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ARTICLE

Analysis of Precursors to Wine Odorant 3-Mercaptohexan-1-ol Using HPLC-MS/MS: Resolution and Quantitation of Diastereomers of 3-*S*-Cysteinylhexan-1-ol and 3-*S*-Glutathionylhexan-1-ol

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A method has been developed and validated for the analysis of the individual diastereomers of 3-*S*-cysteinylhexan-1-ol (Cys-3-MH) and 3-*S*-glutathionylhexan-1-ol (Glut-3-MH) extracted from grape juice and wine. The method uses stable isotope dilution analysis (SIDA) combined with solid-phase extraction (SPE) and reversed-phase high-performance liquid chromatography−tandem mass spectrometry (HPLC-MS/MS) for quantitation. These compounds have been considered as potential precursors to the important wine odorant 3-mercaptohexan-1-ol (3-MH). This constitutes the first analytical method where (1) Glut-3-MH has been accurately quantified in grape juice and wine and (2) the individual Cys- and Glut-3-MH diastereomers were separated and quantified by a single HPLC-MS/MS method. The use of deuterium-labeled internal standards has resulted in an accurate and precise method that can achieve quantitation limits of <0.5 μg/L for the individual Cys- and Glut-3-MH diastereomers in grape juice and white wine. The method has been applied to the determination of 3-MH precursor diastereomers in various white juice and wine samples. Overall, Glut-3-MH was always more abundant than Cys-3-MH in the juices and wines examined, regardless of grape variety. Stereochemically, (*S*)-Glut-3-MH generally dominated over the (*R*)-diastereomer in the juices and wines, but there was not such a marked difference between the distribution of Cys-3-MH diastereomers. These results have important implications for understanding the formation of wine flavor, and the application of this method will allow further exploration of precursors to the varietal thiol 3-MH.

KEYWORDS: Wine aroma; varietal thiols; wine thiol precursors; analysis; SIDA; HPLC-MS/MS

INTRODUCTION

Since their discovery in wine, the volatile thiols 4-mercapto-4-methylpentan-2-one (4-MMP) and 3-mercaptohexan-1-ol (3-MH) have been the focus of a considerable amount of research regarding their contribution to wine aroma. These particular thiols are characterized by extremely low odor detection thresholds; reported thresholds for 4-MMP and racemic 3-MH are 3 ng/L in white and red wines (*1*) and 60 ng/L in aqueous ethanol solution (*2*), respectively. Furthermore, the enantiomers of 3-MH have different reported odor qualities; the (*R*)-form is described as being reminiscent of "grapefruit" and "citrus peel", whereas the (*S*)-form has a characteristic aroma of "passion fruit" (*3*). However, the odor descriptors attributed to 3-MH enantiomers may also relate to the concentration of the solutions used for sensory evaluation, as reported by Fretz et al. for racemic 3-MH (*4*). This may explain why no sensory difference between the enantiomers of 3-MH has also been reported (*5*).

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As impact odorants in several wine varieties, especially Sauvignon Blanc, these thiols exist in grape juices/musts as odorless, nonvolatile conjugates, which are cleaved by the action of yeasts and/or enzymes. Previously, we prepared the two individual diastereomers of 3-*S*-cysteinylhexan-1-ol (Cys-3-MH) and showed that they were cleaved in a stereospecific manner by both apotryptophanase enzyme and *Saccharomyces cerevisiae* yeast (*6*). More recently, we prepared the diastereomerically pure (*R*)-glutathione conjugate and showed that it was converted to (*R*)-Cys-3-MH and (*R*)-3-MH stereospecifically (*7*).

Various methods have been developed to measure the cysteine conjugates of 3-MH in grape juice or must, with the first method developed by Peyrot des Gachons et al. (*8*). This study attempted to measure Cys-3-MH, 4-*S*-cysteinyl-4-methylpentan-2-one (Cys-4-MMP), and 4-*S*-cysteinyl-4-methylpentan-2-ol (Cys-4-MMPOH) indirectly by enzymatic cleavage using a tryptophanase column to yield the free thiols. The samples were subsequently analyzed by gas chromatography−mass spectrometry (GC-MS) using monodeuterated internal standards (*8*). However, this approach was not necessarily specific to cysteine conjugates, as other precursors

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might have contributed to the results. Subsequently, a method was developed by Murat et al. (*9*) for measuring Cys-3-MH directly, which involved a lengthy purification procedure using affinity chromatography. A ^{15}N-labeled internal standard was used, along with derivatization and measurement of the trimethylsilyl (TMS) derivatives using GC-MS (*9*).

