

# **Development and Application of Novel Analytical Methods to the Identification, Formation and Fate of Two Important Wine Aroma Compounds**

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A thesis submitted for the degree of Doctor of Philosophy

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The Australian Wine Research Institute

January 2012



The Australian Wine  
Research Institute



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## Thesis Summary

Wine flavour is complex and encompasses a wide variety of compounds with very different sensorial properties. It is only through detailed investigations, however, that knowledge of aroma compounds is improved. One aroma compound of inconclusive origin and requiring further study was 1,8-cineole, which was especially relevant to the Australian viticultural landscape. Another group of important flavour compounds found in wine that required greater understanding are the sulfur compounds. Varietal thiols, in particular 3-mercaptohexan-1-ol (3-MH), have some of the lowest aroma thresholds of any food or beverage component. Knowledge of thiol precursors is also important for understanding the formation of the corresponding varietal aroma compounds during winemaking, since they are released from odourless precursors in grape juice through fermentation.

To enable a better understanding of the formation and fate of the two important aroma compounds chosen, novel analytical methods using stable isotope dilution analysis (SIDA) were developed and thoroughly validated for the quantification of 1,8-cineole and 3-MH in grapes and wine. In addition, a SIDA method was developed and validated for the analysis of the diastereoisomers of the precursors, 3-S-cysteinylhexan-1-ol (Cys-3-MH) and 3-S-glutathionylhexan-1-ol (Glut-3-MH) to complement the studies on 3-MH.

1,8-Cineole was found to be predominantly present in red wines, being extracted during fermentation rather than forming from terpene precursors as previously proposed. Extension of this research revealed that *Eucalyptus* trees in the vineyard had a strong influence on the concentration of 1,8-cineole in wine. The incorporation of grape leaves and stems, and in particular *Eucalyptus* leaves into red must fermentations significantly elevated the level of 1,8-cineole concentrations in the wine. Additionally, this study revealed a surprising increase in rotundone concentrations when grape leaves and stems were included during fermentation.

An analytical method was developed for 3-MH determination that used conventional electron ionisation GC-MS and eliminated the use of mercuric compounds for thiol isolation. A 3-MH precursor analytical method was also developed which provided the first method where both diastereoisomers of Cys- and Glut-3-MH could be

analysed individually in one run. This was improved further by the addition of Cysgly-3-MH into the method following the identification of this compound in grapes for the first time. These methods were applied to wines and grape juices to investigate factors which might affect their concentrations, such as freezing grapes and juice, grape processing and fruit transportation. These studies highlighted the dynamic nature of these precursor compounds. In addition a new conjugated aldehyde which was the obvious missing link between the reaction of (*E*)-2-hexenal and glutathione in the formation of Glut-3-MH was identified for the first time.

The development of the new analytical methods discussed, together with their applications has contributed considerably to our understanding of various aspects of wine flavour. This work has uncovered the origin of 1,8-cineole in red Australian wine. It has also greatly enhanced our knowledge of 3-MH and its precursors so that we can understand how these compounds are formed and what affects their concentrations in juice and wine. Ultimately, this knowledge will enable wine producers to have greater control over the aroma profile of their wines.

## Declaration

I declare that this thesis is a record of original work and contains no material which has been accepted for the award of any other degree or diploma in any University or other tertiary institution. To the best of my knowledge and belief, this thesis contains no material previously published or written by another person, except where due reference has been made in the text. The publications included in this thesis have not been previously submitted for the award of any degree at the University of Adelaide or other University.

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Dimitra Capone

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Date

## Publications

This thesis is a collection of manuscripts that were published or were submitted for publication in various refereed Journals during candidature. Most of the manuscripts were published in The Journal of Agricultural Food Chemistry (JAFC) and in 2010 the impact factor of JAFC according to ISI statistics was 2.816, and the 5-year impact factor was 3.209. JAFC, a high impact ACS journal, is ranked number 2 for the Agriculture - Multidisciplinary, and is in the top 10 for Chemistry – Applied (number 2 if only considering wine science) and in the top 10 for Food Science & Technology, with the greatest number of published articles and total citations in these subject categories.

The text and figures in these chapters contained different formatting, according to the various journal requirements. A Statement of Authorship, signed by all of the authors, listing individual contributions to the work is included at the beginning of each chapter.

The thesis is based on the following refereed publications.

- Chapter 2. Dimitra L. Capone, Katryna Van Leeuwen, Dennis K. Taylor, David W. Jeffery, Kevin H. Pardon, Gordon M. Elsey, and Mark A. Sefton. Evolution and occurrence of 1,8-cineole (eucalyptol) in Australian wine. *J. Agric. Food Chem.* **2011**, 59: 953-959.
- Chapter 3. Dimitra L. Capone, David W. Jeffery and Mark A. Sefton. Vineyard and fermentation studies to elucidate the origin of 1,8-cineole in Australian red wine. *J. Agric. Food Chem.* **2012**, 60: 2281-2287.
- Chapter 4. Dimitra L. Capone, Mark A. Sefton and David W. Jeffery. 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.
- Chapter 5. Dimitra L. Capone, Mark A. Sefton Capone, Yoji Hayasaka, and David W. Jeffery. 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.* **2011**, *58*: 1390-1395.

