IMPROVED UNDERSTANDING OF VARIETAL THIOL PRECURSORS IN GRAPES AND WINE

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Dimitra Capone, a research scientist from the Australian Wine Research Institute (AWRI), has recently completed a PhD with the University of Adelaide, where she was co-supervised by Professor Dennis Taylor, Dr Mark Sefton and Dr David Jeffery. The results from Dimitra's PhD were prodigious and her thesis earned her a Dean's Commendation for Doctoral Thesis Excellence. What follows is a snapshot of her work on thiol precursors in grapes and wine. For simplicity, there is no discussion of the stereochemistry of these molecules and they are mostly referred to as if they are single components, when in reality 3-MH and its precursors exist as pairs of enantiomers and diastereomers, respectively.

Varietal thiols

Wine contains an abundance of volatile compounds which contribute to its aromas. The origins of certain compounds can be linked to molecules that are present in the grapes, with certain varieties providing distinctive wine aromas. Sauvignon Blanc, with its citrus, passionfruit and boxwood characters, provides a good example of the concept of varietal aromas. These pleasant traits result from the presence of polyfunctional "varietal" thiols, which are among the most potent food odorants known (perceptible at low ng/L concentrations, Table 1). The key thiols responsible for Sauvignon Blanc aromas are 4-mercapto-4-methylpentan-2-one (4-MMP), 3-mercaptohexan-1-ol (3-MH) and 3-mercaptohexyl acetate (3-MHA) (Coetzee and du Toit, 2012). These thiols have also been found in a range of other wines, such as Gewurztraminer, Riesling, Colombard, Merlot, Grenache and Cabernet Sauvignon, although usually not to the same extent as Sauvignon Blanc.

Table 1. Varietal thiol characteristics.

	Aroma detection threshold	Aroma description	Concentration found in wine
4-MMP	3 ng/L	Blackcurrant Box tree Passionfruit	Low ng/L
3-MH	60 ng/L	Grapefruit Passionfruit	Low ng/L to low µg/L
3-MHA	4 ng/L	Passionfruit Box tree Sweaty	Low ng/L to low µg/L

Thiol precursors

While some varietal aroma compounds exist as their aroma-active forms in the grapes and are subsequently extracted during vinification (e.g. methoxypyrazines), it was recently shown that only minor amounts of 3-MH are present in grape juices from a selection of Sauvignon Blanc clones at different stages of ripening (Capone et al., 2011a). In the absence of other identified precursors, it is generally accepted that the varietal thiols found in wine are liberated by yeast enzymes during fermentation from non-volatile precursors present in grapes (except 3-MHA, which is derived from 3-MH). Having an understanding of the grape precursor concentrations and how they vary is therefore an important aspect to consider for optimising wine thiol profiles.

Precursors to 3-MH had been identified as conjugates of glutathione (Glut-3-MH) and cysteine (Cys-3-MH) around a decade ago. Over a number of years the AWRI, among other groups working on the topic, had undertaken research on engineered yeasts which led to improved understanding and superior yields of thiols from their cysteine conjugates. Little was known, however, about the relevance of the glutathione conjugates until some collaborative work revealed for the first time, using model media, that Glut-3-MH could also act as a precursor to 3-MH (approximately 3% conversion), although the cysteine conjugate seemed to be more easily metabolised by yeast (approximately 14% conversion) (Grant-Preece et al., 2010). It was also found that Cys-3-MH arose during fermentation of the pure glutathione conjugate, in all likelihood via a dipeptide intermediate. It appears that yeast first metabolises the glutathione conjugate to its cysteine equivalent before the thiol can be released, but the intermediates for this transformation were not identified at that stage. Other research has subsequently supported the theory that Cys-3-MH is the more easily utilised precursor (e.g. Winter et al., 2011).

Around the same time as this novel fermentation study, the first method was developed for analysing both the known 3-MH precursor types in grape juices and wines, using high performance liquid chromatography coupled to a mass spectrometer (HPLC-MS) (Capone et al., 2010). Precursor concentrations were determined using this method for Pinot Gris, Riesling and Chardonnay, as well as Sauvignon Blanc, with the latter variety generally containing the greatest amounts. There was good comparison of the Cys-3-MH concentrations in Sauvignon Blanc with those in the literature, but the Glut-3-MH results were far in excess of those reported in another study on this grape variety (Table 2). Investigations were continued to determine the reasons for such a difference, along with the effects of viticultural and winemaking practises.

