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Effects of Transporting and Processing Sauvignon blanc Grapes on 3-Mercaptohexan-1-ol Precursor Concentrations

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Supporting Information

ABSTRACT: The effects of different processing treatments on thiol precursor concentrations have been investigated through studies involving transportation of machine-harvested Sauvignon blanc fruit and assessment of different applications of antioxidants, along with juice preparation and enzyme inhibition experiments. The influence these trials had on 3-*S*-cysteinylhexan-1-ol (Cys-3-MH) and 3-*S*-glutathionylhexan-1-ol (Glut-3-MH) concentrations in juices is discussed. Very interesting findings included the large increase in precursor concentrations after transportation, particularly for Cys-3-MH, and the limited formation of Glut-3-MH when grape proteins were precipitated during processing. The various results provided information about the ability to modulate precursor concentrations depending on the processing technique employed. Additionally, a conjugated aldehyde, which is the obvious missing link between the reaction of (*E*)-2-hexenal and glutathione in the formation of Glut-3-MH, has been tentatively identified for the first time. Deuterium-labeled 3-*S*-glutathionylhexanal (Glut-3-MHAl) was produced through the addition of labeled (*E*)-2-hexenal to grapes, followed by grape crushing, and detected in the juice by HPLC-MS/MS, along with the corresponding labeled Glut-3-MH.

KEYWORDS: wine aroma, varietal thiols, 3-mercaptohexan-1-ol, wine thiol precursors, HPLC-MS/MS

INTRODUCTION

Due to the importance of potent, volatile thiols to the characteristic varietal aromas of Sauvignon blanc, a range of studies relating to thiol precursors have been undertaken in recent years. These included the discovery of cysteinylated and glutathionylated precursors to 3-mercapothexan-1-ol (3-MH) and 4-mercapto-4-methylpentan-2-one (4-MMP) in Sauvignon blanc juices.[1−3] Because 3-MH and 4-MMP are released from the precursors during fermentation,[1,4−8] one avenue of investigation focuses on the effects of winemaking processes on precursor concentrations in must or juice.

Murat et al. investigated the localization in grapes and the effect of skin contact in musts on 3-*S*-cysteinylhexan-1-ol (Cys-3-MH) concentrations in Merlot and Cabernet Sauvignon varieties.[9] They found that skin contained much higher amounts of precursor per gram of material and that around 55−65% of Cys-3-MH resided in the skins when accounting for the different proportions from juice and skin. This work also showed that extended skin contact time and higher contact temperatures led to increases in precursor extraction into musts of around 50% or more,[9] which is in accord with the higher proportion of Cys-3MH found in the skins. Peyrot des Gachons et al. undertook a similar study with Sauvignon blanc grapes but included additional cysteine conjugates along with Cys-3-MH and assessed changes during ripening.[10] At maturity, 4-*S*-cysteinyl-4-methylpentan-2-one (Cys-4-MMP) and related 4-methylpentan-2-ol precursors were mainly localized in the juice (around 80% of total based on respective contributions from juice and skin). On the other hand, they found Cys-3-MH was fairly evenly distributed between juice and skin. On a per gram basis of material, Cys-4-MMP-related conjugates were essentially equal between juice and skin, whereas Cys-3-MH was predominantly found in the skins.[10] As with the work of Murat et al., increased skin contact time and temperature for the Sauvignon blanc musts showed improved extraction of precursors. However, differences were noted for Cys-4-MMP-related and Cys-3-MH precursor concentrations based on their localization in the berry. Skin contact time and temperature had a more marked effect on Cys-3-MH concentrations due to the greater abundance of this precursor in the skins.[10] Roland et al. expanded on this localization work by including glutathione conjugates in an assessment of Melon B. and Sauvignon blanc from different regions.[11] The results for Cys-3-MH in Sauvignon blanc were consistent with the earlier work,[10] whereas the glutathione conjugate of 3-MH (Glut-3-MH) was fairly evenly distributed between skin and pulp. Variations in precursor distribution were apparent when Sauvignon blanc samples from different origins were compared, although the skins always contained the greatest amounts. Compared to the pulp, there was up to twice as much Glut-3-MH and around 10 times as much Cys-3-MH in the skin in some instances.[11]

Maggu et al. evaluated the effects of different stages of a winery pressing cycle on Cys-3-MH concentrations (along with other compounds) in press fractions of several Sauvignon blanc juices, as well as the impact of laboratory-scale pressing trials using

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different skin contact times and pressures.[12] Increases in juice precursor concentrations (up to 6-fold relative to free run) were noted for press fractions collected at higher pressures from the winery. Increased precursor concentrations were also observed for juices obtained after extended skin contact time (16 and 32 h) in the laboratory, with increases again typically resulting from higher pressures used for pressing.[12] As those authors pointed out, extended skin contact times and increased pressures used at pressing will affect the extraction of oxidizable phenolics, so any gains in quality from additional varietal thiol precursor extraction may be counteracted. Roland et al. also examined precursor extraction during pressing for Melon B. and Sauvignon blanc, along with the effects of extended skin contact during prefermentative cold soaking for Sauvignon blanc.[11] In general, increases in precursor concentrations were noted for samples collected at the end of pressing compared to those obtained at the beginning, and the relative proportions of Glut-3-MH and Cys-3-MH did not change during pressing in most cases. Prefermentative cold soaking of Sauvignon blanc barely altered the concentrations of either precursor type over the 7 days of the trial,[11] indicating the precursors were extracted relatively quickly into the must.

Roland et al. assessed the effect of grape juice oxidation on varietal thiol cysteine and glutathione conjugates.[13] Melon B. and Sauvignon blanc juices were prepared in the absence of oxygen with exceedingly high levels of metabisulfite to prevent oxidation and benzenesulfinic acid to trap quinones. Subsequent addition of oxygen to the juices did not affect the concentrations of cysteine conjugates or the glutathione conjugate of 4-MMP, but gave a 2.5-fold increase for Glut-3-MH in a Sauvignon blanc juice. Supplementation of the juices with glutathione and (*E*)-2-hexenal was also evaluated after the addition of oxygen, and (*E*)-2-hexenal was determined to be the most limiting component for the formation of Glut-3-MH.[13]

We sought to add to the body of knowledge related to the effects of winemaking processes on thiol precursor concentrations. The primary aim was to verify that Cys-3-MH is endogenous to the berry (from metabolism of Glut-3-MH), whereas the bulk of Glut-3-MH is formed postharvest when processing conditions can have a major impact on juice concentrations. This was addressed through examination of the effects of commercial scale transportation of Sauvignon blanc fruit and investigation of Glut-3-MH formation via the conspicuously absent aldehyde intermediate 3-*S*-glutathionylhexanal, termed here Glut-3-MHAl.

