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Effects on 3-Mercaptohexan-1-ol Precursor Concentrations from Prolonged Storage of Sauvignon Blanc Grapes Prior to Crushing and Pressing

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Supporting Information

ABSTRACT: Formation of wine thiol precursors is a dynamic process, which can be influenced by vineyard and winery processing operations. With the aim of increasing thiol precursor concentrations, a study of the effects of storing machine-harvested Sauvignon blanc grapes prior to crushing and pressing was undertaken on a commercial scale. 3-Mercaptohexan-1-ol (3-MH) precursors, 2-*S*-glutathionylcaftaric acid (grape reaction product, GRP), glutathione (GSH) and a number of C6 compounds were assessed at several time points during the experiment. The concentration of the cysteine precursor to 3-MH doubled within 8 h and tripled after 30 h while the GSH and cysteinylglycine precursors increased in concentration roughly 1.5 times. (*E*)-2-Hexenal and GSH levels decreased as thiol precursors, GRP and C6 alcohols increased during storage. Principal component analysis revealed that precursors contributed to most of the variation within the samples over the storage period, with additional influence, primarily from GSH and GRP, as well as (*E*)-2-hexenal and (*Z*)-3-hexen-1-ol. Early storage time points were associated with higher concentrations of GSH and some unsaturated C6 compounds while longer storage times were most closely associated with higher thiol precursor and GRP concentrations. This study provides a detailed overview of interactions related to thiol precursor formation on a commercial scale and highlights the ability to manipulate precursor concentrations prior to grape crushing.

KEYWORDS: *wine aroma, 3-mercaptohexan-1-ol, wine thiol precursors, storage, C6 volatiles, grape reaction product, analysis, synthesis*

INTRODUCTION

Polyfunctional thiols such as 3-mercaptohexan-1-ol (3-MH) are aroma impact compounds which impart characteristic "tropical", "citrus" and "passionfruit" aromas to wine. The term "varietal thiols" may be used to describe such compounds, since they are frequently associated with the Sauvignon blanc grape cultivar. As such, it is not surprising that varietal thiols are of particular importance to the quality of Sauvignon blanc wines and can influence consumer appreciation of certain wine styles.[1,2] Notwithstanding the large body of work associated with understanding the free forms of varietal thiols which provide the aromas in wine,[3] it is of great interest that varietal thiols arise from odorless, grape-derived precursors.[4]

Studies continue to shed light on the formation and fate of the thiol precursors, which include the cysteine[5] and glutathione[6] conjugates of 3-mercaptohexan-1-ol (Cys−3-MH and Glut−3-MH, respectively) (Figure 1). Most recently, the cysteinylglycine conjugate of 3-MH (Cysgly−3-MH, Figure 1) was identified, providing a logical fit with the other thiol precursors considering its biological relationship to them.[7] As seems to be the case with Glut−3-MH,[8] it may turn out that Cysgly−3-MH acts as a pro-precursor, whereby it is first metabolized to Cys−3-MH, with the free thiol 3-MH being liberated by yeast enzyme activity on the cysteine conjugate.[7,9,10] Additionally, a role has been proposed for a conjugated aldehyde intermediate (Glut−3-MHAl, Figure 1), being the product formed from grape components (*E*)-2-hexenal (an oxidation product) and glutathione (GSH, a natural antioxidant), which must necessarily be reduced (enzymatically) to give Glut−3-MH ordinarily found in grape juices.[9] The contributions that Cysgly−3-MH and Glut−3-MHAl make to wine 3-MH concentrations remained undetermined, but their identification has provided new insight into precursor formation and degradation.

The ability to have control over wine aroma profiles would be highly desirable, and this has led to various undertakings to improve varietal thiol concentrations in wine. One avenue for maximizing wine thiol concentrations involves evaluating the impact that yeasts have on thiol release from grape-derived precursors during winemaking.[3] Indeed, yeasts have been genetically engineered to release larger quantities of thiols during fermentation,[11,12] although these were not commercially available strains. As an alternative, researchers have considered the grapes and their juices or musts, examining the localization of precursors within the berry and effects of skin contact and pressing[13−16] in order to make more effective use of what is derived from the raw material.

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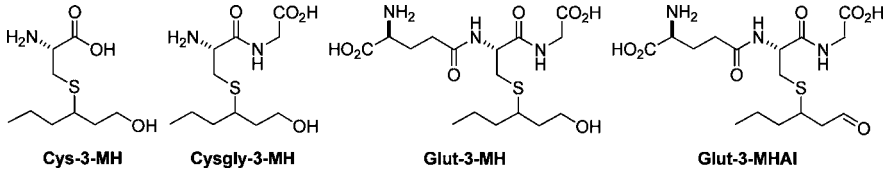

Figure 1. Structures of 3-MH precursors Glut−, Cysgly− and Cys−3-MH found in grape juice, and Glut−3-MHAl which arises from conjugation of GSH and (*E*)-2-hexenal.

Studies relating to the formation of precursors during grape and juice/must processing have revealed that winemaking operations and addition of antioxidants can play a significant role in determining the concentrations of precursors, thereby highlighting their dynamic nature.[7,9,17,18] These are important developments, since they show that precursor profiles can be modified postharvest; they are not simply an indication of what was present in the grape berry. Additionally, the significance of correct harvest timing has been reinforced by monitoring precursors during grape ripening,[18,19] and the varied impacts of harvesting technique have also been reported.[9,20] These outcomes provide opportunities to manipulate precursor concentrations in order to modulate varietal thiols resulting from fermentation.

By considering the potential effects of postharvest operations and with a view to influencing thiol precursor concentrations, we investigated the impact of storing commercial-scale machine-harvested Sauvignon blanc fruit for up to 30 h before crushing and pressing. We determined the evolution of key components such as C6 compounds, glutathione, grape reaction product (GRP) and 3-MH precursors to provide a detailed picture of events surrounding thiol precursor formation. Synthesis of labeled and authentic standards and method development for the analysis of C6 compounds by GC−MS and GRP by HPLC−MS/MS were also undertaken for quantification purposes.

MATERIALS AND METHODS

Materials. Isotopically labeled and unlabeled compounds were previously synthesized according to the procedures of Pardon et al.,[21] Grant-Preece et al.,[8] Kotseridis et al.[22] and Capone et al.[7] The synthesized compounds used were as follows: *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); *S*-[(1*R*/*S*)-1-(2-hydroxyethyl)butyl]-L-cysteinylglycine (Cysgly−3-MH); d_9-(*E*)-2-hexenal. Hexan-1-ol (Ajax Finechem, 98%) was obtained from Rowe Scientific (Lonsdale, SA, Australia), and (*E*)-2-hexenal (98%), (*E*)-2-hexen-1-ol (96%) and (*Z*)-3-hexen-1-ol (98%) were obtained from Sigma-Aldrich (Castle Hill, NSW, Australia). Stock solutions of standards were prepared volumetrically in Milli-Q water (for thiol precursor and GRP analysis) or redistilled ethanol (for C6 analysis) and stored at −20 °C, and working solutions were stored at 4 °C until required. Merck solvents used for synthesis and HPLC−MS analysis were HPLC grade (Rowe Scientific), and all chemicals and reagents were analytical reagent grade (Sigma-Aldrich) unless otherwise stated. *tert*-Butyldimethylsilyl (TBDMS) triflate (98%), 3,4-dihydroxycinnamic (caffeic) acid (99%, predominantly *trans*), (+)-diethyl L-tartrate (98%) and L-glutathione, reduced (97%) were obtained from Alfa Aesar (BioScientific Pty Ltd., Gymea, NSW, Australia), and mushroom polyphenol oxidase (PPO) was purchased from Worthington Biochemical Corporation (Scimar, Templestowe, NSW, Australia). Water was obtained from a Milli-Q purification system (Millipore, North Ryde, NSW, Australia).

Nuclear Magnetic Resonance (NMR) Analysis. Proton (^{1}H) and carbon (^{13}C) NMR spectra were recorded with Bruker Avance III spectrometers operating at 400 or 600 MHz for proton and 100 or 150 MHz for carbon nuclei, respectively. Chemical shifts were recorded as δ values in parts per million (ppm). Spectra were acquired in chloroform-*d* or deuterium oxide (D_2O) at ambient temperature, and resonances were assigned by routine 2D correlation experiments. For ^{1}H NMR spectra, the peak as a result of residual $CHCl_3$ (δ 7.26) or the CH_3 peak of acetonitrile (δ 2.06), added when D_2O was the solvent, was used as the internal reference. For ^{13}C NMR spectra, the central peak of the $CDCl_3$ triplet (δ 77.16) or the CH_3 peak of acetonitrile (δ 1.47), added when D_2O was the solvent, was used as the internal reference.