Chapter 6. Dimitra L. Capone and David W. Jeffery. Effects of transporting and processing Sauvignon blanc grapes on 3-mercaptohexan-1-ol precursor concentrations. *J. Agric. Food Chem.* **2011**, *59*: 4659-4667.

Chapter 7. Dimitra L. Capone, Kevin H. Pardon, Antonio G. Cordente and David W. Jeffery. 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.

Chapter 8. Dimitra L. Capone, Mark A. Sefton and David W. Jeffery. Analytical investigations of wine odorant 3-mercaptohexan-1-ol and its precursors. In "Flavor Chemistry of Wine and Other Alcoholic Beverages". 2011, American Chemical Society: Washington, DC; (Accepted).

Chapter 9. Dimitra L. Capone, Cory A. Black and David W. Jeffery. Effects of 3-mercaptohexan-1-ol precursor concentrations from prolonged storage of Sauvignon blanc grapes prior to crushing and pressing. *J. Agric. Food Chem.* **2012**, *60*: 3515-3523.

An additional 14 related publications co-authored by the candidate are given in the appendices.

## Conferences

### **Crush, 28 to 30<sup>th</sup> September 2011, Adelaide.**

Presented a talk titled 'Studies on 3-mercaptohexan-1-ol and its conjugates in Sauvignon blanc juice and wine'.

### **The International Chemical Congress of Pacific Basin Societies (Pacifichem), 15 to 20<sup>th</sup> December 2010, Honolulu, Hawaii, USA.**

Presented a talk in the session on: "Value-Added Food Products from Fruits and Vegetables" The talk was titled 'Flavour precursors in Sauvignon blanc grape juice: the effect of fruit processing on thiol conjugate concentrations'.

### **University of Adelaide School of Agriculture, Food and Wine Postgraduate symposium, 21 to 22<sup>nd</sup> September 2010, Adelaide.**

Presented a talk titled 'The origin of eucalyptol in Australian wine' and won the "Max Tate Award" for the best presentation at the symposium.

### **ACS Fall National Meeting, 22 to 26<sup>th</sup> August 2010, Boston, MA, USA.**

Presented in the session of "The Chemistry of Alcoholic Beverages" This talk was titled 'Analytical investigations to relate important wine odorant 3-mercaptohexan-1-ol to its precursors'.

### **Separation Science conference, 6 to 7<sup>th</sup> August 2010, Singapore.**

Presented a poster titled 'Identification and analysis of new taint compounds using GC/MS/ODP and GC/MS/SIM'.

### **14<sup>th</sup> Australian Wine Industry Technical Conference, 3 to 8<sup>th</sup> July 2010, Adelaide.**

Presented two talks at a flavour workshop. The presentations were titled 'The origin of eucalyptol in Australian wine' and 'Monoterpenes in wine'. Also presented two posters titled 'Quantification of the precursors to the wine odorant 3-mercaptohexan-1-ol' and 'The origin of eucalyptol in Australian wine'.

## **Panel of supervisors**

### **Dr David Jeffery**

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### **Dr Mark Sefton**

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## Acknowledgements

I thank my supervisors Dr David Jeffery, Dr Mark Sefton and Professor Dennis Taylor for all of their support, advice and encouragement throughout my PhD it was a pleasure working with each and every one of them. I would also like to thank my external advisor Professor James Kennedy for his valuable advice and encouragement.

I also thank the past and present staff at the AWRI for their on-going support and encouragement in particular Dr Leigh Francis, Natoiya Lloyd, Katryna van Leeuwen, Samantha Anderson and the various co-authors on the publications included in this thesis. I would also like to thank Dr Dan Johnson for his efforts in making this PhD possible.

I am extremely grateful to the many wine producers and wine makers across Australia who provided generous support of this research through the contribution of wine and grape samples and through openly providing detailed information about these included in this study. I would particularly like to thank Nick Bruer and Tim McCarthy of Orlando Wines and staff members from Casella Wines, in particular Steve Warne and Frank Mallamace.

On a personal note a special thank you goes to my husband Mauro Capone for his continued encouragement and support during this period. Also thank you to both sides of my family for their encouragement, interest and support. I would like to thank my niece Stefania Mercorella who assisted me with parts of the grape processing during vintage and my parents Niki and Theo Liacopoulos for their continued love and support.

This project was financially supported by:

The Australian Wine Research Institute, a member of the Wine Innovation Cluster in Adelaide, is supported by Australia's grapegrowers and winemakers through their investment body, the Grape and Wine Research Development Corporation, with matching funds from the Australian government.