MATERIALS AND METHODS

Materials. All isotopically labeled and unlabeled compounds were previously synthesized according to the procedures of Pardon et al.[5] and Grant-Preece et al.[6] The compounds used were d_8-(*E*)-2-hexenal; *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); 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 (d_9-Glut-3-MH). Stock solutions of standards were prepared volumetrically in Milli-Q water (for precursors) or ethanol (for d_8-(*E*)-2-hexenal) and stored at −20 °C, and working solutions were stored at 4 °C until required. Solvents used for HPLC-MS/MS analysis 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 and Merck or BDH reagents were purchased from Rowe Scientific (Lonsdale, SA, Australia), and other chemicals were obtained from Sigma-Aldrich (Castle Hill, NSW, Australia).

Grape Samples. *Transport Experiments.* Healthy Sauvignon blanc grape bunch samples (°Brix = 21.4) were hand-harvested from an Adelaide Hills vineyard 2 days prior to commercial harvest. Machine-harvested grape samples (°Brix = 21.7, pH 3.33, titratable acidity = 7.6 g/L as tartaric acid) were obtained from triplicate 2.5 tonne bins (approximately 2000 L) at the time of commercial harvest, and the bins were resampled after being transported approximately 800 km in 12 h to a winery. Samples were taken from several locations within a bin and combined, with equal proportions of juice and berries being collected. Triplicate bins had the following treatments applied in the vineyard: no SO_2/no ascorbate; 50 mg/L SO_2/no ascorbate; 500 mg/L SO_2/no ascorbate; 500 mg/L SO_2/500 mg/L ascorbate; 50 mg/L SO_2/500 mg/L ascorbate; 50 mg/L SO_2/100 mg/L ascorbate; no SO_2/100 mg/L ascorbate. Additions were performed 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. Free and total sulfur levels for each treatment can be found in Supplementary Table 1 in the Supporting Information.

Pressing Experiments. Healthy Sauvignon blanc grape bunch samples (°Brix = 22.8, pH 2.98, titratable acidity = 7.8 g/L as tartaric acid) were hand-harvested from another Adelaide Hills vineyard at commercial harvest and stored overnight at 4 °C in a temperature-controlled room.

Preparation of Juice Samples in the Laboratory. *Transport Experiments.* For hand-harvested samples, 400 plucked and randomized berries (approximately 500 g) containing 20 mg/kg SO_2 added as 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 a 10 mL aliquot of supernatant was prepared for precursor analysis. For machine-harvested samples, approximately 1.5 L of must (juice and berries) was homogenized in a Waring blender (John Morris Scientific, Kent Town, SA, Australia). A portion of the homogenate was centrifuged (Hettich Universal 32R, Adelab Scientific, Thebarton, SA, Australia) at 4000 rpm (2500*g*) for 5 min at 10 °C, and a 10 mL aliquot of the supernatant was prepared for precursor analysis.

Pressing Experiments. Methods for preparing juice for analysis were compared; this included using (1) a small sample press, (2) a stick mixer, and (3) protein precipitation.

(1) Approximately 500 g of whole bunches was pressed with a 2.2 L benchtop sample press (Winequip, Magill, SA, Australia), using a torque wrench to apply pressures of approximately 440 kPa (light pressing, 20 N m wrench setting) and approximately 670 kPa (heavy pressing, 30 N m wrench setting). Each pressure level was applied twice, with mixing of the press load between each application. Prior to applying pressure, the press had the following treatments: no SO_2; 50 mg/kg of SO_2 (added as PMS); 500 mg/kg SO_2 (added as PMS); 10 mg/kg d_8-(*E*)-2-hexenal (5 mg/mL solution in ethanol; these samples underwent light pressing only). The juice from each pressing stage was collected in a measuring cylinder, and the treatments were performed in triplicate. A 10 mL aliquot of each juice was prepared for precursor analysis.

(2) A household stick mixer was used to prepare homogenates in triplicate with the same SO_2 treatments as for the pressing from approximately 300 g of plucked and randomized berries. The homogenates were centrifuged (Beckman J2-21M/E) at 4000 rpm (2830*g*) for 10 min at 15 °C, and a 10 mL aliquot of each supernatant was prepared for precursor analysis. Free and total sulfur levels for these experiments can be found in Supplementary Table 2 in the Supporting Information.

(3) Triplicate samples of approximately 250 g of whole grape bunches were snap-frozen and ground to a fine powder in liquid nitrogen, and 180 mL of methanol/chloroform (2:1 v/v) was added to each powdered