More recently, three additional methods were developed for Cys-3-MH analysis. The method described by Thibon et al. (*10*), which used *S*-benzyl cysteine as internal standard, has been the only example published that can be used to determine the concentrations of the individual diastereomers of Cys-3-MH. The sample preparation was based on the method of Murat et al. (*9*), that is, isolation with affinity chromatography, but using perfluoroacylation instead of TMS derivatization. The derivatives were analyzed by GC using an ion trap tandem MS (GC-MS/MS) (*10*). A similar method was also developed by Subileau et al. (*11*) to quantify the unresolved diastereomers of Cys-3-MH after purification through a Dowex resin, using d_2-Cys-3-MH as an internal standard. The final step was derivatization with ethyl chloroformate and analysis by GC-MS (*11*). A third method, reported by Luisier et al. (*12*), quantified the unresolved diastereomers of Cys-3-MH using d_2-Cys-3-MH as an internal standard. This method was less time-consuming for sample preparation, which involved passing must or wine through a solid-phase extraction (SPE) cartridge. In contrast to the other methods, the samples were analyzed by reversed-phase high-performance liquid chromatography mass spectrometry (HPLC-MS) (*12*), which is more compatible with these nonvolatile, water-soluble compounds.

After evaluating the available methods we chose to combine SPE and HPLC-MS analysis to develop a method that could be used to separate and quantify both diastereomers of Cys-3-MH and, for the first time, the individual diastereomers of Glut-3-MH in a single analysis. The validated method employed the use of deuterium-labeled standards and HPLC-MS/MS of the analytes using multiple reaction monitoring (MRM). A selection of white juice and wine samples was evaluated for precursor content and diastereomer distribution. Synthesis of standards and determination of diastereomer elution order and stereochemical relationship to the free thiol 3-MH were discussed in a separate publication (*7*).

MATERIALS AND METHODS

Materials. Samples were obtained from retail outlets (dry white wine) or supplied by Australian producers (wine and juice). Juice and wine samples from the producers were stored at −20 °C until required for analysis. All isotopically labeled and unlabeled standards were synthesized according to the procedures of Pardon et al. (*6*) and Grant-Preece et al. (*7*). The standards used were *S*-[(1*R*/*S*)-1-(2-hydroxyethyl)butyl]-L-cysteine (referred to as (*R*/*S*)-3-*S*-cysteinylhexan-1-ol or (*R*/*S*)-Cys-3-MH); *S*-[(1*R*/*S*)-1-(2-hydroxyethyl)butyl-1,2,2,3,3,4,4,4-d_8]-L-cysteine (referred to as d_8-(*R*/*S*)-3-*S*-cysteinylhexan-1-ol or d_8-(*R*/*S*)-Cys-3-MH); γ-L-glutamyl-*S*-[(1*R*/*S*)-1-(2-hydroxyethyl)butyl]-L-cysteinylglycine (referred to as (*R*/*S*)-3-*S*-glutathionylhexan-1-ol or (*R*/*S*)-Glut-3-MH); and 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 (referred to as d_9-(*R*/*S*)-3-*S*-glutathionylhexan-1-ol or d_9-(*R*/*S*)-Glut-3-MH). Stock solutions of standards were prepared volumetrically in Milli-Q water and stored at −20 °C, and working solutions were stored at 4 °C until required. All chromatographic solvents were of HPLC grade; 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 were purchased from Rowe Scientific (Lonsdale, SA, Australia), and chemicals were obtained from either Sigma-Aldrich (Castle Hill, NSW, Australia) or BDH (Kilsyth, VIC, Australia). All prepared solutions were % v/v with the balance made up with Milli-Q water, unless otherwise specified. The following SPE cartridges were sourced: Bond Elut LMS, 500 mg, 3 mL, and Bond Elut PPL, 500 mg, 6 mL (Varian, Mulgrave, VIC, Australia); Lichrolut EN, 500 mg, 6 mL (Merck, Darmstadt, Germany); Supelclean ENVI 18, 500 mg, 3 mL (Supelco, Bellefonte, PA); and Strata SDB-L, 500 mg, 6 mL (Phenomenex, Lane Cove, NSW, Australia). The following reversed-phase HPLC columns were evaluated: Synergi Hydro-RP 150 × 2 mm i.d., 4 μm, 80 Å, and Gemini C6-Phenyl 150 × 2 mm i.d., 3 μm, 110 Å (Phenomenex); Alltima C18 250 × 2.1 mm i.d., 5 μm, 100 Å (Grace Davison Discovery Sciences, Baulkham Hills, NSW, Australia).