Table 2. 3-MH precursors (µg/L) in Sauvignon Blanc juice and wine samples.

	Capone et al. (2010) **Juice**	Roland et al. (2010) **Juice**	Capone et al. (2010) **Wine**
Cys-3-MH	21 – 55	8 – 40	1 – 35
Glut-3-MH	245 – 696	1 – 8	138 – 142

Identification of related intermediates

When trying to establish the importance of any precursor contributions to 3-MH found in wine, it certainly helps to have an understanding of all the precursors which can potentially contribute. It was evident from the fermentation work that there could be intermediates in the formation of Cys-3-MH from Glut-3-MH during vinification. The breakdown products of Glut-3-MH may also be present in the grape juice, since Cys-3-MH is naturally found in juices and the biochemical pathway in grapes should also involve dipeptide intermediates. Based on the plant enzymes involved in degradation of glutathione itself, it was hypothesised that one likely intermediate was the cysteinylglycine conjugate of 3-MH (Cysgly-3-MH). This compound was prepared in the laboratory from Glut-3-MH already on hand, using the same type of enzyme found in plants (albeit a commercially available extract from equine kidney). HPLC-MS experiments identified naturally occurring Cysgly-3-MH in Sauvignon Blanc juices for the first time, providing a piece of evidence linking the breakdown of Glut-3-MH to Cys-3-MH (Capone et al., 2011b). This additional component was included in the precursor analytical method, such that Glut-, Cysgly- and Cys-3-MH could now be analysed by a single technique.

Another piece of the puzzle was put in place with experiments to determine the extent to which Glut-3-MH forms as a result of crushing grape berries. Previously, it was more or less assumed that 3-MH precursors were present in the grape berry and were extracted during winemaking. This was intriguing, considering the vastly elevated levels of Glut-3-MH in some other experiments (e.g. freezing grapes as opposed to freezing juice) and the much higher Glut-3-MH results from commercial samples compared to those of Roland et al. (2010) (Table 2). Knowing that (*E*)-2-hexenal, one of the candidate components incorporated into Glut-3-MH along with glutathione, is formed as a result of enzymatic processes with berry damage, it seemed reasonable that if both of these parts of the Glut-3-MH molecule were present and could combine with berry crushing, then additional Glut-3-MH could form during this process. To test this, a synthetic derivative of (*E*)-2-hexenal that isn't naturally present (i.e. has deuterium in place of some hydrogen atoms) was added to whole berries which were then crushed using a bench top sample press. Juice samples were analysed for the incorporation of those deuterium atoms, which established the novel concept that Glut-3-MH was indeed formed during berry crushing (Capone and Jeffery, 2011).

Importantly, along with this known precursor type, the aldehyde intermediate (containing deuterium atoms) which arises from the direct condensation of glutathione with (*E*)-2-hexenal was also indentified – it is this compound (termed Glut-3-MHAl) that must be enzymatically reduced to yield commonly encountered Glut-3-MH. Until now, this new compound had not been considered as a potential precursor to 3-MH, yet if it exists in appreciable quantities (which is unknown at present) it could provide another source of 3-MH during vinification. Together, the identification of Cysgly-3-MH and Glut-3-MHAl has raised understanding of the biochemical transformations involved in the formation of precursors to the important aroma compound 3-MH (Figure 1). As part of this study it was also shown that minimal Glut-3-MH was present after berry crushing if grape enzymes were inhibited, thereby revealing that only a small amount of Glut-3-MH was present in the grape berries themselves. This was somewhat of a revelation and opened the way for seeking new means to manipulate precursor concentrations through post-harvest processing operations.