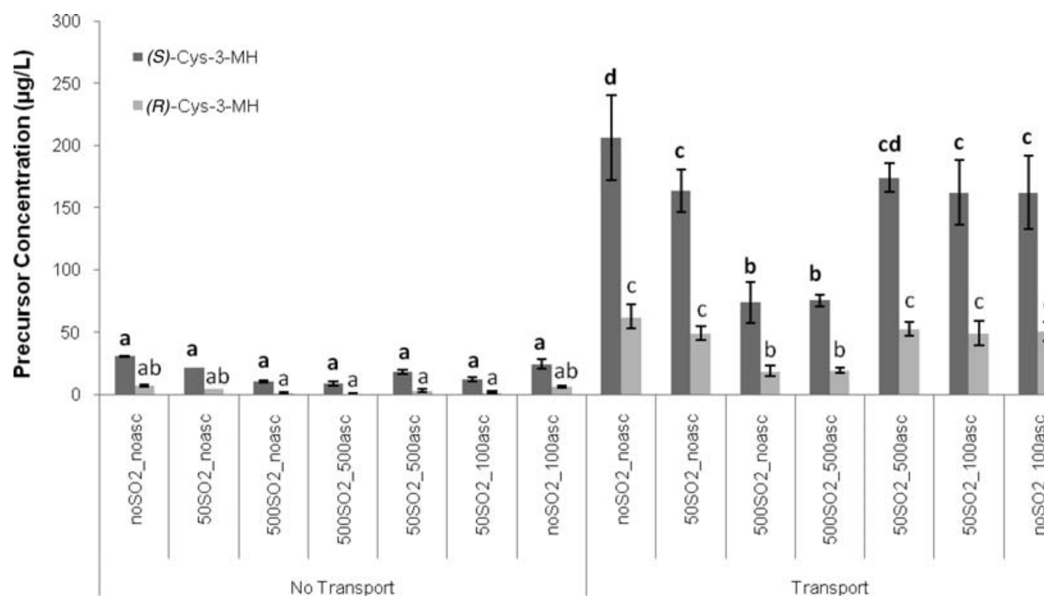

Figure 1. Concentrations of (*R*)- and (*S*)-Cys-3-MH (μg/L) before and after transportation for machine-harvested Sauvignon blanc with different levels of antioxidants. Error bars represent the standard deviation of three replicates. The *x*-axis labels relate to the additions of SO_2 and ascorbic acid, with units of mg/L. There were statistically significant differences ($p < 0.001$) between the treatments and due to the effects of transport. Different letters for the same precursor diastereomer indicate significant differences ($p < 0.05$) between the averages ((*S*)-Cys-3-MH is differentiated with bold letters).

replicate. The mixtures were filtered upon reaching room temperature, and the lower, deep green layer was discarded. The volatiles were removed under reduced pressure at 30 °C and 10 mbar on a rotary evaporator, and a 10 mL aliquot of each aqueous residue was prepared for precursor analysis.

Preparation of Juice and Wine Extracts for Precursor Analysis. Extracts were prepared according to the procedure in Capone et al.,[14] 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 (except for the treatments involving d_8-(*E*)-2-hexenal, for which 10 mL of sample was used with no internal standard added). 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 on a Zymark TurboVap LV evaporator (John Morris Scientific, Chatswood, NSW, Australia) using nitrogen at 25 °C. The sample was 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 a 0.45 μm, 13 mm Acrodisc syringe filter (Pall Gelman Life Sciences, Cheltenham, VIC, Australia) in readiness for HPLC-MS/MS analysis.

HPLC-MS Instrumentation. HPLC-MS/MS analysis was carried out as previously described by Capone et al.,[14] 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 a TurboV source and TurboIonSpray probe (Applied Biosystems/MDS Sciex, Concord, ON, Canada). For enhanced product ion (EPI) experiments, Q1 had unit resolution, the scan rate was set at 1000 amu/s, dynamic fill time was selected for the ion trap, and mass spectra were recorded between m/z 100 and 430 for parent ions of m/z 230.2, 414.3, and 416.4 in separate experiments. The remaining mass spectrometer parameters were the same as previously described.[14]

Statistical Analysis. 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

On the basis of previous studies and with an understanding of the possible enzymatic reactions involved in precursor formation, we aimed to further clarify the origins of Cys-3-MH and Glut-3-MH found in grape juices and musts. We also wanted to ascertain the effects that different commercial fruit processing operations had on thiol precursor concentrations, with a view to understanding how precursor levels could be manipulated.

Effect of Fruit Transportation. A common practice to protect volatile aroma compounds for some styles of white wine is to treat the fruit as delicately as possible, taking care to minimize berry damage between harvesting and crushing at the winery. Anecdotal evidence from winemakers made us aware that long-distance transportation of fruit for extended periods afforded wine with more tropical aromas than if the grapes were crushed locally and the juice was transported. We had other indications that fruit processing was important to thiol precursor concentrations, particularly for Glut-3-MH,[15] and we wanted to work on a commercially relevant scale. To this end, we undertook replicated experiments with different antioxidant treatments to compare fruit obtained immediately after machine harvesting with the same batches of fruit that underwent transportation. Antioxidants were included as they are commonly used during the winemaking process to prevent oxidation, particularly of phenolic material, which would otherwise lead to undesirable browning of white juices and wines. The antioxidant effect would also affect other oxidizable compounds such as glutathione (GSH), which is a naturally present antioxidant that seemingly plays an important role in thiol precursor formation. The fruit was collected in 2.5 tonne bins, some of which were dosed in the

