treatment, highlighting the utility and sensitivity of this GC-MS method. Concentrations of 3-MH were virtually undetectable at veraison and then relatively static, at approximately 100 ng/L, until harvest (Table 3). The dramatic rise observed for the precursors late in the ripening process (Figure 2) was not reflected in the 3-MH concentrations. At maturity, grapes from the different clones were crushed and fermented on a 30 L scale using a single yeast strain. After fermentation and bottling, 3-MH concentrations were determined at modest levels (around 310−420 ng/L, Table 3). The differences between 3-MH concentrations in the individual wines did not appear to be directly related to clone precursor concentrations at harvest, however. That is, juices with higher precursor concentrations did not necessarily lead to wines with higher 3-MH concentrations.

From Grape to Wine. Small-scale winemaking was undertaken in an attempt to correlate precursor and free 3-MH levels under more controlled conditions. Free-run juice precursor concentrations and precursor and free thiol concentrations after wine bottling for the five Sauvignon blanc clones appear in Table 4. The level of ripeness for the clones was very similar at harvest (Supplementary Table 1 in the Supporting Information), thereby revealing an apparent but unconfirmed clonal influence on the precursors present in the respective juices. Clone F7V7 had the greatest amounts of both cysteine and glutathione conjugates in the free run juice, followed by clone H5V10. This could be interesting from a potential flavor perspective, yet these differences appear to be clouded once the juices were fermented and the wines were bottled. In the bottled wines, the greatest amount of 3-MH was found in the wine from clone H5V10, closely followed by F4V6 and then F7V7 (Table 4). The presence of precursors in the finished wines was not surprising, although Cys-3-MH was virtually absent, and the levels of remaining Glut-3-MH were typically lower than found for commercial wines. These results emphasize the potential differences between small-scale and commercial grape processing and fermentation operations and that the relationship between precursors and free thiol is not necessarily straightforward. One of the main discrepancies is the fact that much more precursor is consumed during alcoholic fermentation than free thiol is liberated. Conversion yields of the precursors during fermentation are typically around 10% or less for Cys-3-MH and even lower (<5%) for Glut-3-MH,[3,6,24,30,31] so studies that account for the disappearance of precursors are still required.

Effect of Grape Berry Processing. To assess processing effects, juices from the five Sauvignon blanc clone harvest samples were prepared in two different ways for analysis. One mode of juice preparation involved homogenizing the grape berries with a stick mixer, and the other utilized a 50 kg bag press to obtain free run juice for winemaking. The effect of the processing method can be seen in Table 4, where Glut-3-MH was the main precursor affected, with an average 1.5-fold increase for homogenized samples and significant differences in average Glut-3-MH concentrations between the juice preparation methods for four of the five clone types. The impact of juice preparation method was less apparent for Cys-3-MH in most cases. The stick mixer naturally leads to a great deal of berry damage; we theorize that it gives close to maximum precursor extraction compared to a press and allows for greater potential for oxidation events. Winery press operations may attempt to

Table 4. Total Concentrations of Respective 3-MH Precursor Diastereomers Determined for the Juices at Harvest Prepared Using a Winery Press or Stick Mixer, along with Precursor Concentrations and Free 3-MH in Wine after Bottling for the Five Sauvignon blanc Clones[a]

	precursor totals (μg/L)						free thiol (ng/L)
	Cys-3-MH		Glut-3-MH		Cys-3-MH	Glut-3-MH	3-MH
clone	stick mixer av (SD)[b]	free run av (SD)	stick mixer av (SD)	free run av (SD)	wine	wine	wine
Q9720	50.6 (4.0) bc	44.1 (2.0) b	354.9 (9.7) **d**	167.5 (4.2) **a**	0.9	61.5	313
H5V10	41.9 (3.9) b	47.5 (0.4) b	253.7 (5.4) **bc**	222.3 (0.8) **b**	0.7	112.6	419
F7V7	79.1 (6.0) e	60.3 (1.1) d	355.8 (25.2) **d**	265.3 (16.1) **c**	0.9	113.0	358
5385	47.8 (3.0) b	44.6 (0.6) b	232.4 (19.1) **bc**	183.9 (3.5) **a**	0.8	66.8	313
F4V6	57.2 (1.3) cd	30.8 (0.8) a	345.0 (20.3) **d**	182.4 (3.4) **a**	3.7	99.5	409