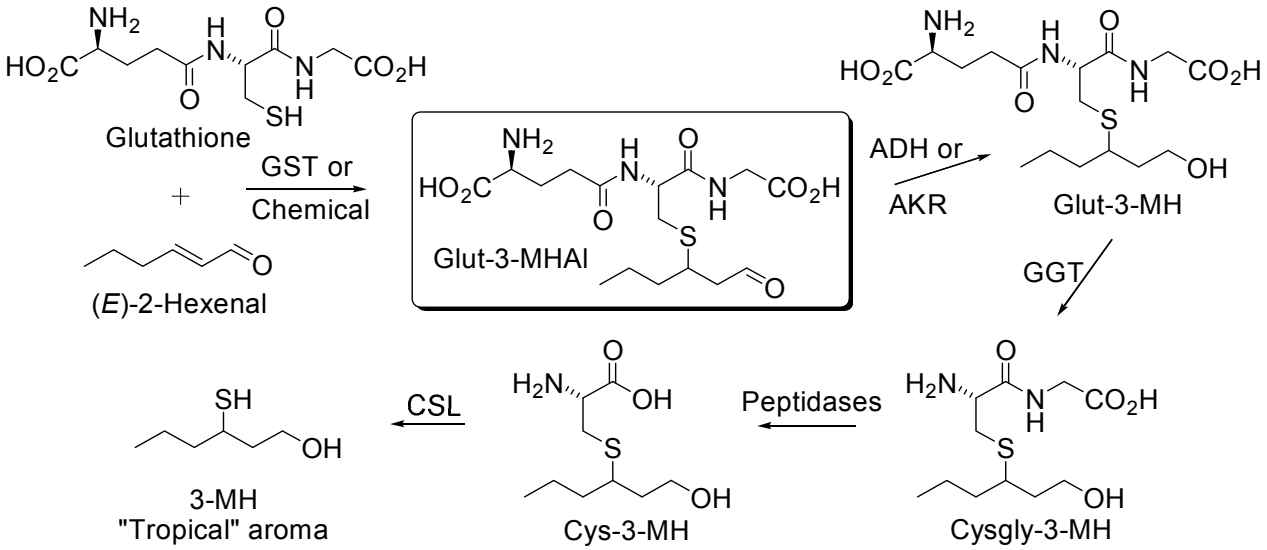

Figure 1. *Formation of aroma compound 3-MH from grape constituents glutathione and (E)-2-hexenal. Enzymes potentially involved are – GST, glutathione S-transferase; ADH, alcohol dehydrogenase; AKR, aldo-keto reductase; GGT, γ-glutamyltranspeptidase; other carboxypeptidases; CSL, carbon-sulfur lyase. The final release of 3-MH is mediated by yeast enzymes but preceding steps may involve plant or microbial enzymes.*

Precursor accumulation pre- and post-harvest

Previous work on Sauvignon Blanc clones in the Adelaide Hills region of South Australia had shown a rise in precursor concentrations during berry ripening, especially in the lead up to harvest (Capone et al., 2011a). An additional study was undertaken with more frequent sampling points to verify the importance of optimal harvest timing on precursor concentrations, so potential wine 3-MH levels could be maximised (Capone et al., 2012a). Samples were collected and analysed from veraison to several weeks past the harvest date – fruit was left on the vine longer than usual to determine the effect of additional hang time (Figure 2). While Cysgly-3-MH was barely detectable at any stage, the other precursors generally increased in concentration during ripening (especially for Glut-3-MH) until TSS reached around 24 °Brix. This was followed by a slight decline in precursor concentrations, but the results were consistent with earlier findings and highlighted the need to harvest at the correct time. This increase has been explained on the basis of loss of cell membrane integrity coupled with a rise in levels of precursor constituents (i.e. glutathione and (*E*)-2-hexenal) as grape maturity is reached. The fluctuation in precursors during ripening was not evaluated further, but it appears to be inversely linked to sugar accumulation. Future experiments could examine the metabolic changes associated with berry ripening and their influence on precursor accumulation. Ultimately, a useful indicator of “flavour ripeness” may evolve from such work.