## **Chapter 1**

### **LITERATURE REVIEW**

This literature review covers the literature up to April 2009, which was within the first 6 months of candidature. The literature beyond this date has been included in the introductions of the publications covered in Chapters 2 to 9.



## Wine Flavour

Volatile compounds affecting aroma are important to a broad range of industries and to date, more than 6000 volatile compounds have been identified in both food and beverages (1). Over 1300 of these compounds have been identified in alcoholic beverages (2) of which more than 800 volatile compounds are found in wine alone (3-5). The number of wine volatile compounds continues to increase as new compounds are continually being identified. Wine flavour is complex and encapsulates a diverse range of compounds which have different sensorial properties. The complexity of wine flavour can be derived from numerous sources, which may include:

- from the grape berry itself,
- volatiles originating from grape-derived non-volatile precursors and being released during processing and/or storage,
- from yeast and bacterial metabolism (fermentation),
- from oakwood contact and
- from oxidative and acid-catalysed chemical reactions upon storage (6).

Detailed reviews have been published discussing the compounds involved in wine flavour from the sources listed above (1,6-9).

### Bound flavour compounds

Grapes contain an array of volatile compounds, often partially or totally bound in a precursor form, which can contribute to a typical wine aroma. Up to now the two main precursor types that have been identified are: glycosides, and cysteine conjugates (the cysteine conjugates will be discussed in a subsequent section). Glycosides are present in large amounts in grape leaves (10). The extensive studies on grape berry glycosides have shown that flavours can be released from crude extracts containing these glycosides, either because of acid hydrolysis (imitating production of aroma compounds that can occur during wine making and storage), or enzyme hydrolysis, or combinations of both (11).

Glycosides contain an aglycone component, derived from several classes of compounds including:

- simple aliphatic compounds (e.g. hexanol, hexenols),
- monoterpenes (mainly linalool, nerol,  $\alpha$ -terpineol and geraniol),
- C<sub>13</sub>-norisoprenoids e.g.  $\beta$ -damascenone,
- volatile phenols (e.g. vanillin) (12) and
- benzene derivatives (e.g. phenylethanol, benzyl alcohol) (11).

Grape glycosides comprise either O- $\beta$ -D-glucosides or disaccharides (5). With respect to the disaccharides, a glucose moiety is further conjugated with one of the following:

- $\beta$ -D-xylopyranose,
- $\beta$ -D-apiofuranose,
- $\alpha$ -L-arabinopyranose,
- $\alpha$ -L-rhamnopyranose or
- $\beta$ -D-glucopyranose (13-15).

In grapes most aglycons are linked to disaccharides, whereas in leaves simple glucosides are mainly present (16).

Acid hydrolysis can occur during fruit juice processing or wine storage through acid catalysed reactions (5). These reactions often occur quite slowly in winemaking and are dependent on pH, temperature and structure of the aglycone moiety.  $\beta$ -Glucosidases such as  $\beta$ -glucoside glucohydrolases can also hydrolyse glycosides. Enzyme hydrolysis requires various enzymes ( $\beta$ -D-glucosidase, diglycosidase) which act in a two step process:

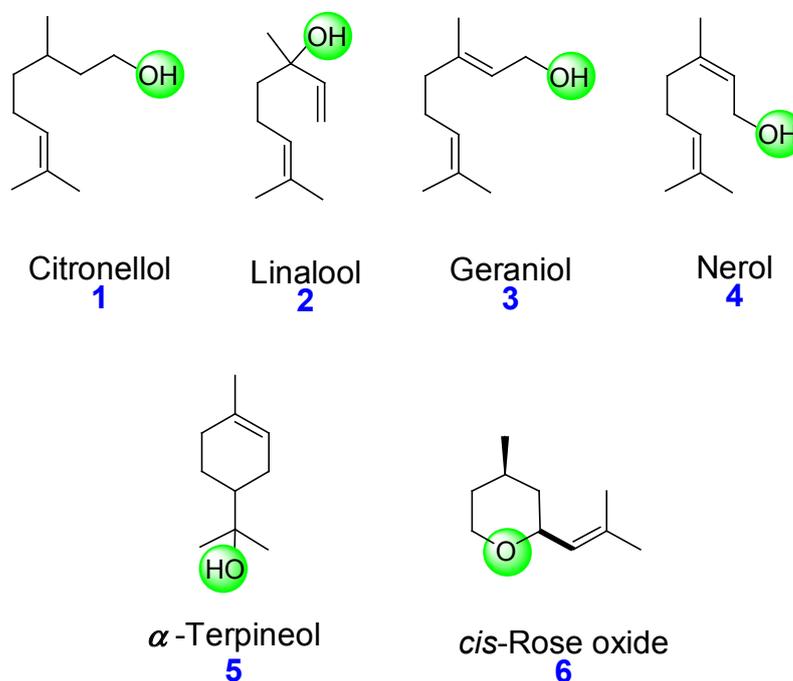
1.  $\alpha$ -L-rhamnosidase,  $\alpha$ -L-arabinosidase,  $\beta$ -D-apiosidase or  $\beta$ -D-xylosidase cleave the terminal sugar and release rhamnose, arabinose, apiose, or xylose and the corresponding  $\beta$ -D-glucosides and
2. liberation of the aglycone part, by the action of a  $\beta$ -D-glucosidase (12).