High-Resolution Mass Spectrometry (HR-MS). Spectra were obtained on a Bruker microTOF-Q II with electrospray ionization (ESI) in positive mode. Samples dissolved in methanol or acetonitrile at concentrations of approximately 1−2 mg/L were analyzed by flow injection.

Melting Points. A Buchi melting point B-540 unit was used, and melting points were uncorrected.

Preparation of (*E*)-2-Hexen-1-ol-1,1,4,4-d_4 (d_4-(*E*)-2-Hexen-1-ol) (3). The synthetic route for this labeled standard is outlined in

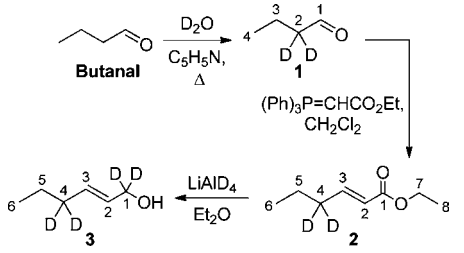

Figure 2. Synthetic approach to d_4-(*E*)-2-hexen-1-ol (**3**) from butanal. Atom numbering shown for compounds relates to the NMR peak assignments.

Figure 2. Butanal-2,2-d_2 (**1**) was prepared based on the method of Bowen et al.[23] Butanal (15 mL, 0.166 mol), D_2O (12 mL, 0.663 mol) and dry pyridine (1.3 mL, 0.016 mol) were heated at 80 °C under N_2 for 48 h. The pale yellow biphasic solution was cooled on ice, and the aqueous layer was syringed out of the flask. Fresh D_2O (10.5 mL, 0.580 mol) and pyridine (1.3 mL, 0.016 mol) were added, and the solution was refluxed for a further 48 h. The solution was again cooled on ice and the aqueous layer removed. This procedure was repeated another time, and the organic layer was separated, dried (Na_2SO_4), filtered and distilled to give **1** as a colorless liquid (4.1 g, 34%) which was 97.7% labeled at position 2 (by ^{1}H NMR). ^{1}H NMR ($CDCl_3$): δ 9.75 (1H, s, H_1), 1.63 (2H, qquin, J = 7.4, 1.0 Hz, H_3), 0.94 (3H, t, J = 7.4 Hz, H_4). ^{13}C NMR ($CDCl_3$): δ 202.75 (C_1), 44.96 (J_{CD} = 19.3 Hz, C_2), 15.21 (C_3), 13.31 (C_4). ESI-HRMS: m/z calcd for $C_4H_6D_2NaO^+$ ($[M + Na]^+$), 97.0593; found, 97.0588.

(*E*)-Ethyl 2-hexenoate-4,4-d_2 (**2**) was prepared by adapting the method of Hebditch et al.[24] A solution of (carbethoxymethylene)-triphenylphosphorane (6.0 g, 17.2 mmol) in dry CH_2Cl_2 (50 mL) was added to butanal-2,2-d_2 (1.28 g, 17.2 mmol) in dry CH_2Cl_2 (50 mL). The reaction mixture was stirred under nitrogen for 42 h and concentrated in vacuo to dryness without heating. Hexane (30 mL)

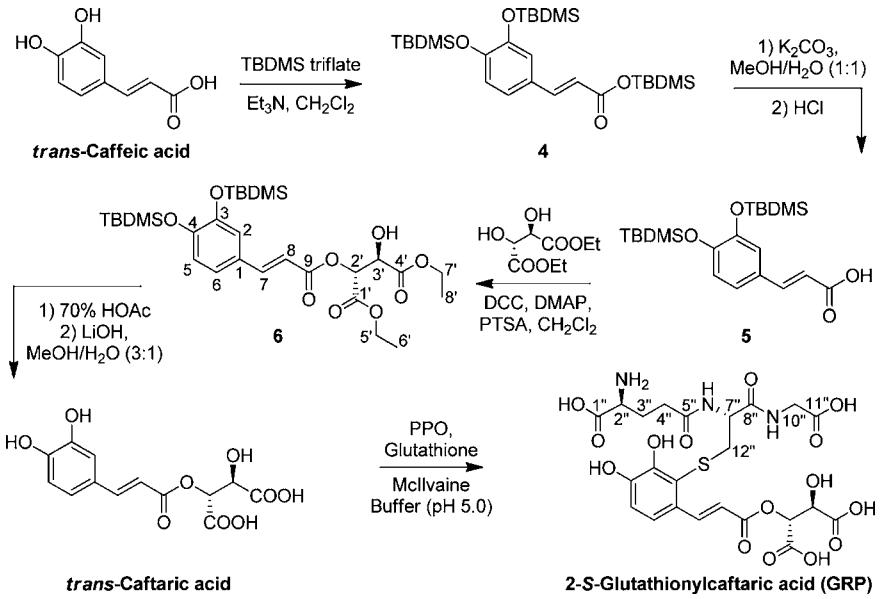

Figure 3. Synthetic approach to GRP from glutathione and *trans*-caffeic acid. Atom numbering shown for compounds relates to the NMR peak assignments.

was added to the resultant pink solid, and the suspension was stirred for 30 min and filtered. The solid was further triturated with hexane (3 × 30 mL) and filtered. The combined filtrates were concentrated in vacuo and purified by silica gel column chromatography (95% pentane/Et_2O, R_f = 0.44) to afford product **2** as a colorless oil (1.9 g, 77%) after solvent removal. ^{1}H NMR ($CDCl_3$): δ 6.89 (1H, d, J = 15.6 Hz, H_3), 5.76 (1H, d, J = 15.6 Hz, H_2), 4.12 (2H, q, J = 7.2 Hz, H_7), 1.42 (2H, q, J = 7.5 Hz, H_5), 1.23 (3H, t, J = 7.2 Hz, H_8), 0.88 (3H, t, J = 7.5 Hz, H_6). ^{13}C NMR ($CDCl_3$): δ 166.71 (C_1), 149.06 (C_3), 121.50 (C_2), 60.09 (C_7), 33.47 (J_{CD} = 19.3 Hz, C_4), 21.15 (C_5), 14.27 (C_8), 13.59 (C_6). ESI-HRMS: m/z calcd for $C_8H_{13}D_2O_2^+$ ([M + H]$^+$), 145.1192; found, 145.1190.

Reduction of ester **2** was based on the procedure of Gassman et al.[25] A suspension of $LiAlD_4$ (208 mg, 5.0 mmol) in anhydrous Et_2O (15 mL) was added dropwise to ester **2** (476 mg, 3.3 mmol) in anhydrous Et_2O (30 mL) at 0 °C. The suspension was stirred for 20 min at room temperature, cooled to 0 °C and quenched by successive dropwise additions of H_2O (1 mL), 10% NaOH (1 mL) and more H_2O (3 mL) and stirred for a further 30 min. The mixture was diluted with Et_2O (20 mL), dried ($MgSO_4$) and filtered. The filter cake was rinsed with Et_2O (20 mL), and the combined filtrates were concentrated in vacuo to yield the crude product as a cloudy oil. Purification by silica gel column chromatography (20% Et_2O/pentane, R_f = 0.49 in 50% Et_2O/pentane) gave the title compound **3** as a pale yellow oil (73 mg, 21%) after solvent removal, with a purity of 64% (by GC–MS). ^{1}H NMR ($CDCl_3$): δ 5.67 (1H, d, J = 15.4 Hz, H_3), 5.62 (d, J = 15.4 Hz, H_2), 1.38 (2H, q, J = 7.4 Hz, H_5), 0.89 (3H, t, J = 7.4 Hz, H_6). ^{13}C NMR ($CDCl_3$): δ 133.46 (C_3), 129.05 (C_2), 63.25 (J_{CD} = 21.7 Hz, C_1), 33.66 (J_{CD} = 19.3 Hz, C_4), 22.22 (C_5), 13.74 (C_6). EI-MS: m/z (%) 104 (M^+, 3), 86 (19), 85 (11), 76 (8), 75(7), 71 (10), 70 (12), 69 (10), 68 (5), 60 (32), 59 (100), 58 (22), 57 (12), 56 (9), 47 (15), 46 (16), 45 (15), 44 (20), 43 (23), 42 (19), 41 (11), 40 (9), 39 (7).