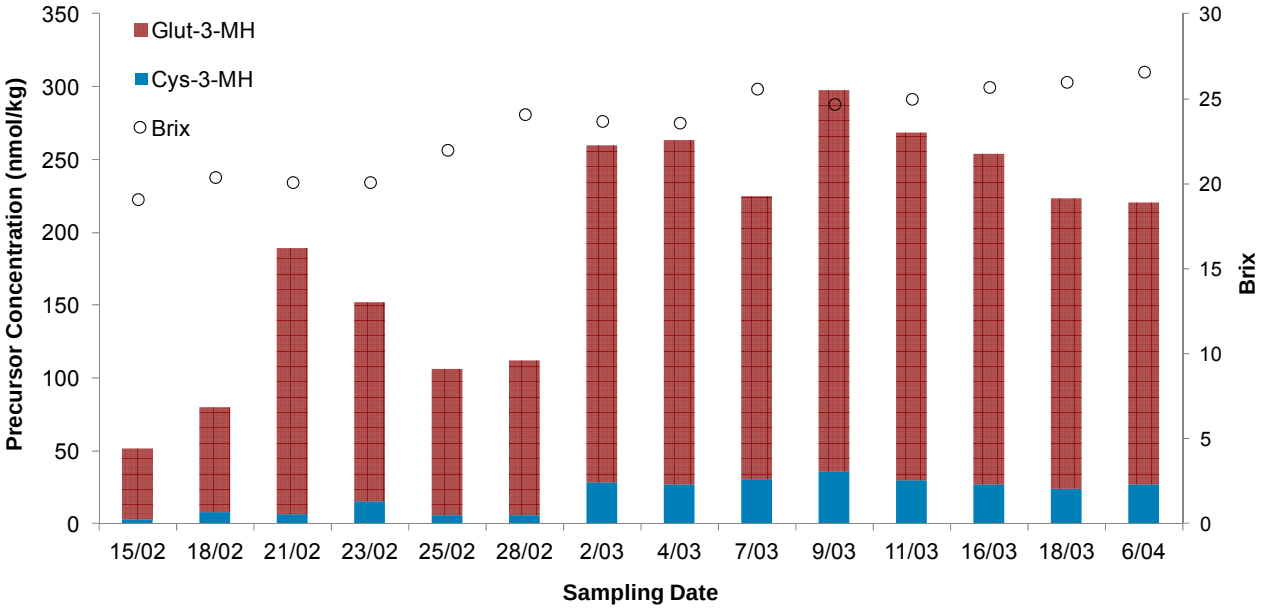

Figure 2. *Concentrations of Cys- and Glut-3-MH diastereomers (nmol/kg) and TSS (°Brix) during ripening of Sauvignon Blanc fruit from Coombe Vineyard, Waite Campus.*

Other research was conducted on a commercial scale to assess the effects of machine harvesting, fruit transportation, antioxidants and storage of fruit prior to crushing and pressing. It appeared that precursors could be affected by processing operations, so it was of clear importance to evaluate commercially relevant operations which could impact on precursor concentrations. One such undertaking involved the application of SO_2 and/or ascorbic acid to replicated 2.5 tonne picking bins of machine-harvested Sauvignon Blanc grapes (Capone and Jeffery, 2011). The different treatments (Figure 3) were sampled in the vineyard and again after being transported 800 km by road. Fruit was also hand-harvested two days before commercial harvest for comparison.

Figure 3. *Diagram of picking bins ready to be transported with a representation of the amounts of antioxidants (in mg/kg) added at the time of harvest.*

Precursor concentrations for the samples obtained before and after transportation were insightful (Figure 4). There was a clear effect of SO_2, which inhibited formation of Glut-3-MH in particular, in both sample sets. Of much greater surprise was the drastic increase in Cys-3-MH (and Glut-3-MH

to a lesser extent) as a result of transportation, to levels not encountered previously. The results reinforced the concept that Glut-3-MH can accumulate post-harvest and can be explained on the basis of Glut-3-MH formation and degradation into Cys-3-MH in the presence of enzymes from the grape berries (and possibly microflora). This experiment also provided measurable quantities of Cysgly-3MH in the transported fruit, and overall highlighted the dynamic nature of precursor formation and the role of post-harvest processing. Although Cysgly-3-MH did not accumulate to the same extent as the other two precursors, its presence was important as it is an intermediate in the breakdown of Glut- to Cys-3-MH, which was clearly occurring in the transported samples. This result is significant, considering that Cys-3-MH appears to be more readily metabolised to 3-MH during vinification, and fits well with anecdotal evidence of increased varietal thiol notes in wine made from transported grapes. Furthermore, hand-harvested samples revealed around 70% lower precursor concentrations, pointing to the impact of machine harvesting with its concurrent berry damage as being another determining factor in the precursor story.