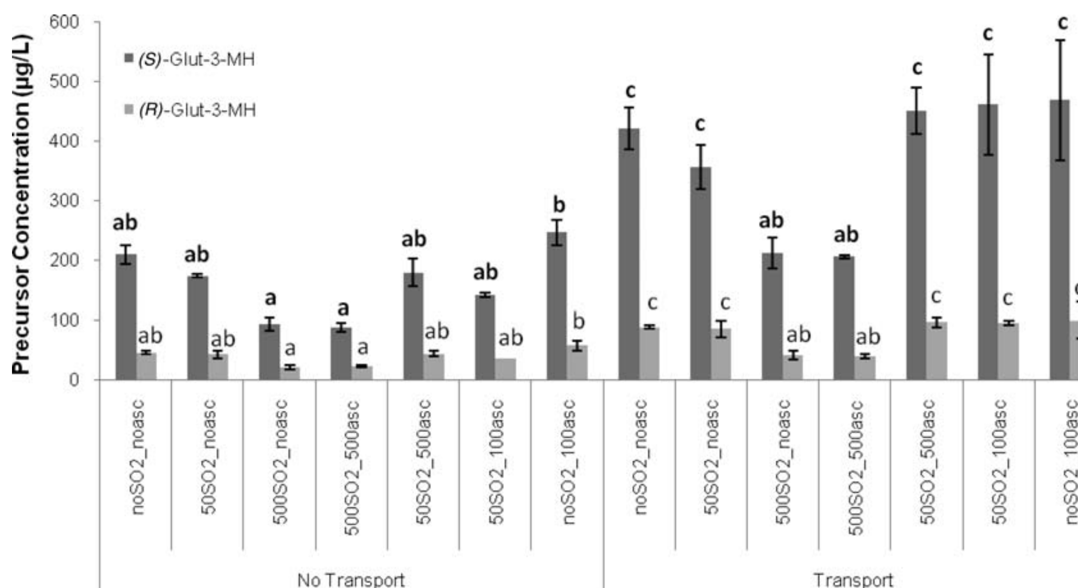

Figure 2. Concentrations of (R)- and (S)-Glut-3-MH (μg/L) before and after transportation for machine-harvested Sauvignon blanc with different levels of antioxidants. Error bars represent the standard deviation of three replicates. The x-axis labels relate to the additions of SO_2 and ascorbic acid, with units of mg/L. There were statistically significant differences ($p < 0.001$) between the treatments and due to the effects of transport. Different letters for the same precursor diastereomer indicate significant differences ($p < 0.05$) between the averages ((S)-Glut-3-MH is differentiated with bold letters).

vineyard with SO_2 and/or ascorbic acid (at standard and very high rates of addition) at the time of harvesting, and samples were brought back to the laboratory for immediate preparation of extracts for precursor analysis. The covered bins were then transported in the early hours of the morning at ambient temperature on flat-bed trucks for approximately 800 km within about 12 h. The trucks were met upon arrival at the winery for sampling of the different treatment bins prior to grape crushing, and the samples were prepared for thiol precursor analysis.

The results obtained for the Cys-3-MH diastereomers are shown in Figure 1. The first observation to note is the stark difference in Cys-3-MH levels between the fruit processed immediately (around 40 μg/L maximum total) and the transported fruit, with up to an approximate 10-fold increase in Cys-3-MH for the transported fruit depending on the treatment (around 270 μg/L maximum total). The general trends related to diastereomer abundance, that is, the (S)-diastereomer being higher in proportion than the (R)-diastereomer, mirrored our previous findings.[14] Further inspection of the results for the transported samples revealed significant differences between the averages for additions of ascorbic acid and SO_2 (Figure 1). The samples with the higher Cys-3-MH concentrations (around 190 μg/L total) were those with lower levels of SO_2, whereas ascorbic acid addition alone or in combination with 50 mg/L of SO_2 seemed to have little impact. The sample without addition of either antioxidant was also high in Cys-3-MH (around 270 μg/L total), but this treatment type would probably not be attractive to winemakers due to the oxidation of phenolics that would have ensued (juice from these treatments was visibly brown, indicating a degree of oxidation).

Interestingly, the samples containing 500 mg/L SO_2 were substantially lower in Cys-3-MH (around 95 μg/L total) compared to the other transport treatments (juice from these treatments was green in color). As this form of precursor is a metabolite of Glut-3-MH, the lower Cys-3-MH levels with high SO_2 are likely due to SO_2 either binding with any (E)-2-hexenal present, preventing enzymatic formation of (E)-2-hexenal, or preventing enzymatic degradation of Glut-3-MH into Cys-3-MH, as detailed further below. Because SO_2 has both antimicrobial and antioxidant capabilities and binds aldehydes as their bisulfite adducts, we could not determine which of these aspects was occurring. Whatever the mechanism, the generation or degradation of Glut-3-MH would be hampered, and this may be reflected in the levels of Cys-3-MH we encountered (Figure 1).

Apart from the 500 mg/L SO_2 treatments, the amounts determined for Cys-3-MH were much greater than those identified and discussed in our previous studies of commercial juices obtained from healthy fruit.[14] This was likely due to the enzymatic degradation of Glut-3-MH into Cys-3-MH, which may have been from the actions of grape berry (endogenous) or grape microflora (exogenous) enzymes. The results were reminiscent of those reported by Thibon et al. for increases in Cys-3-MH in overripe and botrytized fruit, and their description of the enzymes involved is also relevant to our work.[16] Considering Cys-3-MH is probably more readily utilized by yeasts, based on conversion yields of approximately 10% or less for Cys-3-MH and less than 5% for Glut-3-MH,[6−9,17,18] the increase in Cys-3-MH concentrations with transport was a very interesting finding. On the basis of the highest levels of Cys-3-MH we have encountered (Figure 1), a conversion yield on the order of 10% during fermentation could in theory lead to wine concentrations for 3-MH of about 16000 ng/L. This equates to an odor activity value (OAV) of around 270 based on 3-MH threshold data,[19] which is a phenomenal amount of 3-MH even for Sauvignon blanc. This adds credence to the anecdotal evidence from winemakers about greater tropical aromas in wines made from transported fruit.

The Glut-3-MH diastereomer profiles displayed similarities with their Cys-3-MH counterparts, but this time there was only an approximate doubling of Glut-3-MH after transportation (around 570 μg/L maximum total) compared to immediate

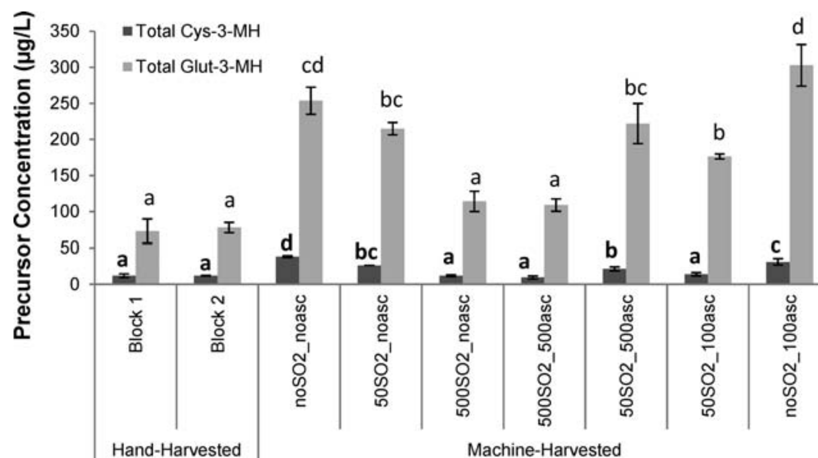

Figure 3. Concentrations of total Cys-3-MH and total Glut-3-MH (μg/L) for hand-harvested Sauvignon blanc grapes and machine-harvested grapes from the same blocks with the addition of antioxidants. Error bars represent the standard deviation of three replicates. The *x*-axis labels relate to the additions of SO_2 and ascorbic acid, with units of mg/L. There were statistically significant differences ($p < 0.001$) between the treatments for both types of precursor. Different letters for the same type of precursor indicate significant differences ($p < 0.05$) between the averages (Cys-3-MH is differentiated with bold letters).