2-*S*-Glutathionylcaftaric Acid (GRP). The synthetic route for this standard is outlined in Figure 3. The di-TBDMS ether of *trans*-caffeic acid **5** was prepared by the method of Bogucki et al.[26] Briefly, *trans*-caffeic acid (1.8 g, 10.1 mmol) in dry CH_2Cl_2 (45 mL) at room temperature under N_2 was reacted with TBDMS triflate (10.3 mL, 45 mmol) and Et_3N (9.2 mL, 66 mmol) for 20 h. The crude product (6.4 g) was obtained as a mixture of brown oil and brown solid which was determined to be 73% trisilyl ether **4** and 23% disilyl ether **5** (by ^{1}H NMR). For characterization purposes, a small amount of crude material was purified by silica column chromatography (10% EtOAc/petroleum ether, R_f = 0.32 for **4**, 0.31 for **5**) to give trisilyl ether **4** as white crystals after solvent removal and standing at −20 °C, mp 157–159 °C. The remainder of crude trisilyl ether **4** (7.4 g, 14 mmol) was dissolved in 2:3 MeOH/H_2O (130 mL) and treated with K_2CO_3 (2.0 g, 14 mmol) at room temperature for 3 h. Product isolation according to Bogucki et al.[26] yielded **5** as a yellow-green solid (4.1 g, 100% from caffeic acid), which was used crude (one spot by TLC) in the following step. A portion was recrystallized from EtOH for the purposes of characterization, yielding a white crystalline solid, mp 158–160 °C [lit. mp 152–155 °C[26]]. The spectroscopic data for compounds **4** and **5** were in complete accord with the published values.[26]

Compound **6** was prepared based on the method of Crosby et al.[27] using diethyl L-tartrate instead of bis(diphenylmethyl) L-tartrate. Briefly, crude disilyl ether **5** (430 mg, 1.0 mmol), diethyl tartrate (460 μL, 2.7 mmol), *N*,*N*′-dicyclohexylcarbodiimide (DCC) (240 mg, 1.2 mmol), 4-dimethylaminopyridine (DMAP) (65.0 mg, 0.5 mmol) and *p*-toluenesulfonic acid (PTSA) (56 mg, 0.3 mmol) in CH_2Cl_2 (20 mL) were stirred overnight at room temperature. Purification by silica column chromatography (90% petroleum ether/EtOAc, R_f = 0.28 in 80% petroleum ether/EtOAc) gave product **6** as a colorless foam after solvent removal (470 mg, 75%). ^{1}H NMR ($CDCl_3$): δ 7.59 (1H, d, J = 15.9 Hz, H_7), 6.99 (1H, dd, J = 7.2, 2.1 Hz, H_6), 6.98 (1H, s, H_2), 6.78 (1H, dd, J = 7.2, 2.1 Hz, H_5), 6.26 (1H, d, J = 15.9 Hz, H_8), 5.57 (1H, d, J = 2.4 Hz, $H_{2'}$), 4.78 (1H, d, J = 1.8 Hz, $H_{3'}$), 4.24 (2H, q, J = 7.2 Hz, $H_{7'}$), 4.21 (2H, m, $H_{5'}$), 3.46 (1H, br s, O*H*), 1.25 (3H, t, J = 7.2 Hz, $H_{8'}$), 1.20 (3H, t, J = 7.2 Hz, $H_{6'}$), 0.95 (9H, s, Si–C(CH_3)$_3$), 0.93 (9H, s, Si–C(CH_3)$_3$), 0.172 (6H, s, Si–CH_3), 0.167 (6H, s, Si–CH_3). ^{13}C NMR ($CDCl_3$): δ 170.74 ($C_{1'}$), 166.81 ($C_{4'}$), 165.79 (C_9), 149.83 (C_4), 147.16 (C_3), 146.63 (C_7), 127.63 (C_1), 122.76 (C_6), 121.11 (C_5), 120.49 (C_2), 113.74 (C_8), 72.89 ($C_{2'}$), 70.67 ($C_{3'}$), 62.41 ($C_{5'}$), 62.01 ($C_{7'}$), 25.85 (Si–C(CH_3)$_3$), 25.81 (Si–C(CH_3)$_3$), 18.44 (Si–C(CH_3)$_3$), 18.37 (Si–C(CH_3)$_3$), 14.07 ($C_{6'}$), 14.05 ($C_{8'}$), −4.09 (Si–CH_3), −4.11 (Si–CH_3), −4.14 (2 × Si–CH_3). ESI-HRMS: m/z calcd for $C_{29}H_{48}NaO_9Si_2^+$ ([M + Na]$^+$), 619.2729; found, 619.2716.

trans-Caffeoyltartaric (caftaric) acid was obtained after cleaving the silyl ethers[27] of **6** and hydrolyzing the ethyl esters.[28] Compound **6** (170 mg, 0.3 mmol) was suspended in 70% acetic acid (13 mL) and refluxed overnight. The resultant solution was dried by coevaporation with toluene in vacuo. The residue was dissolved in EtOAc and extracted with aqueous $NaHCO_3$ (5 × 10 mL). The aqueous layer was acidified with concentrated HCl to pH 1, extracted with EtOAc (4 × 20 mL), and the organic extracts were dried ($MgSO_4$) and concentrated in vacuo. LiOH·H_2O (44 mg, 1.0 mmol) and 3:1

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MeOH/H_2O (1.9 mL) were added to the residue, and the solution was stirred at room temperature for 3 h. The methanol was removed in vacuo, and water (2 mL) was added. The solution was acidified with 1 M HCl to pH 1, saturated with NaCl and extracted with EtOAc (6 × 5 mL). The combined organic extracts were dried ($MgSO_4$) and concentrated in vacuo to give an orange-brown gum (60 mg, 67%), which was used in the next step without further purification (91% pure by ^{1}H NMR). ^{1}H NMR (D_2O): δ 7.51 (1H, d, J = 15.9 Hz, H_7), 6.96 (1H, d, J = 1.9 Hz, H_2), 6.91 (1H, dd, J = 8.3, 1.9 Hz, H_6), 6.80 (1H, d, J = 8.3 Hz, H_5), 6.23 (1H, d, J = 15.9 Hz, H_8), 5.67 (1H, d, J = 2.4 Hz, $H_{2'}$), 4.99 (1H, d, J = 2.4 Hz, $H_{3'}$). ^{13}C NMR (D_2O): δ 174.08 ($C_{1'}$), 171.42 ($C_{4'}$), 168.54 (C_9), 148.35 (C_7), 147.86 (C_4), 144.62 (C_3), 127.16 (C_1), 123.68 (C_6), 116.50 (C_5), 115.64 (C_2), 113.04 (C_8), 74.22 ($C_{2'}$), 70.72 ($C_{3'}$). ESI-HRMS: m/z calcd for $C_{13}H_{12}NaO_9^+$ ([M + Na]$^+$), 335.0380; found, 335.0390.

GRP was prepared using a variation of the method described by Cheynier et al.[29,30] *trans*-Caftaric acid (6.7 mg, 0.02 mmol), GSH (38 mg, 0.11 mmol) and PPO extract (22 mg) were aerated at room temperature for 2 h in a solution of aqueous McIlvaine buffer (10.7 mL, pH 5.0). The orange solution was filtered through a 0.45 μm syringe filter prior to purification by semipreparative HPLC to give the product as a cream colored, crystalline material (9.4 mg, 78%) with a purity of >99% (by HPLC–MS), which was determined to be 95% *trans*-GRP:5% *cis*-GRP. The spectroscopic data for the title compound were entirely consistent with the published values.[29] ESI-HRMS: m/z calcd for $C_{23}H_{28}N_3O_{15}S^+$ ([M + H]$^+$), 618.1241; found 618.1247. HPLC–MS/MS analysis revealed identical fragmentation patterns for *trans*-GRP (t_R = 13.3 min) and *cis*-GRP (t_R = 13.8 min) as reported previously.[31]

Semipreparative HPLC Purification of GRP. An Agilent 1100 HPLC (Agilent, Forest Hill, Australia) equipped with a quaternary pump and diode array detector (DAD) was used. The column was a 250 × 10 mm, 4 μm, 80 Å, Synergi Hydro-RP operated at 25 °C and protected by a guard column of the same material (Phenomenex, Lane Cove, NSW, Australia). Isocratic elution was performed (93% solvent A, 7% solvent B) with 0.1% acetic acid in water (solvent A) and 0.1% acetic acid in acetonitrile (solvent B), at a flow rate of 2.50 mL/min. A 100 μL injection volume was used. DAD signals were recorded at 280 and 320 nm, and spectra were stored between 220 and 700 nm. Fractions were collected manually on the basis of retention time and detector response. Data acquisition and processing were performed using Agilent ChemStation software (revision B.03.01).