### **Important grape derived flavour compounds**

#### **Terpenoids**

Monoterpenes are mainly important to the aroma and flavour of young wines, in particular from Muscat grape varieties (17 and ref. therein). More than 70 monoterpenes have been identified in grapes and/or wine. The most common

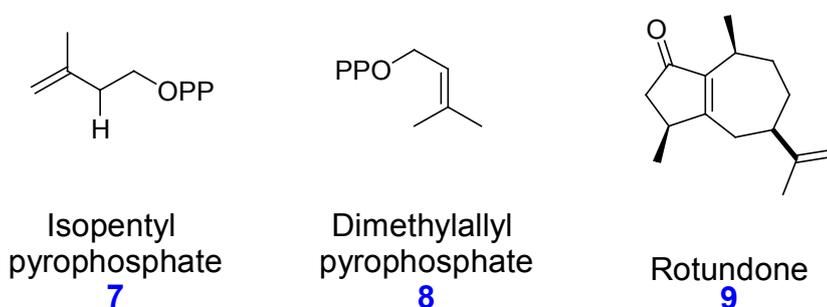
monoterpenes found in grapes and wines are the simple alcohols such as citronellol **1**, linalool **2**, geraniol **3**, nerol **4** and  $\alpha$ -terpineol **5** (**4**) shown in Figure 1. Another group found are the monoterpene ethers, which include rose oxide **6** (Figure 1) and nerol oxide, along with polyhydroxylated monoterpenes which are produced by oxidation of the corresponding monoterpene alcohol (**8**). These terpenes have been known to contribute to the 'floral' and 'fruity' aromas in Muscat and Gewürztraminer wines (**18**) and their aroma detection thresholds vary: 0.2  $\mu\text{g/L}$  for *cis*-rose oxide, 15  $\mu\text{g/L}$  for linalool **2**, 30  $\mu\text{g/L}$  for geraniol **3** and 100  $\mu\text{g/L}$  for citronellol **1** (in 10% ethanol/water solution) (**19**). The aroma detection thresholds of  $\alpha$ -terpineol **5** and nerol **4** are much higher at 250  $\mu\text{g/L}$  and 300  $\mu\text{g/L}$  respectively in an unspecified synthetic medium (**20**). The location of monoterpene alcohols within the components of the grape berry varies for different compounds; for example nerol **4** and geraniol **3** have been found in greater amounts in the skin, whereas linalool **2** seems to be uniform throughout the berry (**8**).



**Figure 1.** The chemical structures of the monoterpene alcohols: citronellol **1**, linalool **2**, geraniol **3**, nerol **4**,  $\alpha$ -terpineol **5** and the monoterpene ether *cis*-rose oxide **6**, commonly found in grapes and/or wine.

## Sesquiterpenes

Sesquiterpenes have not been studied as extensively as the monoterpenes with regard to wine flavour (8). It has been commonly found that the concentration of sesquiterpenes increase during berry maturation as per the monoterpenes (8). The formation of both the monoterpenes and the sesquiterpenes is dependent on the C<sub>10</sub>- and C<sub>15</sub>-precursors which are derived from the C<sub>5</sub>-isoprene precursors isopentenyl pyrophosphate (IPP 7) and dimethylallyl pyrophosphate (DMAPP 8) (Figure 2). The IPP 7 and DMAPP 8 building blocks are extensively transformed in the plant by reactions such as reduction, oxidation, cyclisation and isomerisation to produce various terpenoids (8). In grapes, sesquiterpenes have not been reported to be bound in glycosylated form, in contrast to the monoterpenes (8). The only sesquiterpene identified to date which clearly imparts an aroma to wine is rotundone 9 (21) (Figure 2). The aroma of rotundone 9 is described as ‘pepper’ and its aroma detection threshold was determined to be 16 ng/L in red wine, for those approximately 80% of testers who can detect this compound (21). Rotundone 9 is commonly found in Shiraz wines and has been linked to grapes grown in cooler climates (21).