Table 1. Mass Transitions Chosen for MRM of 3-MH Precursors

analyte	Q1 *m*/*z*	Q3 *m*/*z*	internal standard	Q1 *m*/*z*	Q3 *m*/*z*
(*R*/*S*)-Cys-3-MH	222.2	205.1	d_8-(*R*/*S*)-Cys-3-MH	230.2	213.1
		101.2			109.2
		83.1			90.2
(*R*/*S*)-Glut-3-MH	408.2	333.4	d_9-(*R*/*S*)-Glut-3-MH	417.3	342.5
		279.3			288.5
		262.4			271.2
		162.3			162.1

Preparation of Juice and Wine Extracts for Analysis. An aliquot (100 μL) of an aqueous solution containing both diastereomers of d_8-(*R*/*S*)-Cys-3-MH and d_9-(*R*/*S*)-Glut-3-MH (final concentrations of 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), 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 with a gentle stream of nitrogen to approximately 0.5 mL for HPLC-MS/MS analysis.

HPLC-MS Instrumentation. All HPLC-MS analyses were carried out 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 TurboIonSpray probe (Applied Biosystems/MDS Sciex, Concord, ON, Canada). Data acquisition and processing were performed using Applied Biosystems/MDS Sciex Analyst software (version 1.5).

HPLC Conditions. The column was a 250 × 2.1 mm i.d., 5 μm, 100 Å Alltima C18 (Grace Davison Discovery Sciences) operated at 25 °C and protected by a 4 × 2 mm i.d. C18 guard column (Phenomenex). The solvents were 0.5% aqueous formic acid (solvent A) and 0.5% formic acid in acetonitrile (solvent B), with a flow rate of 0.300 mL/min. The gradient for solvent B was as follows: 0 min, 5%; 10 min, 15%; 20 min, 30%; 21 min, 80%; and 25 min, 80%. The column was equilibrated with 5% B for 10 min prior to an injection. A 10 μL injection volume was used for each sample.

Mass Spectrometer Conditions. All mass spectrometric data were obtained in positive-ion mode. Nitrogen was used for curtain gas, 103.4 kPa; nebulizing gas, 344.7 kPa; drying gas, 344.7 kPa; and collision gas, high. The ion spray voltage, declustering potential, source temperature, and collision energy were set at 5500 V, 45 V, 500 °C, and 20 eV, respectively. For MRM, Q1 and Q3 had unit resolution and the transitions chosen (**Table 1**) had a dwell time of 100 ms each. MRM parameters were optimized with infusion MS/MS experiments of pure synthetic reference compounds (approximately 1 mg/L) diluted with 40% aqueous acetonitrile containing 0.5% formic acid, using an infusion pump operating at 5 μL/min.

Analytical Method Validation. The analytical method was validated by a series of standard additions of unlabeled Cys- and Glut-3-MH diastereomers (duplicate analyses of 0, 0.25, 2.5, 5, 12.5 25, 50, and 100 μg/L of each diastereomer) to (1) a Riesling juice (Brix 18°, pH 2.97, titratable acidity = 5.5 g/L, SO_2 (free) = 9 mg/L, SO_2 (total) = 32 mg/L) and (2) a fresh dry white wine (10.8% ethanol, pH 3.44, titratable acidity = 5.9 g/L, SO_2 (free) = 20 mg/L, SO_2 (total) = 172 mg/L). To determine the precision of the analysis, seven replicate samples were spiked at two different concentrations, 5 and 50 μg/L, for the individual Cys-3-MH and Glut-3-MH diastereomers. The samples were extracted and analyzed according to the method.

For quantifying the analytes in batches of unknown samples, duplicate standards were prepared at the same time as the juice and wine samples, by adding internal standard (100 μL) to solutions of the synthesized analytes in water (9.9 mL), with individual analytes at concentrations of 0, 5, 25, 50,