Grape Samples. Healthy Sauvignon blanc grapes (°Brix = 20.2, pH 3.37, titratable acidity = 8.9 g/L) were machine-harvested from the Riverina region in country NSW into duplicate 2.5 tonne bins (approximately 2000 L, 10 bins in total) at the time of commercial harvest. Antioxidants (50 mg/L SO_2 and 100 mg/L ascorbate) were applied to the grape bins by dissolving the required amount of potassium metabisulfite (PMS) or ascorbic acid in 1 L of water and adding half the solution to the bottom of an empty bin and the remainder to the top of the full bin in the vineyard. The grape bins were placed in a temperature-controlled room set at 10 °C and samples were taken from several locations within duplicate bins and combined, with equal proportions of juice and berries (approximately 2 L) being collected each time over a 30 h period. Samples from the bins were collected at the time of harvest and again at 2, 8, 14, 24, and 30 h postharvest. The grapes from each bin were then crushed/destemmed and pressed at the winery and samples were obtained from the storage tank. Free and total sulfur levels, pH, TA, ascorbate, glutathione, °Brix and temperature of the fruit in each grape bin for each time point can be found in Supplementary Tables 1 and 2 in the Supporting Information.

Preparation of Juice Samples. Approximately 1.5 L of must (juice and berries) from the machine-harvested samples was homogenized in a Waring blender (John Morris Scientific, Kent Town, SA, Australia). A portion of the homogenate was centrifuged (Eppendorf 5810 R CF, Eppendorf South Pacific Pty. Ltd., North Ryde, NSW, Australia) at 3500 rpm (2465g) for 5 min at 10 °C, and a 10 mL aliquot of the supernatant was prepared for precursor analysis. The remainder of the juice was frozen and was used for other analyses.

Basic Analyses. Glutathione and ascorbic acid were determined by AWRI Commercial Services, and free and total sulfur levels were obtained by the aspiration method.[32]

Quantitative HPLC–MS Analysis of 3-MH Precursors and GRP. *Sample Preparation.* Extracts were prepared for HPLC–MS/MS precursor analysis according to the procedure of Capone et al.[18] Samples for GRP analysis were prepared by adding labeled standard (50 μL) containing d_8-Cys–3-MH and d_9-Glut–3-MH (20 mg/L of each) to 450 μL of sample to be analyzed. The samples were thoroughly mixed and filtered through Acrodisc syringe filters (0.45 μm, 13 mm, Pall Gelman Life Sciences, Cheltenham, VIC, Australia) for HPLC–MS/MS analysis. Both labeled precursor standards were assessed for calibration of GRP.

HPLC–MS Instrumentation. HPLC–MS/MS analysis was carried out as previously described by Capone et al.[7,33] using 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, Ontario, Canada).

Mass Spectrometer Conditions. Multiple reaction monitoring (MRM) was conducted under the conditions previously described.[7,33] The mass spectrometer parameters were modified to incorporate the mass transitions for GRP (m/z 618.1 → 543.1, 618.1 → 489.1 and 618.1 → 264.1) based on infusion of the synthetically prepared reference material. *trans*-GRP was quantified using mass transition m/z 618.1 → 543.1, whereas *cis*-GRP was quantified using m/z 618.1 → 489.1.

Method Validation for GRP. The analytical method was validated by a series of duplicate standard additions of GRP (0, 1, 5, 25, 50, 100, 200, and 300 mg/L; combined isomer concentration is specified for all validation and calibration samples) to a Sauvignon blanc juice (°Brix = 21.7, pH 3.26, titratable acidity = 8.9 g/L, subsequently found to contain 70.4 and 2.8 mg/L of *trans*-GRP and *cis*-GRP, respectively) and Milli-Q water. To determine the precision of the analysis, seven replicate samples were spiked with GRP at 25 mg/L. The limit of detection (LOD) and limit of quantitation (LOQ) were determined by visual evaluation. 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-to-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 GRP in batches of unknown samples, duplicate standards in water were prepared at the same time as the juice samples with GRP at concentrations of 0, 25, 100, and 200 μg/L. To ensure that the accuracy of the analysis was maintained, duplicate control juice samples, spiked with 0 and 25 mg/L of GRP, were included with every set of samples to be quantified. All validation and calibration samples were prepared and analyzed according to the method.

GC–MS Analysis of C6 Compounds. *Method Optimization.* Assessment of parameters was based primarily on previous work.[17,34−37] Model wine (5 or 10% v/v ethanol) (100 mL) was spiked with unlabeled C6 compounds (Table 1) at concentrations of 0, 10, and 500 μg/L, and the mixtures were shaken. Aliquots (10 mL) were transferred into 20 mL amber screw cap vials for SPME–GC–MS analysis. Various preconditioned SPME fibers were trialed on these samples. The fibers investigated were polydimethylsiloxane/divinylbenzene (PDMS/DVB) 65 μm, polydimethylsiloxane (PDMS) 100 μm and divinylbenzene/carboxen/polydimethylsiloxane (DVB/CAR/PDMS, 2 cm) 50/30 μm (Supelco, Bellefonte, PA) at the recommended operating temperatures for each fiber. Once the best fiber was determined, different sampling parameters were investigated in Sauvignon blanc juice, and white and red cask wines spiked at 0, 10, or 500 μg/L with unlabeled C6 compounds. The parameters were no dilution, no salt and no agitation; diluting the extract by 50, 90 and 99% with Milli-Q water (v/v); salting the sample with either 1 or 2 g of NaCl;

Table 1. Quantifier and Qualifier Ions (m/z) and Associated Dwell Times (ms) for the Analytes and Their Corresponding Deuterium Labeled Internal Standards Assessed by GC−MS in SIM Mode

	m/z (dwell)		
compound[a]	quantifier	qualifiers	
d_9-(*E*)-2-hexenal	89 (25)	107 (25)	73 (25)
(*E*)-2-hexenal	97 (25)	98 (25)	83 (25)
d_{13}-hexan-1-ol	78 (20)	96 (20)	64 (20)
hexan-1-ol	69 (20)	84 (20)	56 (20)
(*Z*)-3-hexen-1-ol	82 (30)	69 (30)	67 (30)
d_4-(*E*)-2-hexen-1-ol	86 (20)	85 (20)	74 (20)
(*E*)-2-hexen-1-ol	82 (20)	67 (20)	57 (20)

[a]The labeled internal standard used for calibration is listed directly above the analyte(s).

and inclusion of agitation (400 rpm, agitation on 10 s and off 1 s) during fiber extraction.

GC−MS Instrumentation. Samples were analyzed with an Agilent 6890N gas chromatograph (Santa Clara, CA, USA) fitted with a Gerstel MPS2 autosampler (Lasersan Australasia Pty Ltd., Robina, QLD, Australia) and coupled to an Agilent 5973N mass spectrometer. The gas chromatograph was fitted with a 60 m J & W DB-Wax fused silica capillary column (0.25 mm i.d., 0.25 μm film thickness). The carrier gas was helium (BOC gases, Ultra High Purity), and the flow rate was 1.5 mL/min. The oven temperature started at 45 °C, was held at this temperature for 5 min, then increased at 2 °C/min to 100 °C, then increased at 15 °C/min to 240 °C and held at this temperature for 8 min. The injector was held at 240 °C throughout the run, and the transfer line was also maintained at this temperature. Positive ion electron ionization mass spectra at 70 eV were recorded in the range m/z 35−350 for scan runs.

Optimized Method for Preparation of Juice and Wine Extracts. An aliquot (1 mL) of juice or wine was added into a 20 mL glass screw cap amber SPME vial along with 9 mL of Milli-Q water. An aliquot (100 μL) of an ethanol solution containing deuterium labeled C6 standards (d_{13}-hexan-1-ol, 6052 μg/L; d_9-(*E*)-2-hexenal, 30260 μg/L; d_4-(*E*)-2-hexen-1-ol, 10400 μg/L) was added, the sample was mixed, 2 g of NaCl was added, and the contents was shaken by hand and sealed for GC−MS analysis.

Quantitative GC−MS Analysis. Quantitation was carried out using the GC−MS system with a 60 m DB-Wax column as described above. The autosampler was fitted with a 65 μm PDMS/DVB SPME fiber. The sample was extracted at 40 °C for 30 min while agitating at 500 rpm (10 s on, 1 s off) and desorbed in the inlet for 15 min. The splitter, at 90:1, was opened after 36 s. Injection was done in pulsed/splitless mode with an inlet pressure of 45.0 psi maintained until splitting. The injection liner was a Supelco injection sleeve made of deactivated borosilicate glass, 0.75 mm i.d. Other chromatographic parameters such as inlet and transfer line temperatures and oven temperature program were the same as described above. For quantitation, mass spectra were recorded in selected ion monitoring (SIM) mode. Table 1 displays the ions monitored for the analytes and deuterium labeled standards as well as the respective dwell times. The ion used for quantitation was typically chosen as having the best signal-to-noise ratio and the least interference from other wine components, while the other ions were used as qualifiers.