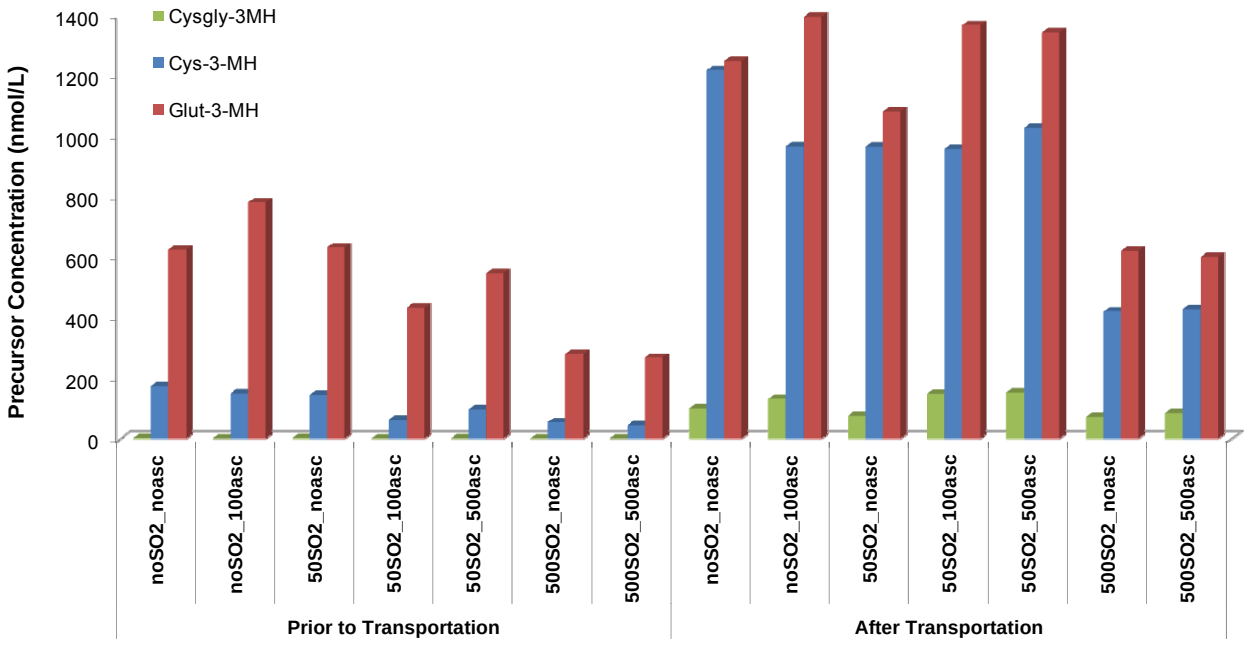

Figure 4. *Precursor concentrations (nmol/L) for treatments dosed with antioxidants and sampled in the vineyard at the time of harvest and again after transportation to the winery.*

Considering the intriguing results from the transport study, a commercial scale experiment was conducted the following vintage to determine whether time was the critical factor in determining the juice precursor profile. Machine-harvested Sauvignon Blanc fruit was stored in 2.5 tonne picking bins in a temperature controlled room at 10 °C (fruit reached a minimum of 24 °C) and samples were obtained periodically over a 30 hour period (Capone et al., 2012b). Samples from the different time points were analysed for Cys-, Cysgly- and Glut-3-MH, as well as for (*E*)-2-hexenal and related C_6 compounds, and grape reaction product (GRP). These additional analytes, which are related to the precursor concentrations since they involve an alternative route for consumption of Glut-3-MH components (*E*)-2-hexenal and glutathione, were assessed in order to understand their relationship to the dynamics of precursor formation.

The precursors evolved over the time course of the storage experiment, increasing in all cases, especially during the first hours (Figure 5). In particular, Cys-3-MH doubled in concentration within 8 hours and had tripled by 30 hours. The other two precursors increased in concentration by about 1.5 times during the storage time, but as with the transportation study, Cysgly-3-MH was found in much lower concentrations compared to its counterparts. Overall, increases in Glut-3-MH concentrations were consistent with the transport results, but Cys-3-MH was not as dramatically affected during storage as it was from transportation. This difference may arise due to the effects

of agitation, maceration, temperature and aeration during transportation impacting on key enzymatic reactions. Despite this difference, relatively short term storage of harvested fruit (e.g. 8 hours) still led to important increases in precursor concentrations, providing greater potential to release 3-MH during vinification.