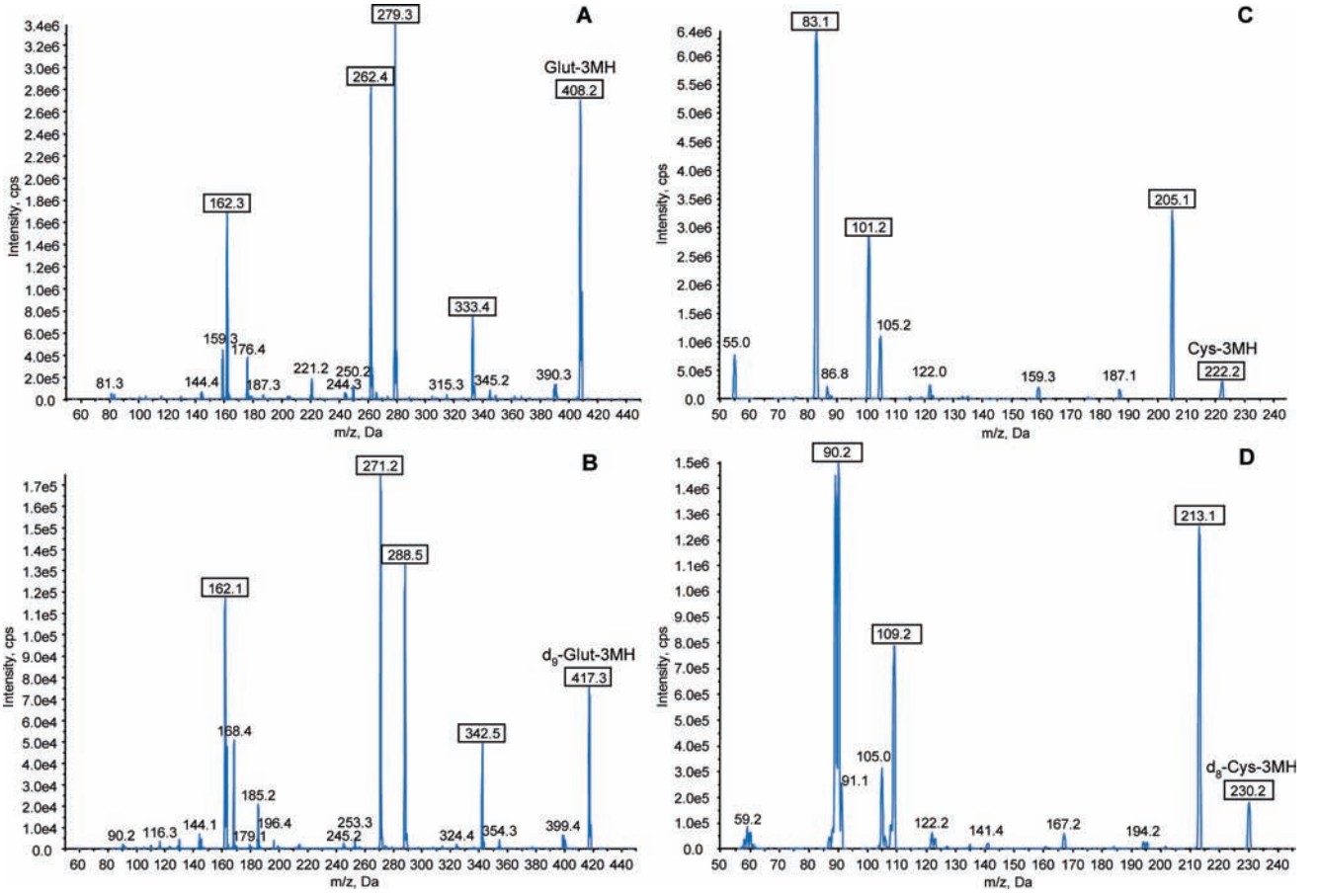


Figure 1. Mass spectra of (**A**) (*R*/*S*)-Glut-3-MH, (**B**) d_9-(*R*/*S*)-Glut-3-MH, (**C**) (*R*/*S*)-Cys-3-MH, and (**D**) d_8-(*R*/*S*)-Cys-3-MH obtained from infusions of standards. The boxed numbers relate to the transitions chosen for the HPLC-MS/MS method (see **Table 1**).

100, and 250 μg/L (with an additional level of 600 μg/L for Glut-3-MH). To ensure that the accuracy of the analysis was maintained, duplicate control samples, spiked with 0 and 25 μg/L of the individual diastereomers, were included with every set of samples to be quantified.

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 limit of detection (LOD) and limit of quantitation (LOQ) for each precursor diastereomer were determined by multiplying the standard error of the *y*-intercept by 3.3 (for LOD) and 10 (for LOQ) and dividing these values by the slope of the calibration curve for each standard. The standard error of the mean (SEM) for HPLC retention times and method repeatability samples was determined by dividing standard deviations by the square root of the number of replicate injections. Statistical analyses were performed with Microsoft Excel 2003, with the LINEST function used to obtain calibration curve slopes and intercepts, and their associated standard errors.

RESULTS AND DISCUSSION

Optimization of MS Parameters. Synthesis of labeled and unlabeled thiol precursors was undertaken according to the methods of Pardon et al. (*6*) and Grant-Preece et al. (*7*). Separate infusion MS/MS experiments of dilute, aqueous solutions (diastereomeric mixtures) of labeled and unlabeled conjugates were performed in positive ionization mode to obtain and optimize the fragmentation pattern of the analytes by collision-induced dissociation (CID). The mass spectra obtained from the infusions for labeled and unlabeled (*R*/*S*)-Cys- and (*R*/*S*)-Glut-3-MH can be seen in **Figure 1**. For (*R*/*S*) Cys-3-MH, the fragment ion *m*/*z* 205 resulted from the neutral loss of ammonia (17 Da) from the protonated molecular ion, the fragment ion *m*/*z* 101 resulted from the neutral loss of cysteine (121 Da) from the protonated molecular ion, and the fragment ion *m*/*z* 83 resulted from the neutral loss of water (18 Da) from fragment ion *m*/*z* 101. For (*R*/*S*)-Glut-3-MH, the fragment ion *m*/*z* 333 resulted from the neutral loss of glycine (75 Da) from the protonated molecular ion, the fragment ion *m*/*z* 279 resulted from the neutral loss of glutamate (129 Da) from the protonated molecular ion, the fragment ion *m*/*z* 262 resulted from the loss of ammonia (17 Da) from fragment ion *m*/*z* 279, and the fragment ion *m*/*z* 162 resulted from the neutral loss of hexenal (100 Da) from fragment ion *m*/*z* 262. The labeled analogues fragmented in an identical manner, albeit with differences in the masses of various fragments due to the labeled positions. The infusions resulted in the choice of mass transitions selected for MRM experiments (**Table 1**) and optimization of the MS parameters.