Analytical Method Validation. The analytical method was validated by a series of duplicate standard additions of unlabeled C6 compounds shown in Table 1 (0, 5, 10, 25, 50, 100, 250, and 500 μg/L) to a Sauvignon blanc juice (°Brix = 19.1, pH 3.72, titratable acidity = 5.0 g/L, subsequently found to contain 122.5, 47.5, 12.5, and 67.5 μg/L of (*E*)-2-hexenal, hexan-1-ol, (*Z*)-3-hexen-1-ol and (*E*)-2-hexen-1-ol, respectively), a commercial fresh dry white bag-in-box wine (9.5% ethanol, pH 2.98, subsequently found to contain 199.2 and 2.9 μg/L of hexan-1-ol and (*Z*)-3-hexen-1-ol, respectively), and a commercial dry red bag-in-box wine (12.5% ethanol, pH 3.16, subsequently found to contain 242.3 and 14.4 μg/L of hexan-1-ol and (*Z*)-3-hexen-1-ol, respectively). To determine the precision of the analysis, seven replicate samples were spiked with C6 compounds at two different concentrations (10 μg/L and 250 μg/L). LOD and LOQ values for the C6 analytes 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. For quantifying the analytes in batches of unknown samples, duplicate standards in model wine (10% aqueous ethanol, saturated with potassium hydrogen tartrate, pH adjusted to 3.2 with tartaric acid) were prepared at the same time as the juice samples with C6 compounds at concentrations of 0, 10, 100, and 500 μg/L. To ensure that the accuracy of the analysis was maintained, duplicate control wine samples, spiked with 0 and 50 μg/L of C6 compounds (total of four control samples), were included with every set of samples to be quantified. All validation and calibration samples were prepared and analyzed according to the optimized method.

Statistical Analysis. The results reported for the calibration of the methods were derived from the average of two replicate measurements for each concentration of analyte (and seven replicates for repeatability samples). The LINEST function in Microsoft Excel 2007 was used to obtain calibration function slopes and intercepts and their associated standard errors. The effects of the trial were analyzed using principal component analysis (PCA) and one-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

SIDA Method for C6 Compounds. *GC−MS Method Optimization.* As part of this study, we developed a stable isotope dilution analysis (SIDA) method for the quantitative analysis of C6 compounds listed in Table 1. The analysis of various C6 compounds in grape juice or wine by SPME−GC−MS has been recently reported by others,[17,34,35] and these reports formed the basis for rapid optimization of analytical parameters including SPME fiber type, headspace sampling and separation of analytes. As such, a PDMS/DVB fiber was chosen[34] with extraction and desorption parameters virtually identical to Pérez Olivero and Pérez Trujillo.[35] Separation was performed on a DB-Wax column[17,35] with a comparable temperature program to that reported previously.[17] Dilution of samples with Milli-Q water improved sensitivity in accord with previous findings.[36,37] For the SIDA method the synthesis of polydeuterated standards which were not commercially available was necessary. d_9-(*E*)-2-Hexenal was on hand from previous work[8] whereas d_4-(*E*)-2-hexen-1-ol was prepared from butanal by the route depicted in Figure 2.

C6 Method Validation. The standard addition curves obtained for the C6 compounds under consideration were linear throughout the concentration range (0−500 μg/L), with coefficients of determination (R^2) shown in Table 2 for each of the compounds in a white juice, a white wine and a red wine. Method sensitivity in each matrix was evaluated, and calculated LOQs and LODs for each compound are shown in Table 2. The precision of the analysis was determined by spiking seven replicate samples with internal standard at two concentrations of each of the C6 compounds. Spikes at 10 and 250 μg/L (existing analyte concentrations in each matrix are specified in Materials and Methods) gave respective relative standard deviations as shown in Table 2, which were <5% in all cases. Although the method was applied to white juice in this study, we validated the method in white and red wine as a matter of course, thereby identifying that the method was applicable to these wine matrices.

Table 2. Validation Data for SIDA of C6 Compounds by SPME−GC−MS

analyte	R^2	RSD[a] 10 μg/L	RSD[a] 250 μg/L	LOD[b]	LOQ[c]
Sauvignon Blanc Juice					
(*E*)-2-hexenal	0.999	3.3	1.7	0.23	0.69
hexan-1-ol	1.000	3.2	0.40	0.04	0.11
(*Z*)-3-hexen-1-ol	1.000	0.80	0.46	0.12	0.35
(*E*)-2-hexen-1-ol	1.000	0.70	0.64	0.19	0.58
White Wine					
(*E*)-2-hexenal	0.999	2.1	1.8	0.19	0.56
hexan-1-ol	1.000	1.8	1.3	0.10	0.27
(*Z*)-3-hexen-1-ol	1.000	1.8	0.94	0.15	0.46
(*E*)-2-hexen-1-ol	1.000	2.2	0.55	0.22	0.66
Red Wine					
(*E*)-2-hexenal	0.998	1.6	1.7	0.11	0.32
hexan-1-ol	1.000	1.5	1.1	0.13	0.40
(*Z*)-3-hexen-1-ol	0.999	1.3	0.46	0.20	0.61
(*E*)-2-hexen-1-ol	1.000	1.0	0.95	0.09	0.26

[a]RSD, % relative standard deviation for repeatability (N = 7). [b]LOD, limit of detection (μg/L). [c]LOQ, limit of quantitation (μg/L).

Synthesis and Analysis of GRP. Grape reaction product (GRP) was synthesized in five steps from *trans*-caffeic acid (Figure 3). To begin, *trans*-caftaric acid was prepared by DCC-coupling[27] silyl protected *trans*-caffeic acid **5**[26] with diethyl tartrate, followed by deprotection of the phenols[27] and hydrolysis of the ethyl esters.[28] GRP was then afforded by reacting PPO with *trans*-caftaric acid in the presence of GSH and air.[29,30] GRP was characterized by NMR, HPLC−MS and MS/MS experiments and infused into the MS to obtain the MRM transitions (listed under Materials and Methods) used for quantitative determinations. An existing thiol precursor method[33] was validated for analysis of GRP in a similar manner to that reported previously for Cysgly−3-MH.[7] Calibrations were assessed with synthetic GRP and precursor labeled internal standards (d_8-Cys−3-MH and d_9-Glut−3-MH) ordinarily employed for analysis of 3-MH precursors using the HPLC−MS/MS method.[33] The standard addition functions for GRP in Sauvignon blanc juice or water were linear throughout the concentration range (0−200 mg/L combined isomer total) with coefficients of determination (R^2) greater than 0.99. *trans*-GRP eluted around 13.3 min and *cis*-GRP eluted around 13.8 min using the identical gradient as originally developed for 3-MH precursors.[33] The precision of the method was evaluated with repeatability samples at 25 mg/L (i.e., 23.75 and 1.25 mg/L of *trans*- and *cis*-GRP, respectively) for a juice found to contain 70.4 and 2.8 mg/L of *trans*- and *cis*-GRP, respectively, affording relative standard deviations of <17%. The LOD and LOQ were 0.3 and 0.95 mg/L, respectively, for *trans*-GRP, and 0.08 and 0.25 mg/L, respectively, for *cis*-GRP. This level of sensitivity was appropriate for the samples being assessed, and no further improvement was attempted.

Precursor Evolution during Storage. Our previous work on Sauvignon blanc grape ripening and processing identified that loss of berry integrity (i.e., berry softening or damage) could influence 3-MH precursor concentrations.[9,18] In particular, depending on the level of antioxidant addition, transportation of machine-harvested grapes led to important increases in Glut−3-MH and Cys−3-MH,[9] while Cysgly−3-MH appeared to be a transient intermediate.[7] It was apparent that 3-MH precursor concentrations could be manipulated through processing operations and application of antioxidants, so the next step was to investigate whether storing fruit on a commercial scale for a period of time had a similar effect. This could have considerable economic and quality implications for winemakers if precursor profiles of fruit with low varietal thiol potential could be optimized. The effect of storage was evaluated with machine-harvested Sauvignon blanc fruit held in a temperature-controlled room at 10 °C immediately after harvest, with samples taken over a 30 h period for 3-MH precursor analysis, among others.