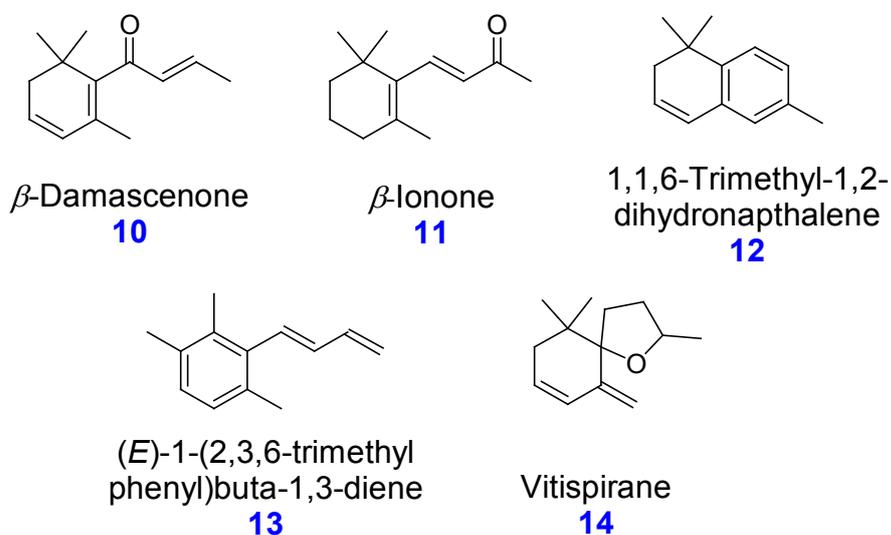
**Figure 2.** The chemical structures of the monoterpene building blocks IPP 7 & DMAPP 8 and the sesquiterpene rotundone 9.

## C<sub>13</sub>-norisoprenoids

Norisoprenoids occur in grapes and wine as glycosidically bound derivatives similar to monoterpenes. The proposed formation of C<sub>13</sub>-norisoprenoids is by “oxidative cleavage of the carotenoid molecule between the C<sub>9</sub> and C<sub>10</sub> positions” during grape ripening (16). There have been numerous identifications of C<sub>13</sub>-norisoprenoids in grapes but only a few of them have been confirmed to be important to the flavour of wine (19). These are β-damascenone 10, β-ionone 11, 1,1,6-trimethyl-1,2-dihydronaphthalene (TDN 12), and (*E*)-1-(2,3,6-trimethylphenyl)buta-1,3-diene (TPB

**13)** (Figure 3).  $\beta$ -Damascenone **10** is an important compound not only to grapes and wines but many other fruit and vegetables, coffee and beer (8).

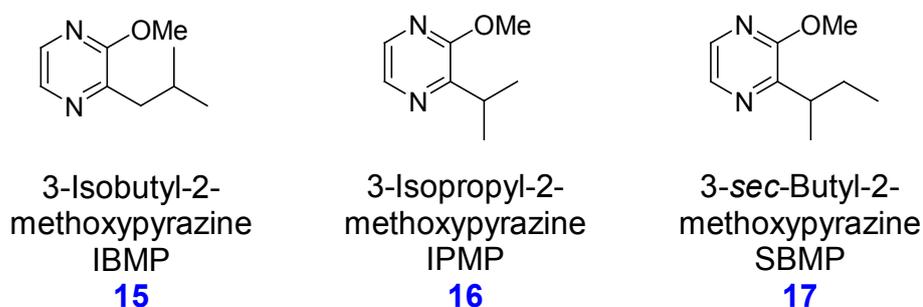
The characteristic aroma of  $\beta$ -damascenone **10** changes with concentration. It has an aroma threshold of 50 ng/L in a model wine and at high concentrations it is described as 'rose', 'honey' and 'apple', while at low concentrations it is described as 'lemon balm' (22,23).  $\beta$ -Ionone **11**, like  $\beta$ -damascenone **10**, is also an important compound to many fruits.  $\beta$ -Ionone **11** is described as 'sweet' or 'violet' and has an odour detection threshold of 90 ng/L (in 10% v/v aqueous ethanol) (24). The concentrations of both  $\beta$ -damascenone **10** and  $\beta$ -ionone **11** are commonly found above their detection thresholds in grapes and have been shown to positively contribute to many varieties of wine (8 and ref. therein). TDN **12** is described as having a 'kerosene-like' aroma with an aroma detection threshold of 20  $\mu$ g/L in a hydro-alcoholic solution (10%) (25). Higher amounts of both TDN **12** and vitispirane **14** (Figure 3) have been generally associated with bottle aged Riesling wines. Vitispirane **14** is described as 'eucalyptus' or 'camphoraceous' but has a relatively high aroma detection threshold of 800  $\mu$ g/L in wine (2 and ref. therein). TPB **13** has been identified from glycoside hydrolysates of Cabernet Sauvignon and Shiraz grapes and leaves. It was detected in aged white wine, particularly Semillon and has also been implicated as a white wine bottle aged character (26). With an aroma threshold of 40 ng/L in a neutral white wine, it's aroma is described as 'floral' and 'geranium' at low concentrations and as 'insecticide' and 'plastic' at levels above 270 ng/L (27).