processing (around 300 μg/L maximum total). The effects of transportation were still significant, however (Figure 2). It appears that Glut-3-MH in the transported samples had time to be enzymatically converted to Cys-3-MH. If no such conversion had taken place, the Glut-3-MH concentrations encountered after transportation would most certainly have been much higher. These results highlight the dynamic nature of these precursors, where there is the propensity to form more precursor depending on the processing methodology. We had previously shown that Cys-3-MH was formed during fermentation of Glut-3-MH,[6] and now we have apparently observed the same effect in uninoculated juice.

In contrast to the situation with Cys-3-MH after transportation, ascorbic acid seemed to have an impact on Glut-3-MH levels (Figure 2). The optimal conditions for maximum Glut-3-MH concentrations were the treatments involving ascorbic acid with 50 mg/L of SO_2. The treatment without addition of antioxidants was comparable but, as explained above, oxidation of phenolics in this case makes the treatment undesirable. Furthermore, some SO_2 use is necessary when employing ascorbic acid to reduce the hydrogen peroxide formed in the presence of oxygen.[20] As with Cys-3-MH in the transported samples, the highest levels of SO_2 coincided with significantly lower amounts of Glut-3-MH, and this can be similarly explained due to one or more factors: inhibition of the formation of (*E*)-2-hexenal via the lipoxygenase (LOX)/hydroperoxide lyase (HPL) pathway[21] by preventing oxidation of the parent fatty acid;[22] binding of the aldehyde formed via LOX/HPL as the bisulfite adduct;[23] and inhibition of other enzymatic reactions such as GST-mediated conjugation of GSH with the aldehyde.[24] Any one of these points could lead to disruption of Glut-3-MH formation. Roland et al. have suggested that (*E*)-2-hexenal was the limiting component in Glut-3-MH conjugate formation in their studies,[13] which is supported by our observations. Conjugate formation requires both (*E*)-2-hexenal and GSH, but the protective actions of SO_2 and ascorbic acid on the glutathione present in the juice were seemingly outweighed by the competing effects of (*E*)-2-hexenal binding or inhibition of formation for the high SO_2 treatments. Kobayashi et al. have revealed the role of several enzymes in thiol precursor formation,[25] and this fits with our observations too, in the event that Glut-3-MH formation relies heavily on an enzymatic conjugation step, which is inhibited by SO_2. The field of thiol precursor research is progressing well with each discovery, but clearly there is a lot more to understand about the role of chemical and enzymatic reactions within the grape and wine biochemical system.

Hand versus Machine Harvesting. Along with the samples used for the transport study, we also collected hand-harvested samples several days before the vineyard was machine-harvested. The effect of grape processing can be clearly seen in Figure 3, with approximately 70% less Glut-3-MH and 65% less Cys-3-MH compared to machine-harvested fruit without the addition of antioxidants. Indeed, hand-harvested fruit yielded precursor average values slightly lower than the 500 mg/L SO_2 treatments, although the difference was not significant. In the case of hand harvesting it would appear that minimal berry damage relative to machine harvesting has resulted in less formation of the precursors. Considering that the samples were processed in the laboratory in an identical manner and within a comparable time frame, the effects of machine harvesting appear to manifest themselves relatively quickly. These results may be relevant to decisions about how carefully fruit is treated during harvesting and highlight another factor that can affect precursor concentrations.

Effects of Processing on Precursors in Juice. Replicated experiments were conducted with different levels of SO_2 to compare juices obtained from homogenates (prepared with a stick mixer) with juices from a small benchtop sample press. At the same time we investigated the effect of inhibiting enzymes by precipitating proteins during sample preparation. The results from these studies are shown in Figure 4 as the totals of Cys-3-MH and Glut-3MH. The first three bars are the results obtained for the stick mixer, the next three are those obtained from combining the light- and hard-pressing results (approximately 70% of precursors were found in the light-press fractions, and this was consistent across the treatments), and the last bar is for the

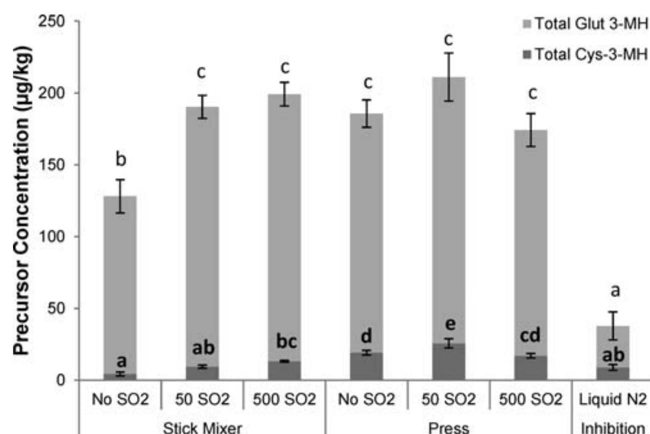

Figure 4. Effect of mode of juice preparation on thiol precursor concentrations (μg/L) using a stick mixer or benchtop sample press with different additions of SO_2, and inhibiting enzymes through cryogenic processing and protein precipitation. Error bars represent the standard deviation of three replicates. The x-axis labels relate to the additions of SO_2, with units of mg/L. There were statistically significant differences ($p < 0.001$) between the treatments for both types of precursor. Different letters for the same type of precursor indicate significant differences ($p < 0.05$) between the averages (Cys-3-MH is differentiated with bold letters).

enzyme inhibition experiment. From stick mixing and combined pressings assessments, it appears that in the presence of SO_2, both types of processing are adequate to obtain juices for analysis. There appears to be no need to collect the light- and hard-press stages separately, but a hard-press level is certainly advisible. The greater ease of the stick mixer process means this would be the more favored method, but some SO_2 is required. The lower result in the absence of SO_2 with stick mixing probably relates to phenolic oxidation and binding of GSH, limiting formation of Glut-3-MH to some extent.