The results in Figure 4 illustrate how 3-MH precursors (combined diastereomer totals) evolved over the 30 h time period of the experiment. There was a significant effect as a result of storage (p < 0.001) whereby precursor levels increased as storage progressed, especially during the first few hours of

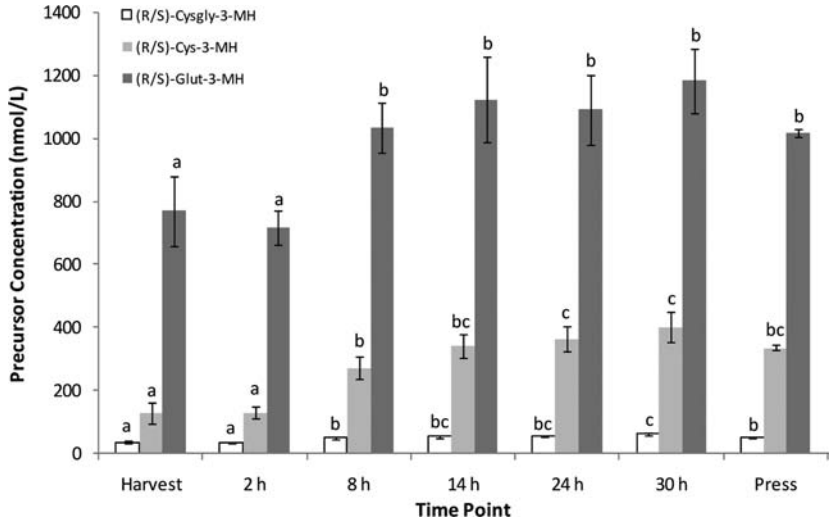

Figure 4. Mean concentrations (nmol/L) of Cysgly−, Cys−, and Glut−3-MH (combined diastereomer totals) determined at various time points during storage of machine-harvested Sauvignon blanc grapes. Error bars represent the standard deviation of five replicates for the harvest and storage time points and three replicates for press samples. There were statistically significant differences (p < 0.001) due to the effects of storage time for each precursor type. Different letters for the same precursor type indicate significant differences (p < 0.05) between the means.

Table 3. Mean Concentrations of C6 Compounds (μg/L) and GRP Isomers (mg/L) Determined at Various Time Points during Storage of Machine-Harvested Sauvignon Blanc Grapes[a]

	C6 compounds, av (SD) (μg/L)[b]				GRP, av (SD) (mg/L)[b]	
time point	(*E*)-2-hexenal	hexan-1-ol	(*Z*)-3-hexen-1-ol	(*E*)-2-hexen-1-ol	*trans*	*cis*
harvest	14.4 (7.0) bc	17.2 (5.6) a	11.2 (2.0) c	27.2 (6.6) c	31.6 (11.2) a	1.0 (0.3) a
2 h	16.0 (6.8) c	12.0 (2.1) a	8.4 (1.3) ab	20.2 (3.6) abc	24.1 (6.0) a	0.7 (0.2) a
8 h	10.4 (2.8) abc	14.9 (0.7) a	9.0 (0) bc	19.0 (1.4) ab	45.8 (18.8) ab	1.5 (0.6) ab
14 h	12.6 (6.6) bc	13.9 (4.0) a	7.5 (1.0) ab	15.6 (2.7) ab	86.6 (17.9) c	2.5 (0.5) cd
24 h	5.4 (0.8) ab	15.0 (2.7) a	6.7 (0.7) a	14.4 (1.8) a	73.8 (18.7) bc	2.1 (0.5) bc
30 h	6.0 (1.4) ab	42.2 (15.9) b	7.8 (1.2) ab	22.9 (5.6) bc	80.4 (9.4) c	3.0 (0.6) d
press	2.6 (0.4) a	16.2 (0.8) a	7.0 (0) ab	22.0 (1.0) abc	36.1 (3.7) a	1.5 (0.1) abc

[a]There were statistically significant differences ($p < 0.001$, except for (*E*)-2-hexenal, $p = 0.002$) due to storage for each compound type. [b]av, average, and SD, standard deviation, for five replicates (three replicates for press samples); different letters down a column indicate significant differences ($p < 0.05$) between the averages.

the experiment. The most noticeable impact was seen for Cys−3-MH, with an approximate 2-fold increase in concentration after 8 h (time point 2) and a 3-fold increase by 30 h (time point 5). Glut− and Cysgly−3-MH increased roughly 1.5-fold over the duration of the storage period, although Cysgly−3-MH was found in concentrations that were much lower than the other precursor types. This points to the transient nature and minimal accumulation of the dipeptide conjugate, as discussed previously.[7]

While the increase in Glut−3-MH was consistent with our previous transport study, Cys−3-MH levels were not nearly as affected by extended storage as they were with transportation (which produced around a 10-fold increase after about 12 h).[9] Considering the likely role that enzymes play in the formation and degradation of these precursors, this difference may be a result of the effects of agitation, maceration, aeration and elevated temperatures arising during transportation of fruit compared to storage in a temperature-controlled room. Nonetheless, the results in Figure 4 clearly show that storing fruit on a commercial scale, even for a short period of time prior to pressing, leads to increased 3-MH precursor concentrations, which may in turn yield higher 3-MH concentrations in the finished wine.[13,19]

The increases in 3-MH precursor concentrations observed during storage were in good accord with small-scale skin contact trials where Cys−3-MH was assessed,[13−15] but contrasted with results for Cys− and Glut−3-MH evaluated over 7 days, where no changes were observed.[16] Based on our understanding, we suggest that any increases in precursor concentrations during storage of harvested fruit (i.e., juice and grape skins) are not necessarily a result of improved extraction alone; the most important contributions may be from precursor formation and degradation.[7,9,17,18]

Changes to C6 Compounds and GRP. Formation of Glut−3-MH, and therefore Cysgly− and Cys−3-MH, requires the conjugation of GSH with (*E*)-2-hexenal followed by reduction of the intermediate aldehyde.[9] There are a number of important enzymatic steps and oxidation events which impact the availability of GSH and (*E*)-2-hexenal, so these and related compounds were evaluated during the storage period to assess their relationship to 3-MH precursor concentrations on a commercial scale. Table 3 displays the mean concentrations of C6 compounds and GRP isomers, along with statistical differences as a result of storage time. In general, the evolution of C6 compounds was consistent with the profiles presented by Joslin and Ough.[38] (*E*)-2-Hexenal, and to a lesser extent (*Z*)-3-hexen-1-ol, decreased during the storage period, hexan-1-ol fluctuated during the early stages and peaked after 30 h of storage and (*E*)-2-hexen-1-ol steadily decreased until 30 h of storage, whereupon it reached close to its initial concentration. Both isomers of GRP were typically seen to increase during the storage period in accord with previous findings for winery samples,[15] peaking at around 14 h of storage.

These observations are consistent with the formation and transformation of the compounds as enzymatic and oxidative processes occur, with a central role for GSH in conjugating with the oxidation products. In particular, unsaturated C6 compounds are present or form after grape damage[38] and then react, being incorporated into precursor conjugates or ending in more reduced forms, while GRP isomers form as caftaric acid is oxidized in the juice.[15] The data were consistent with the observations of Roland et al.,[17] highlighting the importance to thiol precursor formation of having (*E*)-2-hexenal readily available early during juice preparation in the presence of GSH. We did not, however, encounter the high (*E*)-2-hexenal levels (approximately 200 μg/L) suggested as necessary for Glut−3-MH formation in that work.[17] As further evidenced by our study, it is not until the latter stages of juice processing that GSH begins to be consumed to a greater extent in the reaction forming GRP,[15] showing that the timing of different enzymatic oxidation events (i.e., formation of (*E*)-2-hexenal earlier and GRP later) can impact the formation of thiol precursors through availability of required constituents.

The PCA biplot (Figure 5) supported the interpretation of the data relative to changes due to storage time based on the analyte concentrations in Table 3 (converted to nmol/L) and precursor concentrations in Figure 4. PC1 accounted for 69% of the variation, and PC2 corresponded to 17% (Figure 5). Based on the loadings and explained variance, thiol precursors, GSH and GRP contributed most to the variation, with some input from unsaturated C6 compounds. Early storage time points (i.e., harvest and 2 h) were associated with higher concentrations of GSH, (*Z*)-3-hexen-1-ol and (*E*)-2-hexenal, while longer storage times (i.e., 14 to 30 h) were characterized by higher amounts of thiol precursors and GRP. A high concentration of the fully reduced C6 compound, hexan-1-ol, was also closely associated with the 30 h sampling time point while press samples were mainly associated with relatively high thiol precursor concentrations.