Method Optimization. Stable isotope dilution analysis (SIDA) was chosen because labeled analogues of the compounds of interest are the best choice of internal standard to compensate for analyte losses and matrix effects (*13*). The use of SIDA significantly reduces variability that can occur during sample preparation, injection, and detection, leading to a more reliable determination. Furthermore, whereas the peaks for labeled internal standard and analyte are only sometimes resolved chromatographically, they can be unambiguously resolved by a mass spectrometer.

The HPLC parameters were optimized for separation of the individual diastereomers of Cys-3-MH and Glut-3-MH. Initially, acetic acid was used to acidify the aqueous and acetonitrile mobile phases, and a number of columns were evaluated with different gradients. The 150 mm Synergi Hydro and C6-Phenyl columns

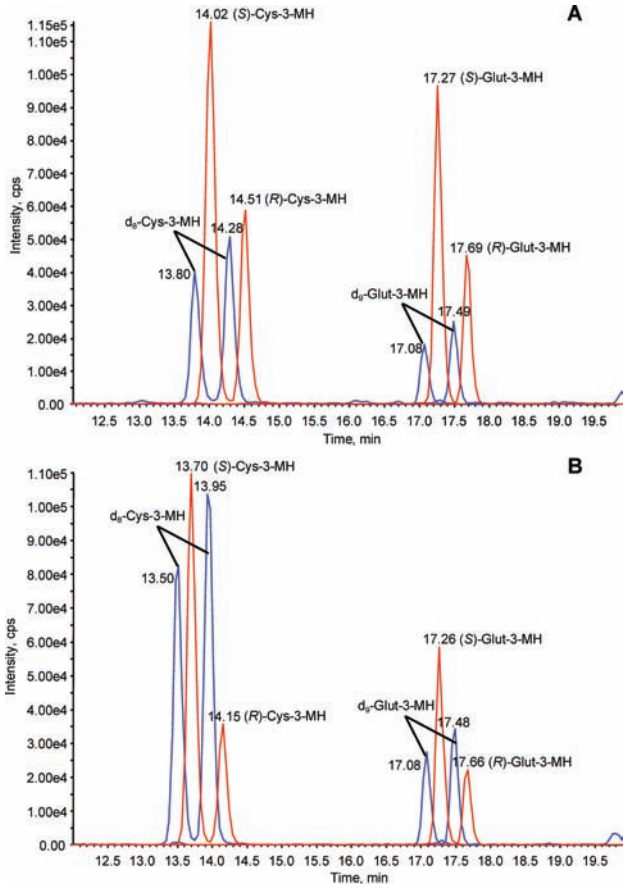


Figure 2. Representative HPLC-MS/MS chromatogram overlays of (**A**) a Sauvignon Blanc juice and (**B**) a Sauvignon Blanc wine.

showed little to no separation of diastereomers. Swapping to the longer Alltima C18 column led to improvements in diastereomer resolution, and a switch to formic acid to acidify the mobile phases led to more symmetrical peak shapes. Further gradient optimization enabled separation of precursor diastereomers in a relatively short analysis time (see **Figure 2**). Assignment of elution order of the precursor diastereomers of 3-MH was reported in Grant-Preece et al. (*7*).

For optimization of the extraction procedure, various cartridges were trialed with different degrees of success. Generally, there was variability in the retention of analytes upon loading, where the two precursor types behaved slightly differently depending on the nature of the SPE sorbent (data not shown). After the sample was loaded onto the SPE cartridge, investigations were carried out to determine the effect of a water wash prior to sample elution and whether the concentration step after analyte elution was necessary. Although the water wash led to slightly cleaner samples, overall it was not beneficial as the recovery of compounds decreased due to loss of some analytes in the washings (data not shown). As a result, the water wash was not included in the final optimized method. Concentration of the extract after sample elution was required, however, for optimum sensitivity of the analysis. Ultimately, Strata SDB-L gave the best results when evaluating the overall recovery of the different analytes from the various sorbents, although Bond Elut PPL performed almost as well, followed closely by Lichrolut EN. The remaining cartridges investigated (Supelclean ENV 18 and Bond Elut LMS) behaved similarly to each other, with an approximate 40% reduction in analyte recovery compared to Strata SDB-L.