The most interesting thing to note, however, is the much lower concentration of Glut-3-MH with the enzyme inhibition experiment (Figure 4). In an effort to prevent enzymatic activity, grapes were frozen in liquid nitrogen, pulverized to a fine powder, and added to methanol/chloroform to precipitate the proteins. Whereas there was no significant difference in the average amounts of Cys-3-MH compared to the stick mixer results, there was around 6 times less Glut-3-MH compared to the processing experiments involving SO_2. In fact, there was a significant difference between the averages for enzyme inhibition and all of the other treatments for Glut-3-MH. This result is strongly indicative that a large proportion of Glut-3-MH is formed during berry processing, which helps explain the low Glut-3-MH results presented by Roland et al. with their specific juice elaboration method.[13] That is, if enzymatic reactions in juices are prevented or oxygen is limited during formation of crucial components (e.g., (E)-2-hexenal), then low Glut-3-MH concentrations are the result. This knowledge led us to believe that Cys-3-MH, a breakdown product of Glut-3-MH, is endogenous to the berry and relatively unaffected by grape processing (except for long-distance transportation, for instance), whereas the bulk of Glut-3-MH is formed postharvest when processing conditions can have a major impact on juice concentrations. Interestingly, further work from Roland et al. revealed the predominance of Glut-3-MH over Cys-3-MH for the first time in a study of French grape varieties,[11] which was in accord with our findings for Australian juices.[14] In light of their previous results,[13] this led them to suggest that the procedures used to obtain a grape must might alter the extraction and distribution of precursors,[11] which contrasts somewhat with our proposal that Glut-3-MH forms during processing. To further show the effect of processing on Glut-3-MH, an additional replicated experiment was performed to verify the formation of GSH conjugates upon grape crushing and to investigate the missing intermediate between Glut-3-MH found in juice and the conjugation of GSH to (E)-2-hexenal.

Glut-3-MHAl as an Intermediate to Glut-3-MH. To observe products from the conjugation of GSH to (E)-2-hexenal during grape crushing, we employed a deuterium-labeled aldehyde and HPLC-MS/MS analysis to monitor product ions revealing where the labeled aldehyde was incorporated. d_8-(E)-2-Hexenal was added to whole grape berries before they were crushed in a press, and the juices were prepared and analyzed without the addition of internal standard. After evaluation of the mass spectra (Figure 5), we can provide evidence for Glut-3-MHAl, from conjugation of GSH and (E)-2-hexenal, as an intermediate in the formation of Glut-3MH for the first time. For samples crushed in the presence of labeled (E)-2-hexenal we could identify the labeled aldehyde intermediate d_8-Glut-3-MHAl (m/z 414) along with the labeled alcohol d_8-Glut-3-MH (m/z 416). The amounts were not quantified, but there was roughly 3−5 times more aldehyde than alcohol, assuming the same ionization efficiency for both compounds (data not shown). No labeled Cys-3-MH (m/z 230) was identified in these experiments.

The fragmentation patterns of the conjugates were easily recognized on the basis of the fragmentation pattern of the related d_{10}-Glut-3-MH, which was detailed in Capone et al.[14] The tentatively assigned d_8-Glut-3-MHAl is 2 mass units less than d_8-Glut-3-MH, which itself is 2 mass units less than the known d_{10}-analogue. For the fragmentation pattern of d_8-Glut-3-MHAl in Figure 5A: fragment ion m/z 396 resulted from a loss of water (18 Da) from the protonated molecular ion m/z 414; fragment ion m/z 339 was from the neutral loss of glycine (75 Da) from the protonated molecular ion; fragment ion m/z 285 resulted from the neutral loss of glutamate (129 Da) from the protonated molecular ion; fragment ion m/z 267 resulted from the neutral loss of water (18 Da) from fragment ion m/z 285; fragment ion m/z 268 resulted from neutral loss of ammonia (17 Da) from fragment ion m/z 285; fragment ion m/z 250 resulted from the neutral loss of water (18 Da) from fragment ion m/z 268; and fragment ion m/z 162 resulted from the neutral loss of d_8-hexenal (106 Da) from the fragment ion m/z 268. The fragmentation pattern for d_8-Glut-3-MH (Figure 5B) was very similar to that of the aldehyde and identical to that of d_{10}-Glut-3-MH reported previously.[14]

This result provided evidence for the obvious missing link in the formation of Glut-3-MH from (E)-2-hexenal and GSH. We assume that chemical or enzymatic conjugation of (E)-2-hexenal, formed enzymatically from linolenic acid, with GSH in the juice yields Glut-3-MHAl, which is converted to the alcohol Glut-3-MH. The process for conversion of Glut-3-MHAl to Glut-3-MH would necessarily involve grape berry or microflora reduction enzymes, probably from the alcohol dehydrogenase (ADH) or aldo-keto reductase (AKR) families.[26−31] The process of conversion to Glut-3-MHAl would also occur in the grape berry as a stress response, most likely by GST enzymes,[25] and the aldehyde would be reduced enzymatically to the more stable alcohol Glut-3-MH. It is suggested that Glut-3-MH is converted predominantly to Cys-3-MH in the grape berry, on the