**Figure 3.** Chemical structures of various norisoprenoids found in wine.

## Methoxypyrazines

Methoxypyrazines have been linked to the varietal aroma of Cabernet Sauvignon, Sauvignon blanc and Semillon wines. The aroma of pyrazines is described as 'vegetative', 'earthy', 'herbaceous' or 'bell pepper' (28). Of the pyrazines, the most relevant are 3-isobutyl-2-methoxypyrazine (IBMP) **15**, 3-isopropyl-2-methoxypyrazine (IPMP) **16** and 3-sec-butyl-2-methoxypyrazine (SBMP) **17** (Figure 4). They are extremely potent and have very low aroma detection thresholds. The aroma detection threshold of IBMP **15** is 10 ng/L and 16 ng/L in white and red wine, respectively (29,30) and it has been found at levels of 3.6 - 56.3 ng/L in Cabernet Sauvignon wines (31). The aroma detection threshold of IPMP **16** is 1 - 2 ng/L in water, white and red wine. Since it is rarely found above this level in wine, it is thought to be less important than IBMP **15** (8). The aroma detection threshold of SBMP **17** is 1 - 2 ng/L in water (24) and as with IPMP **16**, this compound is not normally found above its threshold and again is thought to be less important than IBMP **15** (32 and ref. therein). Higher concentrations have been found in cooler climates and with low levels of light exposure in the vine canopy during grape ripening (2 and ref. therein). Methoxypyrazines have been located in the skins of the grape berries (33).



**Figure 4.** Chemical structures of various methoxypyrazines; IBMP **15**, IPMP **16** and SBMP **17** found in wine.

## Organo-sulfur compounds

Sulfur compounds can provide positive or negative aromas and flavours to wine. Various off-flavours in wines can be produced by fermentation-derived sulfur compounds. Some examples, covered in a subsequent section, include the smell of 'rotten eggs' which is caused by H<sub>2</sub>S and the smell of 'onions', 'green asparagus', 'burnt rubber' or 'garlic' caused by methyl and ethyl sulfides, disulfides and thiols

(23). In contrast, polyfunctional thiols convey pleasant and important varietal aromas in wine.

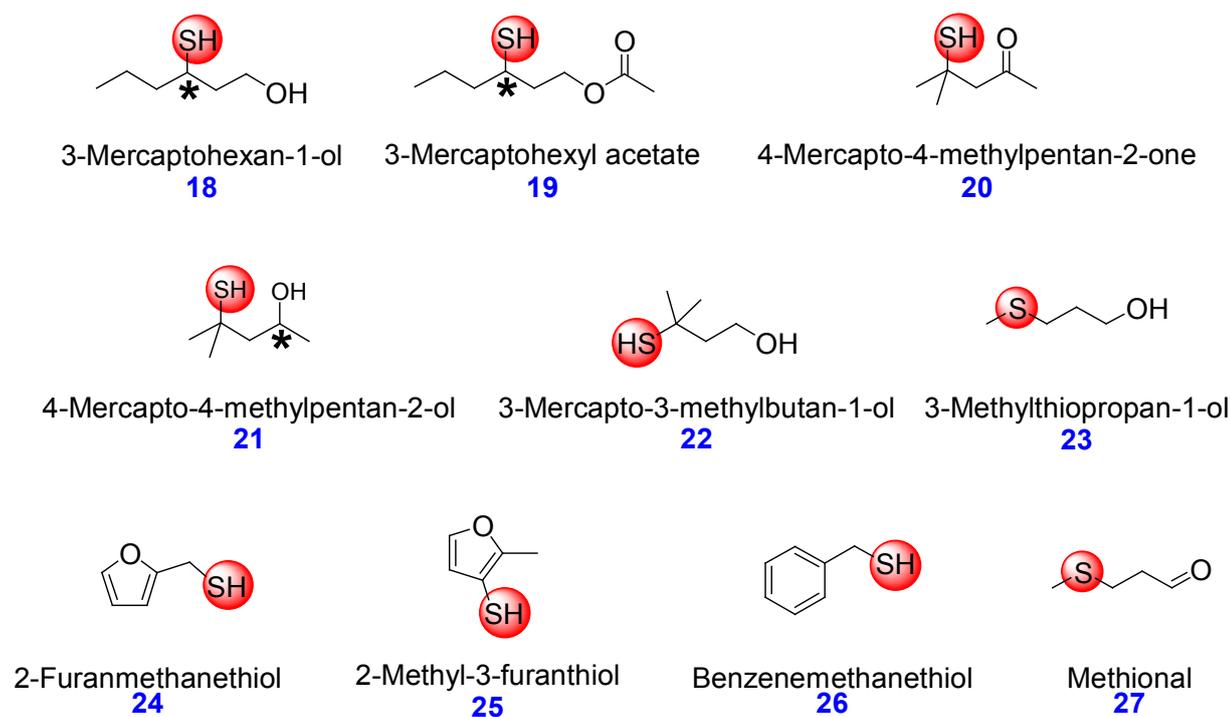
Compounds now recognised as grape-derived thiols were first identified in the 1980s in various fruits such as blackcurrant, grapefruit, passionfruit or guava and found in Sauvignon blanc for the first time in 1995 (23,34). Important wine thiols have some of the lowest aroma thresholds found in food. The first of the thiols which exhibited a typical Sauvignon blanc aroma in wine was identified as 4-mercapto-4-methylpentan-2-one (4-MMP **20**) (34) (Figure 5). It's characteristic aroma has been described as 'blackcurrant', 'boxwood' and 'broom' and it has an aroma threshold of 3.3 ng/L in white and red wine (34). A year later, an additional important thiol exhibiting varietal character in Sauvignon blanc wine was identified as 3-mercaptohexyl acetate (3-MHA) **19** (35) (Figure 5). The aroma of 3-MHA **19** is described as 'grapefruit', 'passionfruit' and 'box tree' with an aroma detection threshold of 4 ng/L in 12% aqueous ethanol (35). Soon after the identification of 3-MHA, 3-mercaptohexan-1-ol (3-MH) **18** (Figure 5) was also found in Sauvignon blanc (36). Its aroma is described similarly to 3-MHA **19** as 'passionfruit' or 'grapefruit' with a detection threshold of 60 ng/L in 12% aqueous ethanol (36). Although these thiols generally have pleasant aromas, they can impart unpleasant 'sweaty' aromas analogous to that of 'cat's urine' at very high concentrations (37). Table 1 summarises the organo-sulfur compounds that are important to wine flavour, (adapted from a table in reference 8). Of these compounds **18** to **20** are generally considered to be the most important.