Method Validation. The standard addition curves obtained for individual Cys- and Glut-3-MH diastereomers were linear throughout the concentration range, with a coefficient of determination (R^2) ranging from 0.9845 to 0.9998 in a Riesling juice and from 0.9849 to 0.9979 in a dry white wine (**Table 2**). Slopes of the validation curves were virtually equal and independent of the matrix (data not shown). For simplicity and to obtain a true zero level, water was chosen as the matrix for generating calibration curves when analytes in batches of samples were quantified.

Table 2. Validation Parameters for Analysis of Cys- and Glut-3-MH by HPLC-MS/MS

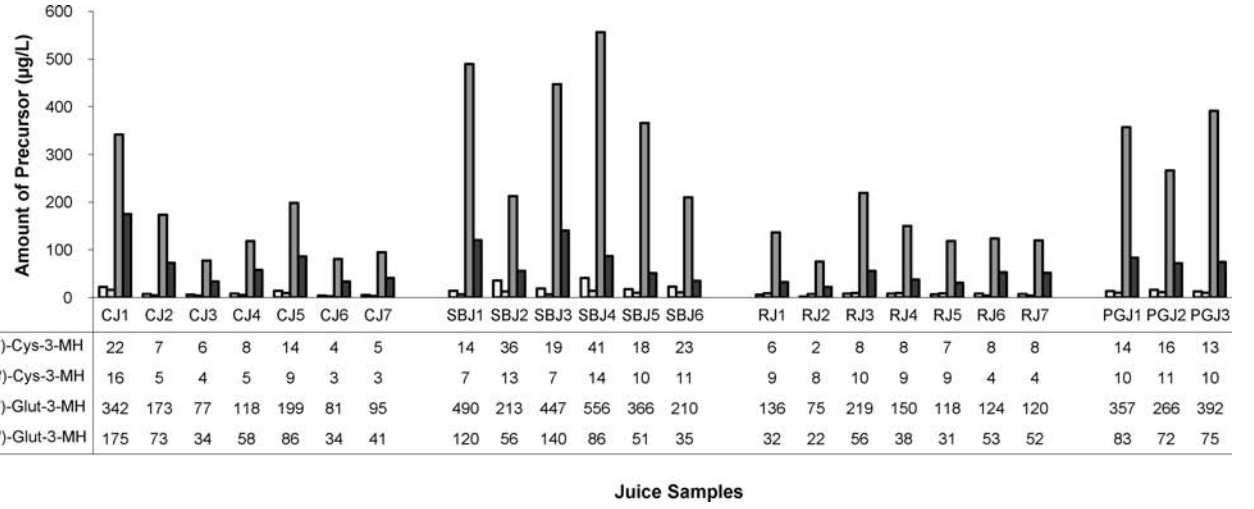
		SEM[a]					
compound	curve R^2	5 μg/L	50 μg/L	LOD[b]	LOQ[c]	av RT[d]	RT SEM[e]
			Riesling Juice				
Cys-3MH							
(*S*)-	0.9998	0.20	0.73	0.01	0.03	14.16	0.10
(*R*)-	0.9998	0.22	0.71	0.02	0.06	14.62	0.10
Glut-3-MH							
(*S*)-	0.9845	0.40	0.79	0.09	0.28	17.27	0.08
(*R*)-	0.9937	0.34	0.52	0.08	0.26	17.70	0.07
			Dry White Wine				
Cys-3MH							
(*S*)-	0.9979	0.34	0.95	0.04	0.12	14.07	0.05
(*R*)-	0.9971	0.05	0.19	0.04	0.12	14.54	0.04
Glut-3-MH							
(*S*)-	0.9849	0.49	0.72	0.13	0.39	17.31	0.05
(*R*)-	0.9963	0.23	0.60	0.08	0.24	17.74	0.04

[a] SEM, standard error of the mean (μg/L) for repeatability ($N = 7$). [b] LOD, limit of detection (μg/L). [c] LOQ, limit of quantitation (μg/L). [d] av RT, average retention time (minutes) of validation samples ($N = 30$). [e] RT SEM, analyte retention time standard error of the mean ($N = 30$).

The precision of the analysis, determined by spiking seven replicate samples with internal standard at two concentrations (5 and 50 μg/L) of the individual Cys-3-MH and Glut-3-MH diastereomers, is given in **Table 2**. The resulting standard errors ranged from 0.20 to 0.40 μg/L in a Riesling juice and from 0.05 to 0.49 μg/L in a dry white wine for the diastereomers at 5 μg/L (**Table 2**). Standard errors for replicates spiked with the diastereomers at 50 μg/L ranged from 0.52 to 0.79 μg/L in a Riesling juice and from 0.19 to 0.95 μg/L in a white wine. The method sensitivity was excellent, with calculated LOQs for the diastereomers of 0.03−0.28 μg/L in a Riesling juice and slightly higher at 0.12−0.39 μg/L for a dry white wine.