This study provides a comprehensive overview of reactions related to thiol precursor formation. It appears that enzymatic reduction of C6 compounds is relatively facile and occurs at various stages of juice storage. Such a generalization may extend to the reduction of Glut−3-MHAl to produce Glut−3-MH,

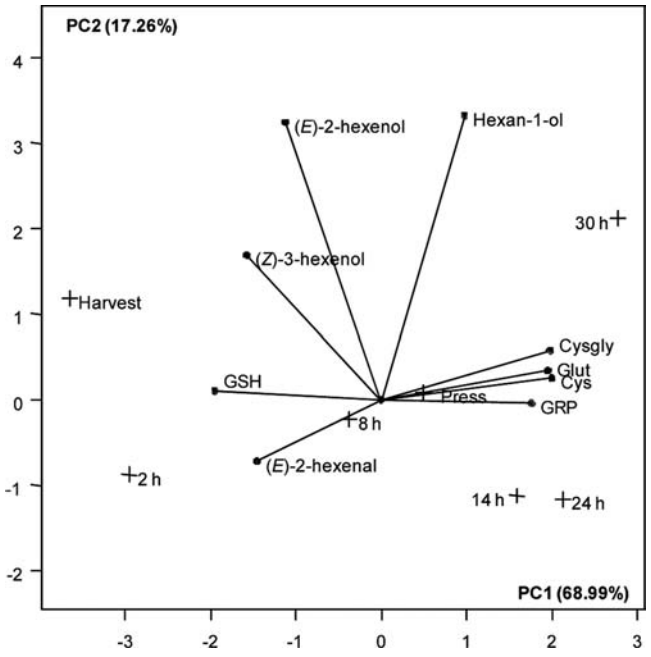

Figure 5. PCA biplot of analyte concentrations (nmol/L) at different storage time points. Cys, Cysgly and Glut refer to the 3-MH precursors.

meaning that oxidation reactions are of primary importance. Early formation of (*E*)-2-hexenal with an availability of GSH led to conjugation and ultimately Glut−3-MH production, which continued during a period of storage. The conjugation step is quite possibly enzymatic,[39,40] and enzymatic degradation of Glut−3-MH,[41] leading to important increases of Cys−3-MH, also occurred during storage. After some time, GSH concentrations were further decreased as GRP formed, thereby diverting GSH away from thiol precursor formation. This was compounded by a decline in (*E*)-2-hexenal over time as it was enzymatically reduced via intermediates through to hexan-1-ol as the end product, which would also have an impact on the ability to form additional thiol precursors.

Aspects which influence enzymatic reactions, such as judicious application of antioxidants at critical points during processing, should be investigated, but it appears from this study that maximum thiol precursor and GRP concentrations coincided after about 14 h of storage. This highlighted the ability to manipulate precursor concentrations postharvest, and could signify a chemical component in the conjugation of GSH and (*E*)-2-hexenal, in the same manner that GSH and caftaric acid *o*-quinone are coupled, once the reactive electrophiles are formed enzymatically. Importantly, precursor concentrations were preserved during pressing, whereas GRP concentrations decreased, most likely as a result of further oxidation. Additional GSH was released during pressing, yet (*E*)-2-hexenal was at its lowest concentration, indicating that thiol precursor formation may be minimal after removal of the grape solids.

■ ASSOCIATED CONTENT

Supporting Information

Tables displaying temperatures and basic chemical data at different time points for the stored samples. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interest.

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■ ABBREVIATIONS USED

3-MH, 3-mercaptohexan-1-ol; Cys−3-MH, 3-*S*-cysteinylhexan-1-ol; Glut−3-MH, 3-*S*-glutathionylhexan-1-ol; Cysgly−3-MH, 3-*S*-cysteinylglycinehexan-1-ol; Glut−3-MHAl, 3-*S*-glutathionylhexanal; GSH, glutathione; GRP, grape reaction product; PPO, polyphenol oxidase; TBDMS, *tert*-butyldimethylsilyl; DCC, *N*,*N*′-dicyclohexylcarbodiimide; DMAP, 4-dimethylaminopyridine; PTSA, *p*-toluenesulfonic acid; PMS, potassium metabisulfite; MRM, multiple reaction monitoring; LOD, limit of detection; LOQ, limit of quantitation; PCA, principal component analysis; SIDA, stable isotope dilution analysis

■ REFERENCES

(1) King, E. S.; Osidacz, P.; Curtin, C.; Bastian, S. E. P.; Francis, I. L. Assessing desirable levels of sensory properties in Sauvignon blanc wines − consumer preferences and contribution of key aroma compounds. *Aust. J. Grape Wine Res.* **2011**, *17*, 169−180.

(2) Lund, C. M.; Thompson, M. K.; Benkwitz, F.; Wohler, M. W.; Triggs, C. M.; Gardner, R.; Heymann, H.; Nicolau, L. New Zealand Sauvignon blanc distinct flavor characteristics: sensory, chemical, and consumer aspects. *Am. J. Enol. Vitic.* **2009**, *60*, 1−12.

(3) Roland, A.; Schneider, R.; Razungles, A.; Cavelier, F. Varietal thiols in wine: discovery, analysis and applications. *Chem. Rev.* **2011**, *111*, 7355−7376 and references therein.

(4) Peña-Gallego, A.; Hernández-Orte, P.; Cacho, J.; Ferreira, V. S-Cysteinylated and S-glutathionylated thiol precursors in grapes. A review. *Food Chem.* **2012**, *131*, 1−13 and references therein.

(5) Tominaga, T.; Peyrot des Gachons, C.; Dubourdieu, D. A new type of flavor precursors in *Vitis vinifera* L. cv. Sauvignon blanc: *S*-cysteine conjugates. *J. Agric. Food Chem.* **1998**, *46*, 5215−5219.

(6) Peyrot des Gachons, C.; Tominaga, T.; Dubourdieu, D. Sulfur aroma precursor present in *S*-glutathione conjugate form: identification of *S*-3-(hexan-1-ol)-glutathione in must from *Vitis vinifera* L. cv. Sauvignon blanc. *J. Agric. Food Chem.* **2002**, *50*, 4076−4079.

(7) Capone, D. L.; Pardon, K. H.; Cordente, A. G.; Jeffery, D. W. Identification and quantitation of 3-*S*-cysteinylglycinehexan-1-ol (Cysgly-3-MH) in Sauvignon blanc grape juice by HPLC−MS/MS. *J. Agric. Food Chem.* **2011**, *59*, 11204−11210.

(8) Grant-Preece, P. A.; Pardon, K. H.; Capone, D. L.; Cordente, A. G.; Sefton, M. A.; Jeffery, D. W.; Elsey, G. M. Synthesis of wine thiol conjugates and labeled analogues: fermentation of the glutathione