Table 1. Odours of some important volatile organo-sulfur compounds.

Compound	Odour description	Odour threshold (ng/L)*	References
3-Mercaptohexan-1-ol <b>18</b>	Passionfruit, grapefruit	60	(36)
3-Mercaptohexyl acetate <b>19</b>	Grapefruit, passionfruit, box tree	4	(35)
4-Mercapto-4-methylpentan-2-one <b>20</b>	Blackcurrant, boxwood, broom	0.8	(34)
4-Mercapto-4-methylpentan-2-ol <b>21</b>	Citrus zest, passionfruit, box tree, boom	55	(1,38)
3-Mercapto-3-methylbutan-1-ol <b>22</b>	Cooked leaks	1500	(1,11,36)
3-methylthiopropen-1-ol <b>23</b> (Methionol)	Sweet potato, cauliflower, soup, meat like	1 x 10 <sup>6</sup>	(1,11,39)
2-Furanmethanethiol <b>24</b>	Roasted Coffee, meat, bread, popcorn	0.4	(40,41)
2-Methyl-3-furanthiol <b>25</b>	Meaty, savoury, fried	5	(1,11,22,39)
Bezenemethanethiol <b>26</b>	Empyreumatic, burnt, match	0.3	(42)
Methional <b>27</b>	Baked potatoes	500	(43)

\* Thresholds determined in hydroalcoholic solutions

3-MH **18**, 4-MMP **20** and 3-MHA **19** are not only varietal impact aroma compounds of Sauvignon blanc but also other wine varieties. They have been isolated from wines made from many different cultivars of *Vitis vinifera*, such as Scheurebe, Gewurztraminer, Riesling, Colombard, Petit Manseng, Semillon, Cabernet Sauvignon, and Merlot (19,36,38,44-46).



**Figure 5.** Chemical structures of various organo-sulfur compounds found in wine. The stars in structures **18**, **19** and **21** indicate the position of the chiral centres of the compound.

In Sauvignon blanc wines from the Bordeaux and Sancerre region, the amount of 4-MMP **20** ranged from 4 to 24 ng/L, the amount of 3-MH **18** ranged from 733 to 12,822 ng/L and the amount of 3-MHA **19** ranged from 212 to 724 ng/L (47). As can be seen from the values obtained in these wines relative to their aroma detection threshold, these compounds are important to wine aroma. [Values found in the literature have been updated in Chapter 4 and 8].

Stereochemistry is another key feature of wine thiol characteristics. For example, 3-MH **18** exists as two possible enantiomers, as does its O-acetate derivative 3-MHA. Chirality is important as it results in different interactions with biological aroma receptors and therefore both enantiomers can often have different aroma descriptors

and thresholds (8). The chiral centres are indicated with stars on the structures in Figure 5. Both enantiomers of 3-MH **18** and 3-MHA **19** are present in Sauvignon blanc and Semillon wines. The aroma detection thresholds for (*R*)- and (*S*)-forms of 3-MH **18** were similar (50 and 60 ng/L, respectively, in model wine) while there is a larger disparity in the detection thresholds for the acetates; the (*R*)-form being higher (9 ng/L) than the (*S*)-form (2.5 ng/L) (48). Furthermore, the aroma descriptors vary, as summarised in Table 2 along with their thresholds (adapted from 8):