[a] There was a statistically significant difference ($p < 0.001$) as a result of preparation method for both precursor types. [b] Av, average concentration of three samples; SD, standard deviation of average for three samples; averages followed by the same letter are not significantly different ($p = 0.05$) when values for a single type of precursor are compared (Glut-3-MH differentiated with bold letters).

Table 5. Total Concentrations of Cys-3-MH and Glut-3-MH Precursors Determined for Sauvignon blanc Juices That Were Analyzed Fresh and after Freezing, along with Juices Prepared from Frozen and Thawed Grape Berries[a]

	precursor totals, av (SD)[b] (μg/L)					
	Cys-3MH			Glut-3-MH		
clone	fresh juice	frozen juice	frozen grapes	fresh juice	frozen juice	frozen grapes
F4V6	57.2 (1.2) cd	47.7 (1.6) abc	61.4 (3.7) d	345.0 (20.4) **b**	341.0 (9.3) **b**	1608.7 (34.1) **f**
5385	47.8 (3.0) abc	36.8 (3.4) a	41.9 (1.3) ab	232.4 (19.0) **a**	197.4 (22.5) **a**	795.2 (45.1) **c**
H5V10	41.9 (3.9) ab	35.4 (4.2) a	73.7 (8.0) e	253.7 (5.4) **a**	250.9 (8.9) **a**	1079.0 (21.9) **d**
Q9720	50.6 (4.0) bcd	43.1 (1.2) ab	52.3 (2.6) bcd	354.9 (9.7) **b**	326.4 (12.8) **b**	1291.0 (26.2) **e**

[a] There was a statistically significant difference ($p < 0.001$) as a result of storage for both precursor types. [b] Av, average concentration of three samples; SD, standard deviation of average for three samples; averages followed by the same letter are not significantly different ($p = 0.05$) when values for a single type of precursor are compared (Glut-3-MH differentiated with bold letters).

limit oxidation, yet some oxidation is required upon berry crushing to form (*E*)-2-hexenal.[32] Therefore, it would seem that techniques which aim to prevent oxidation through careful juice preparation are in effect preventing the formation of Glut-3-MH upon crushing, thereby yielding low Glut-3-MH concentrations. This could be one of the reasons for the differences in Glut-3-MH concentrations between our results from commercial and experimental juices and the work of Roland et al.,[25] although the magnitude of the differences is not fully explained by our observations. The scale of the studies also seems to have an impact, with commercial operations generally yielding higher juice precursor levels.

Impact of Freezing on Precursor Concentrations. Although fresh fruit was used for all of our investigations, we wanted to assess the impact of freezing grape bunches and grape juice on precursor levels. Freezing may be required for large vintage trials, when instrument access is impeded, or when fruit samples have to be sent to a phylloxera-free zone and freezing is unavoidable for quarantine reasons. We found a marked difference in precursor concentrations between freezing grapes compared to freezing juice, most notably for Glut-3-MH (Table 5). Freezing juice had virtually no impact, whereas freezing grapes had a dramatic effect on Glut-3-MH, increasing the apparent concentration by up to 5-fold compared to analysis of juice from fresh homogenates. Interestingly, although Cys-3-MH concentrations were also affected, fresh juice average precursor values were not significantly different from either frozen juice or juice prepared from frozen and thawed berries in most cases. These results point to berry damage as the cause of the differences, where glutathione conjugate formation rather than improved extraction from frozen and thawed berries was the major contributor. Together with our observations for the clones during berry ripening, these data led us to believe that Cys-3-MH is already present in the berry, whereas Glut-3-MH is primarily formed after berry damage. The current and previous[19] results for Glut-3-MH in commercial samples are generally much higher than other reports for Glut-3-MH,[25,31] whereas our Cys-3-MH determinations are fully in accord with other studies, as discussed in Capone et al.[19] This does not seem to signify differences in analytical techniques but rather indicates an effect of juice processing on Glut-3-MH determinations.