Regarding the other analytes, these were affected by formation and transformation reactions associated with enzymatic and oxidative processes. As such, (*E*)-2-hexenal decreased during storage as it was incorporated into precursors and enzymatically reduced to other C_6 compounds, while GRP increased as a result of caftaric acid oxidation, thereby consuming glutathione. Both these aspects had the net effect of diverting the necessary components away from Glut-3-MH formation as storage time progressed. This highlights the need to have (*E*)-2-hexenal (which requires some oxygen to form) available near the beginning of post-harvest processing in the presence of glutathione, so any operation which interferes with this aspect (e.g. reductive handling, excessive SO_2) early in the process will likely lead to lower thiol precursor concentrations in the juice. On the contrary, highly oxidative processing at an early stage will allow greater conjugation of glutathione with caftaric acid, potentially also impeding precursor formation. Once Glut-3-MH has formed, however, facilitating its enzymatic breakdown to Cys-3-MH could be encouraged through a period of extended storage.

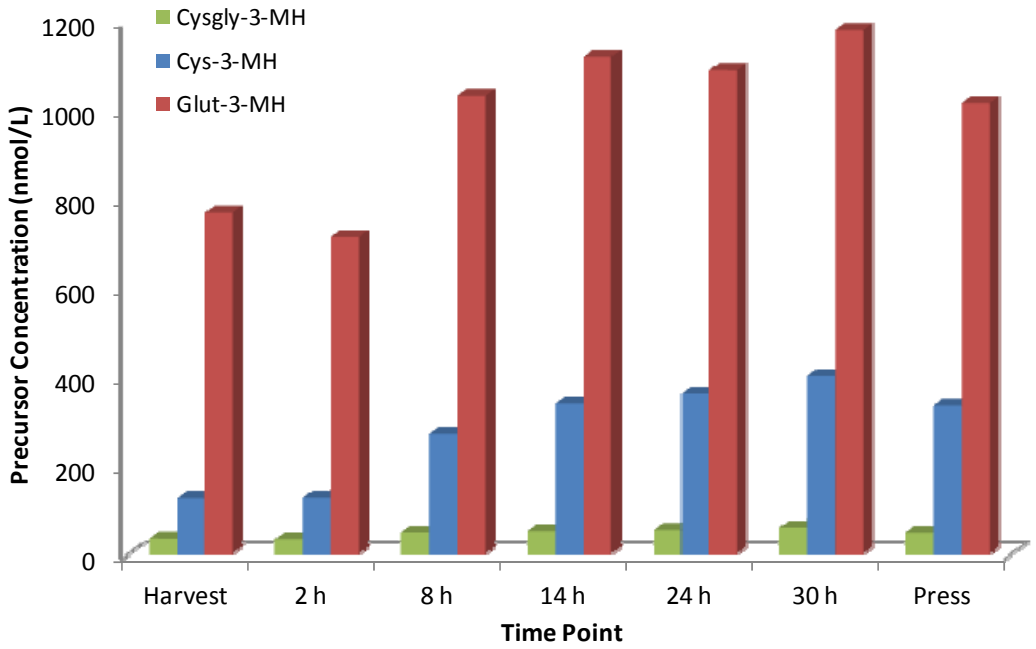

Figure 5. *Evolution of 3-MH precursor concentrations (nmol/L) during temperature controlled storage of machine-harvested Sauvignon Blanc grapes.*

Conclusion

This insightful series of studies has provided deeper insight into biochemical aspects of 3-MH precursor formation. The work has yielded unique knowledge about the dynamic nature of thiol precursors during berry development and post-harvest operations and has foreshadowed the ability of winemakers to control precursor profiles in the juice in order to realise quality improvements through modulation of varietal thiol concentrations in wine. This is a precursor of more good things to come, with further challenges to be resolved in the varietal thiol/precursor research space.

Biography

David W. Jeffery is Lecturer in Wine Science at the School of Agriculture, Food and Wine, Waite Campus, The University of Adelaide.

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