conjugate of 3-mercaptohexan-1-ol yields the corresponding cysteine conjugate and free thiol. *J. Agric. Food Chem.* **2010**, *58*, 1383−1389.
(9) Capone, D. L.; Jeffery, D. W. Effects of transporting and processing Sauvignon blanc grapes on 3-mercaptohexan-1-ol precursor concentrations. *J. Agric. Food Chem.* **2011**, *59*, 4659−4667.
(10) Winter, G.; Van Der Westhuizen, T.; Higgins, V. J.; Curtin, C.; Ugliano, M. Contribution of cysteine and glutathione conjugates to the formation of the volatile thiols 3-mercaptohexan-1-ol (3MH) and 3-mercaptohexyl acetate (3MHA) during fermentation by *Saccharomyces cerevisiae*. *Aust. J. Grape Wine Res.* **2011**, *17*, 285−290.
(11) Swiegers, J. H.; Capone, D. L.; Pardon, K. H.; Elsey, G. M.; Sefton, M. A.; Francis, I. L.; Pretorius, I. S. Engineering volatile thiol release in *Saccharomyces cerevisiae* for improved wine aroma. *Yeast* **2007**, *24*, 561−574.
(12) Roncoroni, M.; Santiago, M.; Hooks, D. O.; Moroney, S.; Harsch, M. J.; Lee, S. A.; Richards, K. D.; Nicolau, L.; Gardner, R. C. The yeast IRC7 gene encodes a β-lyase responsible for production of the varietal thiol 4-mercapto-4-methylpentan-2-one in wine. *Food Microbiol.* **2011**, *28*, 926−935.
(13) Murat, M.-L.; Tominaga, T.; Dubourdieu, D. Assessing the aromatic potential of Cabernet Sauvignon and Merlot musts used to produce rose wine by assaying the cysteinylated precursor of 3-mercaptohexan-1-ol. *J. Agric. Food Chem.* **2001**, *49*, 5412−5417.
(14) Peyrot des Gachons, C.; Tominaga, T.; Dubourdieu, D. Localization of *S*-cysteine conjugates in the berry: effect of skin contact on aromatic potential of *Vitis vinifera* L. cv. Sauvignon blanc must. *Am. J. Enol. Vitic.* **2002**, *53*, 144−146.
(15) Maggu, M.; Winz, R.; Kilmartin, P. A.; Trought, M. C. T.; Nicolau, L. Effect of skin contact and pressure on the composition of Sauvignon blanc must. *J. Agric. Food Chem.* **2007**, *55*, 10281−10288.
(16) Roland, A.; Schneider, R.; Charrier, F.; Cavelier, F.; Rossignol, M.; Razungles, A. Distribution of varietal thiol precursors in the skin and the pulp of Melon B. and Sauvignon blanc grapes. *Food Chem.* **2011**, *125*, 139−144.
(17) Roland, A.; Vialaret, J.; Razungles, A.; Rigou, P.; Schneider, R. Evolution of *S*-cysteinylated and *S*-glutathionylated thiol precursors during oxidation of Melon B. and Sauvignon blanc musts. *J. Agric. Food Chem.* **2010**, *58*, 4406−4413.
(18) Capone, D. L.; Sefton, M. A.; Jeffery, D. W. 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. *J. Agric. Food Chem.* **2011**, *59*, 4649−4658.
(19) Kobayashi, H.; Takase, H.; Kaneko, K.; Tanzawa, F.; Takata, R.; Suzuki, S.; Konno, T. Analysis of *S*-3-(hexan-1-ol)-glutathione and *S*-3-(hexan-1-ol)-L-cysteine in *Vitis vinifera* L. cv. Koshu for aromatic wines. *Am. J. Enol. Vitic.* **2010**, *61*, 176−185.
(20) Allen, T.; Herbst-Johnstone, M.; Girault, M.; Butler, P.; Logan, G.; Jouanneau, S.; Nicolau, L.; Kilmartin, P. A. Influence of grape-harvesting steps on varietal thiol aromas in Sauvignon blanc wines. *J. Agric. Food Chem.* **2011**, *59*, 10641−10650.
(21) Pardon, K. H.; Graney, S. D.; Capone, D. L.; Swiegers, J. H.; Sefton, M. A.; Elsey, G. M. Synthesis of the individual diastereomers of the cysteine conjugate of 3-mercaptohexanol (3-MH). *J. Agric. Food Chem.* **2008**, *56*, 3758−3763.
(22) Kotseridis, Y.; Ray, J. L.; Augier, C.; Baumes, R. Quantitative determination of sulfur containing wine odorants at sub-ppb levels. 1. Synthesis of the deuterated analogues. *J. Agric. Food Chem.* **2000**, *48*, 5819−5823.
(23) Bowen, R. D.; Suh, D.; Terlouw, J. K. Reactions of ionized dibutyl ether. *Org. Mass Spectrom.* **1994**, *29*, 791−805.
(24) Hebditch, K. R.; Nicolau, L.; Brimble, M. A. Synthesis of isotopically labelled thiol volatiles and cysteine conjugates for quantification of Sauvignon blanc wine. *J. Labelled Compd. Radiopharm.* **2007**, *50*, 237−243.
(25) Gassman, P. G.; Valcho, J. J.; Proehl, G. S.; Cooper, C. F. Reaction of 1-chloro-2-alkylcycloalkenes with organolithium reagents. A novel cyclopropanation reaction involving the generation of carbenes from vinyl halides. *J. Am. Chem. Soc.* **1980**, *102*, 6519−6526.
(26) Bogucki, D. E.; Charlton, J. L. A non-enzymatic synthesis of (*S*)-(−)-rosmarinic acid and a study of a biomimetic route to (+)-rabdosiin. *Can. J. Chem.* **1997**, *75*, 1783−1794.
(27) Crosby, D. C.; Lei, X.; Gibbs, C. G.; McDougall, B. R.; Robinson, W. E.; Reinecke, M. G. Design, synthesis, and biological evaluation of novel hybrid dicaffeoyltartaric/diketo acid and tetrazole-substituted L-chicoric acid analogue inhibitors of human immunodeficiency virus type 1 integrase. *J. Med. Chem.* **2010**, *53*, 8161−8175.
(28) Liu, Z.-C.; Chen, C.-H.; Wang, H.-W.; Huang, Y.-C.; Kao, M.-J.; Lim, T.-S.; Luh, T.-Y. Hydrogen-bonding induced cooperative effect on the energy transfer in helical polynorbornenes appended with porphyrin-containing amidic alanine linkers. *Chem. Asian J.* **2010**, *5*, 1425−1438.
(29) Cheynier, V. F.; Trousdale, E. K.; Singleton, V. L.; Salgues, M. J.; Wylde, R. Characterization of 2-*S*-glutathionyl caftaric acid and its hydrolysis in relation to grape wines. *J. Agric. Food Chem.* **1986**, *34*, 217−221.
(30) Cheynier, V. F.; Van Hulst, M. W. J. Oxidation of *trans*-caftaric acid and 2-*S*-glutathionylcaftaric acid in model solutions. *J. Agric. Food Chem.* **1988**, *36*, 10−15.
(31) Cejudo-Bastante, M. J.; Pérez-Coello, M. S.; Hermosín-Gutiérrez, I. Identification of new derivatives of 2-*S*-glutathionylcaftaric acid in aged white wines by HPLC-DAD-ESI-MS^n. *J. Agric. Food Chem.* **2010**, *58*, 11483−11492.
(32) Iland, P.; Bruer, N.; Edwards, G.; Weeks, S.; Wilkes, E. Sulfur dioxide (SO_2) by the aspiration method. In *Chemical analysis of grapes and wine: techniques and concepts*; Patrick Iland Wine Promotions Pty Ltd: Campbelltown, South Australia, 2004; pp 54−58.
(33) Capone, D. L.; Sefton, M. A.; Hayasaka, Y.; Jeffery, D. W. 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. *J. Agric. Food Chem.* **2010**, *58*, 1390−1395.
(34) Kalua, C. M.; Boss, P. K. Sample preparation optimization in wine and grapes: dilution and sample/headspace volume equilibrium theory for headspace solid-phase microextraction. *J. Chromatogr., A* **2008**, *1192*, 25−35.
(35) Pérez Olivero, S. J.; Pérez Trujillo, J. P. A new method for the determination of carbonyl compounds in wines by headspace solid-phase microextraction coupled to gas chromatography−ion trap mass spectrometry. *J. Agric. Food Chem.* **2010**, *58*, 12976−12985.
(36) Capone, D. L.; Van Leeuwen, K. A.; Pardon, K. H.; Daniel, M. A.; Elsey, G. M.; Coulter, A. D.; Sefton, M. A. Identification and analysis of 2-chloro-6-methylphenol, 2,6-dichlorophenol and indole: causes of taints and off-flavours in wines. *Aust. J. Grape Wine Res.* **2010**, *16*, 210−217.
(37) Capone, D. L.; Van Leeuwen, K.; Taylor, D. K.; Jeffery, D. W.; Pardon, K. H.; Elsey, G. M.; Sefton, M. A. Evolution and occurrence of 1,8-cineole (eucalyptol) in Australian wine. *J. Agric. Food Chem.* **2011**, *59*, 953−959.
(38) Joslin, W. S.; Ough, C. S. Cause and fate of certain C6 compounds formed enzymatically in macerated grape leaves during harvest and wine fermentation. *Am. J. Enol. Vitic.* **1978**, *29*, 11−17.
(39) Kobayashi, H.; Takase, H.; Suzuki, Y.; Tanzawa, F.; Takata, R.; Fujita, K.; Kohno, M.; Mochizuki, M.; Suzuki, S.; Konno, T. Environmental stress enhances biosynthesis of flavor precursors, *S*-3-(hexan-1-ol)-glutathione and *S*-3-(hexan-1-ol)-L-cysteine, in grapevine through glutathione *S*-transferase activation. *J. Exp. Bot.* **2011**, *62*, 1325−1336.
(40) Thibon, C.; Cluzet, S.; Merillon, J. M.; Darriet, P.; Dubourdieu, D. 3-Sulfanylhexanol precursor biogenesis in grapevine cells: the stimulating effect of *Botrytis cinerea*. *J. Agric. Food Chem.* **2011**, *59*, 1344−1351.
(41) Thibon, C.; Dubourdieu, D.; Darriet, P.; Tominaga, T. Impact of noble rot on the aroma precursor of 3-sulfanylhexanol content in *Vitis vinifera* L. cv Sauvignon blanc and Semillon grape juice. *Food Chem.* **2009**, *114*, 1359−1364.