Combining precursor analysis by HPLC-MS/MS with reliable quantitation of derivatized 3-MH by GC-MS has enabled us to gain a better understanding of the concentrations and profiles of precursors and free thiol. However, we did not find a correlation between precursors and 3-MH for ferments conducted on different scales. An interesting observation was that 3-MH and its precursors were present in high quantities not only in Sauvignon blanc but also in other varieties. Furthermore, large amounts of precursors remain in bottled wine, which could have an effect on 3-MH concentrations during aging or upon consumption via in-mouth release. Through this study we have shown various factors that can affect precursor concentrations and showed that 3-MH can be detected in juices using our modified GC-MS method. Determinations during grape

ripening revealed low concentrations of precursors until approximately 2 weeks before harvest, followed by a drastic increase of precursors in the harvest samples, demonstrating the impact that harvest timing can have on precursor concentrations. We have also shown the effects of storage and processing on precursors, where freezing grape berries as opposed to juice and the mode of juice preparation had large effects on Glut-3-MH concentrations. These are important results and highlight that care needs to be taken when interpreting data, comparing results from different studies, and determining appropriate sample preparation techniques.

It is now apparent that Glut-3-MH formation, and subsequent metabolism to Cys-3-MH in grape berries, can occur as a stress response,[33] as is the case for glutathione conjugation in other plants.[34,35] On the other hand, Dixon et al. consider that glutathione conjugate formation arises during the winemaking process.[36] We have shown that Cys-3-MH concentrations are relatively unaffected by some storage and processing operations, whereas Glut-3-MH concentrations are markedly altered. This leads us to believe that for healthy grape berries, most of the Cys-3-MH identified in juice is already present in the berry, whereas most of the Glut-3-MH is formed in the juice, with the conjugation step occurring through chemical and/or biological processes. Although further exploration is required, this hypothesis is supported by the work of Roland et al., who showed that addition of oxygen to juice prepared under highly reductive conditions (which would limit the usual formation of (*E*)-2-hexenal) increased Glut-3-MH levels but did not affect Cys-3-MH.[25] This highlights the importance of oxygen in the enzymatic formation of (*E*)-2-hexenal, which necessarily precedes formation of thiol conjugates. Under ordinary juice preparation conditions, when (*E*)-2-hexenal is not limiting, large amounts of Glut-3-MH can be formed as we have shown.

■ ASSOCIATED CONTENT

Ⓢ Supporting Information. Table and figures displaying basic analytical data for Sauvignon blanc clone juices and wines. This material is available free of charge via the Internet at http://pubs.acs.org.

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■ ABBREVIATIONS USED

Cys-3-MH, 3-*S*-cysteinylhexan-1-ol; Glut-3-MH, 3-*S*-glutathionylhexan-1-ol; SIDA, stable isotope dilution analysis; SPE, solid phase extraction; GC-MS, gas chromatography−mass spectrometry; HPLC-MS/MS, high-performance liquid chromatography−tandem mass spectrometry; 3-MH, 3-mercaptohexan-1-ol; SIM, selected ion monitoring; SPME, solid-phase microextraction; LOD, limit of detection; LOQ, limit of quantitation; EI, electron ionization; PFBBr, 2,3,4,5,6-pentafluorobenzyl bromide; PFBn, 2,3,4,5,6-pentafluorobenzyl; EDTA 2Na, ethylenediaminetetraacetic acid disodium salt; DBU, 1,8-diazabicyclo [5.4.0]undec-7-ene.

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