Evaluation of Diastereomer Distribution in Juice and Wine. After the validation was complete, the method was applied to a range of juice and wine samples (**Figure 2** provides an example of each matrix) to determine the distribution of the Cys- and Glut-3-MH diastereomers in these media (**Figures 3** and **4**). It was immediately obvious from **Figure 3** that in every juice sample examined the Glut-3-MH diastereomers (140−640 μg/L combined isomer total) were up to 35 times more abundant than their Cys-3-MH counterparts (10−55 μg/L combined isomer total). Additionally, these presumed 3-MH precursors, commonly associated with Sauvignon Blanc grapes, were found in the greatest quantities in juices of that variety.

Although no quantitative data for Glut-3-MH have been reported previously, the concentrations of Cys-3-MH found in grape juices have been reported by several other groups. The levels of Cys-3-MH encountered in the current study (10−55 μg/L combined isomer total) are broadly similar to those previously determined using direct measurements. Cys-3-MH was found to range from approximately 11 to 35 μg/L in Sauvignon Blanc juice (*11*), from 48 to 78 μg/L in Sauvignon Blanc juice and from 4 to 10 μg/L in Semillon juice (1100−2000 μg/L in Sauvignon



	CJ1	CJ2	CJ3	CJ4	CJ5	CJ6	CJ7	SBJ1	SBJ2	SBJ3	SBJ4	SBJ5	SBJ6	RJ1	RJ2	RJ3	RJ4	RJ5	RJ6	RJ7	PGJ1	PGJ2	PGJ3
(*S*)-Cys-3-MH	22	7	6	8	14	4	5	14	36	19	41	18	23	6	2	8	8	7	8	8	14	16	13
(*R*)-Cys-3-MH	16	5	4	5	9	3	3	7	13	7	14	10	11	9	8	10	9	9	4	4	10	11	10
(*S*)-Glut-3-MH	342	173	77	118	199	81	95	490	213	447	556	366	210	136	75	219	150	118	124	120	357	266	392
(*R*)-Glut-3-MH	175	73	34	58	86	34	41	120	56	140	86	51	35	32	22	56	38	31	53	52	83	72	75

Figure 3. Concentrations (μg/L) of the diastereomers of Cys- and Glut-3-MH found in various juice samples. CJ, Chardonnay juice; SBJ, Sauvignon Blanc juice; RJ, Riesling juice; PGJ, Pinot Grigio juice.

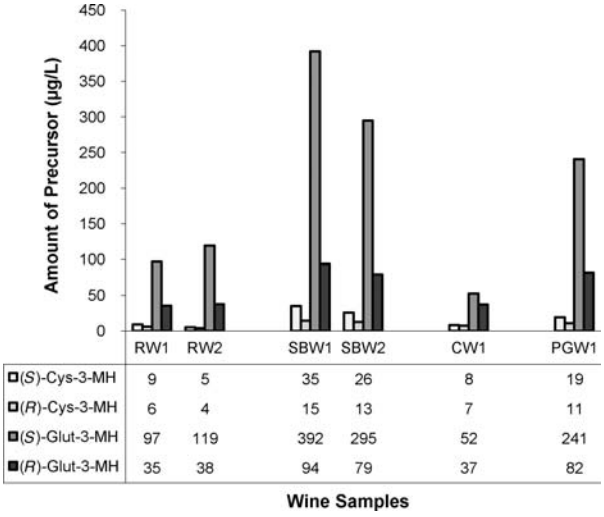
Blanc juice and 520−680 μg/L in Semillon juice from *Botrytis*-affected grapes) (*10*), and from 30 to 85 μg/L (approximately 370 μg/L for a single sample of a different vintage) in 20 clones of healthy Petite Arvine grapes (*12*). Indirect assessment of Cys-3-MH concentrations by assaying 3-MH, formed from enzymatic treatment of a must extract, yielded concentrations for Cys-3-MH of approximately 10−82 μg/L during Sauvignon Blanc berry ripening (*8*) and 35−132 μg/L of Cys-3-MH in Sauvignon Blanc musts prepared with different skin contact times and temperatures (*14*). Considering the amounts of Cys-3-MH that have been reported, the results of the current study add weight to the theory that the cysteine conjugates are minor components (*11*) which arise from their glutathione congeners.