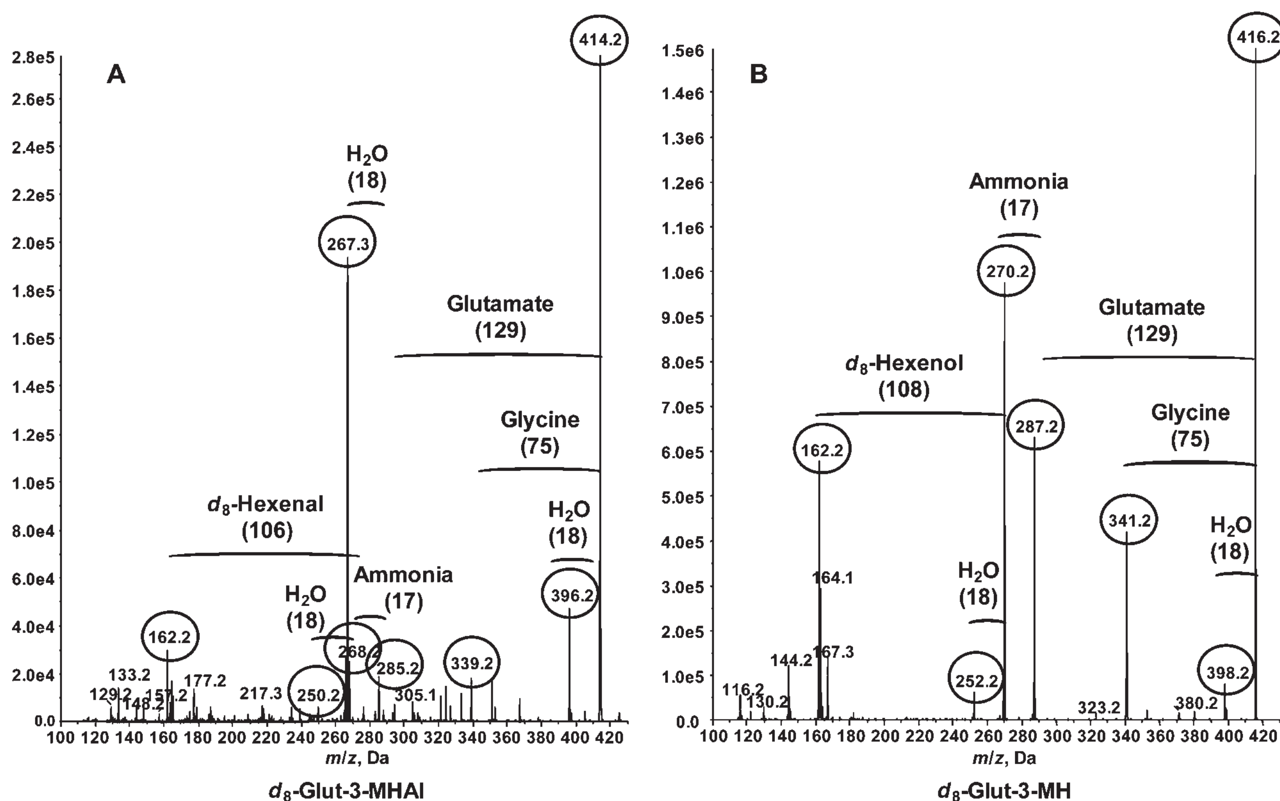

Figure 5. Enhanced product ion mass spectra of new, tentatively assigned intermediate d_8-Glut-3MHAl (A) and related d_8-Glut-3-MH (B), obtained from crushing grapes in the presence of d_8-(*E*)-2-hexenal.

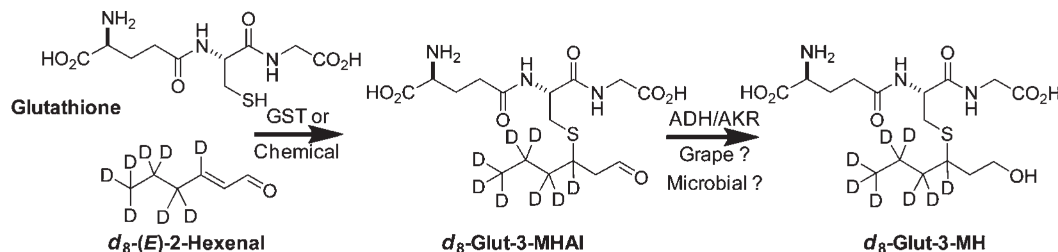

Figure 6. Proposed formation pathway of d_8-Glut-3-MH from d_8-(*E*)-2-hexenal and glutathione upon grape crushing, showing tentatively assigned d_8-Glut-3MHAl as an intermediate. GST, glutathione *S*-transferase; ADH, alcohol dehydrogenase; AKR, aldo-keto reductase.

basis of our findings. In juice, however, once C6 compounds have formed enzymatically (or labeled aldehyde has been added with crushing), the conjugation may be enzymatic or chemical, and this still needs to be clarified. Regardless of the mode of conjugation in juice, which appears to be quite rapid, enzymatic reduction would still be a requirement. Glut-3-MHAl, not accounted for until now, may be a transient intermediate, but it seems to constitute another potential precursor of 3-MH in wine. The role of this aldehyde precursor in enzymatic transformations related to Glut-3-MH also needs to be clarified to determine if there is a cysteinylated counterpart, for instance.

Our studies have shown that a range of factors have the potential to affect thiol precursor concentrations. A comparison of pressing and using a stick mixer to obtain juices for analysis gave similar results for both methods with the addition of 50 mg/L SO_2. We demonstrated that inhibiting enzyme activity by precipitating grape proteins dramatically limited the formation of Glut-3-MH during crushing, whereas the Cys-3-MH concentration remained essentially unaffected. Significant effects caused by transportation were found for Sauvignon blanc grapes, with increased precursor concentrations for both conjugate types. Cys-3-MH was increased more by transportation than Glut-3-MH, however, which is an important finding as this conjugate is probably more easily utilized by yeast. We observed the effect of antioxidants SO_2 and ascorbic acid in the transportation studies, where a combination of both appeared to be optimum. When levels of SO_2 were high (500 mg/L), a clear suppression of conjugate formation was observed. Furthermore, there was an important difference in precursor concentrations depending on the method of harvesting; hand-harvested fruit had less than half the precursor levels of machine-harvested grapes. Experiments also revealed the presence of labeled Glut-3-MHAl arising from conjugation of labeled (*E*)-2-hexenal with GSH. This enabled us to tentatively assign the identity of d_8-Glut-3-MHAl for the first time, along with the related d_8-Glut-3-MH, arising from grapes crushed in the presence of d_8-(*E*)-2-hexenal. The processes involved in the conjugation are most likely

enzymatic in nature but may involve a chemical contribution that is yet to be determined (Figure 6). The importance of Glut-3-MHAl to wine 3-MH concentrations needs to be examined, and studies are required to quantify it in juices. Nonetheless, considering that yeasts have reductase activity, it is conceivable that Glut-3-MHAl could be reduced to Glut-3-MH during vinifcation, thereby increasing the pool of potential 3-MH in wine. These results fit well with recent studies and provide further insights into understanding the formation of thiol precursors. Knowledge of the relationships between important varietal aroma compounds such as 3-MH and its conjugates could lead to the development of options for optimizing varietal thiol profiles in wines through manipulation of the precursors during grape processing.

ASSOCIATED CONTENT

Supporting Information. Tables of free and total sulfur levels. This material is available free of charge via the Internet at http://pubs.acs.org.

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ABBREVIATIONS USED

3-MH, 3-mercaptohexan-1-ol; 4-MMP, 4-mercapto-4-methylpentan-2-one; Cys-3-MH, 3-*S*-cysteinylhexan-1-ol; Cys-4-MMP, 4-*S*-cysteinyl-4-methylpentan-2-one; Glut-3-MH, 3-*S*-glutathionylhexan-1-ol; Glut-3-MHAl, 3-*S*-glutathionylhexanal; PMS, potassium metabisulfite; EPI, enhanced product ion; OAV, odor activity value; LOX, lipoxygenase; HPL, hydroperoxide lyase; GSH, glutathione; GST, glutathione *S*-transferase; ADH, alcohol dehydrogenase; AKR, aldo-keto reductase.

REFERENCES

(1) 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.

(2) 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.

(3) Fedrizzi, B.; Pardon, K. H.; Sefton, M. A.; Elsey, G. M.; Jeffery, D. W. First identification of 4-*S*-glutathionyl-4-methylpentan-2-one, a potential precursor of 4-mercapto-4-methylpentan-2-one, in Sauvignon Blanc juice. *J. Agric. Food Chem.* **2009**, *57*, 991–995.

(4) 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.

(5) 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.

(6) 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.

(7) 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.

(8) Roland, A.; Schneider, R.; Guernevé, C. L.; Razungles, A.; Cavelier, F. Identification and quantification by LC-MS/MS of a new precursor of 3-mercaptohexan-1-ol (3MH) using stable isotope dilution assay: Elements for understanding the 3MH production in wine. *Food Chem.* **2010**, *121*, 847–855.

(9) 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.

(10) 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.

(11) 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.

(12) 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.

(13) 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.

(14) 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.

(15) 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**doi: 10.1021/jf200116q.

(16) 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.

(17) Dubourdieu, D.; Tominaga, T.; Masneuf, I.; Peyrot des Gachons, C.; Murat, M. L. The role of yeasts in grape flavor development during fermentation: the example of Sauvignon blanc. *Am. J. Enol. Vitic.* **2006**, *57*, 81–88.

(18) Subileau, M.; Schneider, R.; Salmon, J.-M.; Degryse, E. New insights on 3-mercaptohexanol (3MH) biogenesis in Sauvignon Blanc wines: Cys-3MH and (*E*)-hexen-2-al are not the major precursors. *J. Agric. Food Chem.* **2008**, *56*, 9230–9235.

(19) Tominaga, T.; Furrer, A.; Henry, R.; Dubourdieu, D. Identification of new volatile thiols in the aroma of *Vitis vinifera* L. var. Sauvignon blanc wines. *Flavour Fragrance J.* **1998**, *13*, 159–162.

(20) Waterhouse, A. L.; Laurie, V. F. Oxidation of wine phenolics: a critical evaluation and hypotheses. *Am. J. Enol. Vitic.* **2006**, *57*, 306–313.

(21) Kalua, C. M.; Boss, P. K. Comparison of major volatile compounds from Riesling and Cabernet Sauvignon grapes (*Vitis vinifera* L.) from fruitset to harvest. *Aust. J. Grape Wine Res.* **2010**, *16*, 337–348.

(22) Cayrel, A.; Crouzet, J.; Chan, H. W. S.; Price, K. R. Evidence for the occurrence of lipoxygenase activity in grapes (variety Carignane). *Am. J. Enol. Vitic.* **1983**, *34*, 77–82.

(23) de Azevedo, L. C.; Reis, M. M.; Motta, L. F.; da Rocha, G. O.; Silva, L. A.; de Andrade, J. B. Evaluation of the formation and stability of hydroxyalkylsulfonic acids in wines. *J. Agric. Food Chem.* **2007**, *55*, 8670–8680.

(24) Leung, K.-H.; Post, G. B.; Menzel, D. B. Glutathione *S*-sulfonate, a sulfur dioxide metabolite, as a competitive inhibitor of glutathione *S*-transferase, and its reduction by glutathione reductase. *Toxicol. Appl. Pharmacol.* **1985**, *77*, 388–394.

(25) 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.

(26) 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.

(27) Molina, I.; Nicolas, M.; Crouzet, J. Grape alcohol dehydrogenase. I. Isolation and characterization. *Am. J. Enol. Vitic.* **1986**, *37*, 169–173.

(28) Herraiz, T.; Herraiz, M.; Reglero, G.; Martin-Alvarez, P. J.; Cabezudo, M. D. Changes in the composition of alcohols and aldehydes of C6 chain length during the alcoholic fermentation of grape must. *J. Agric. Food Chem.* **1990**, *38*, 969–972.

(29) Kuhn, A.; van Zyl, C.; van Tonder, A.; Prior, B. Purification and partial characterization of an aldo-keto reductase from *Saccharomyces cerevisiae*. *Appl. Environ. Microbiol.* **1995**, *61*, 1580–1585.

(30) Fauconnier, M. L.; Mpambara, A.; Delcarte, J.; Jacques, P.; Thonart, P.; Marlier, M. Conversion of green note aldehydes into alcohols by yeast alcohol dehydrogenase. *Biotechnol. Lett.* **1999**, *21*, 629–633.

(31) Deytieux, C.; Geny, L.; Lapaillerie, D.; Claverol, S.; Bonneu, M.; Doneche, B. Proteome analysis of grape skins during ripening. *J. Exp. Bot.* **2007**, *58*, 1851–1862.