Although the isomer distribution varied, the (*S*)-isomer was generally present in greater quantities than the (*R*)-isomer for both Cys- and Glut-3-MH (**Figure 3**). This inequality was most evident with the Glut-3-MH diastereomers, where the distribution in juices ranged in favor of the (*S*)-diastereomer from approximately 2:1 in Chardonnay to 7:1 in Sauvignon Blanc. The Cys-3-MH distribution in juices ranged from about 1:1 in Chardonnay to 3:1 in favor of the (*S*)-diastereomer in Sauvignon Blanc. Thibon et al. also found that (*S*)-Cys-3-MH was slightly more abundant (1.2:1) in Sauvignon Blanc and Semillon musts, becoming more dominant (2.3:1) in *Botrytis*-affected juices of those two varieties (*10*).

The distribution of (*S*)-Glut-3-MH/(*S*)-Cys-3-MH in the juices varied considerably, ranging from 6:1 in a Sauvignon Blanc to 37:1 in a Riesling. In contrast, the distribution of (*R*)-Glut-3-MH/(*R*)-Cys-3-MH was lower, ranging from 3:1 to 20:1 in a Sauvignon Blanc juice.

It was interesting to note that there were significant amounts of Cys-3-MH (9−50 μg/L combined total) and Glut-3-MH (90−480 μg/L combined total) present in various bottled wine samples (**Figure 4**). Similar to juices, the highest wine precursor amounts were found in Sauvignon Blanc. Wine precursor diastereomer ratios showed relationships similar to those described for the juices. The (*S*)-stereochemistry was dominant for the remaining Glut-3-MH, whereas the Cys-3-MH diastereomer distribution was more even. As for the juices, the ratio (*S*)-Glut-3-MH/(*S*)-Cys-3-MH was larger than that observed for the related (*R*)-diastereomers.

Although this was a limited data set, the amounts of each precursor type and diastereomer distribution, in particular, the ratio of (*S*)-Glut-3-MH to (*R*)-Glut-3-MH, appeared to relate to grape



	RW1	RW2	SBW1	SBW2	CW1	PGW1
(*S*)-Cys-3-MH	9	5	35	26	8	19
(*R*)-Cys-3-MH	6	4	15	13	7	11
(*S*)-Glut-3-MH	97	119	392	295	52	241
(*R*)-Glut-3-MH	35	38	94	79	37	82

Figure 4. Concentrations (μg/L) of the diastereomers of Cys- and Glut-3-MH found in six wine samples. RW, Riesling wine; SBW, Sauvignon Blanc wine; CW, Chardonnay wine; PGW, Pinot Grigio wine.

variety. A more detailed study, however, is required to examine the relationship between grape juice precursor concentrations, grape variety, and the amounts of precursors remaining in the finished wine.

These four juice components (individual diastereomers of Cys- and Glut-3-MH) are potential precursors to 3-MH found in wine, so varying diastereomer ratios in grapes could yield varying amounts of each 3-MH enantiomer in the resultant wine. In all likelihood the Glut-3-MH diastereomers arise from conjugation of the grape components glutathione and 2-hexenal, with the minor cysteine conjugates arising from metabolism of their glutathione counterparts. Observing such a marked distribution of the glutathione diastereomers indicates this results from reaction stereospecificity during their formation, perhaps determined by double-bond geometries of 2-hexenal in an enzymatic process.

This simple method for the determination of individual Cys- and Glut-3-MH diastereomers is suitable for large sample numbers and will allow a raft of important studies to be undertaken. In particular, it is now possible to investigate the link between the stereochemistry of glutathione and cysteine conjugate diastereomers

and the respective enantiomers of important wine thiol 3-MH. The localization of precursor types within the grape berry and the impact of vineyard and winery practices on precursor concentrations could also be further explored. Furthermore, an evaluation of the contribution of Glut-3-MH to the formation of 3-MH during fermentation could lead to the selection of yeast strains that are better able to utilize glutathione precursors, in a similar manner to the yeasts engineered to release enhanced amounts of varietal thiols from their cysteine conjugates. Ultimately, knowledge about these thiol precursors could lead to modulation of the aroma profile of wine varieties, including those not necessarily dominated by tropical and citrus notes.

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; HPLC-MS/MS, high-performance liquid chromatography−tandem mass spectrometry; 3-MH, 3-mercaptohexan-1-ol; 4-MMP, 4-mercapto-4-methylpentan-2-one; Cys-4-MMP, 4-*S*-cysteinyl-4-methylpentan-2-one; Cys-4-MMPOH, 4-*S*-cysteinyl-4-methylpentan-2-ol; GC-MS, gas chromatography−mass spectrometry; TMS, trimethylsilyl; GC-MS/MS, gas chromatography−tandem mass spectrometry; MRM, multiple reaction monitoring; LOD, limit of detection; LOQ, limit of quantitation; SEM, standard error of the mean; CID, collision-induced dissociation.

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