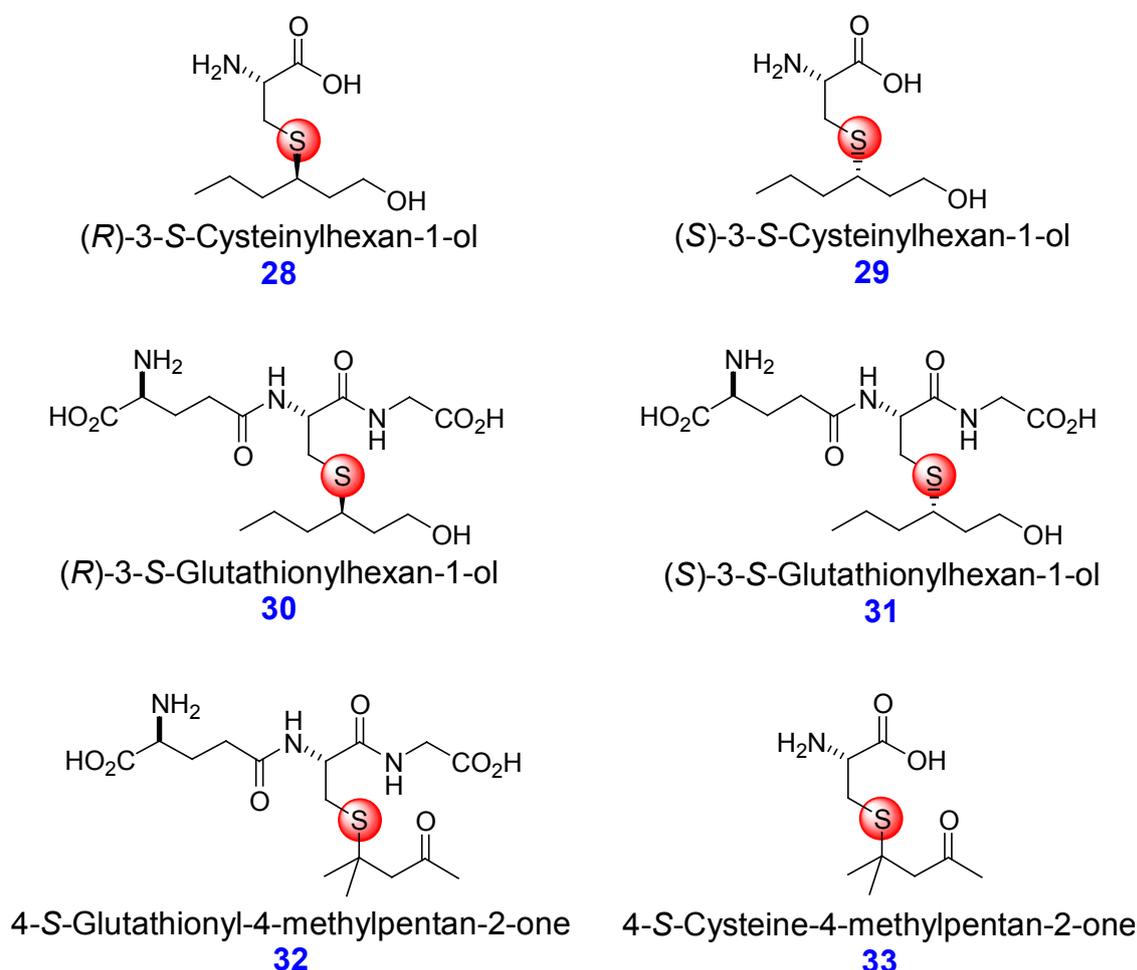
Table 2. Odour threshold and quality for different enantiomers of 3-MH and 3-MHA

Compound	Chirality	Odour threshold (ng/L)	Odour description
3-Mercaptohexan-1-ol	<i>R</i>	50	Grapefruit, citrus peels
	<i>S</i>	60	Passionfruit
3-Mercaptohexyl acetate	<i>R</i>	9	Passionfruit
	<i>S</i>	2.5	Boxwood

For wines with 3-MH **18** present, the amounts of each of the enantiomers were quantified in order to evaluate the contribution of the compound to the sensory properties of the wine assessed. The (3*R*)-3-MH:(3*S*)-3-MH ratios in dry white wines which included Sauvignon blanc and Semillon, were compared to those in sweet wines made from grapes of the same variety affected by *Botrytis cinerea* and these were approximately 1:1 and 3:7, respectively (48). This difference in the ratios of the 3-MH **18** enantiomers may help explain why the aromas of the dry white wines are equally linked with 'passionfruit' and 'grapefruit', whereas sweet wines have a much stronger 'passionfruit' aroma.

Varietal thiols 3-MH and 4-MMP are considered to be formed from odourless grape derived precursors. Putative precursors to these compounds were first identified as S-cysteinyl conjugates of 3-MH **28**, **29**, 4-MMP **32** and 4-MMPOH in a Sauvignon blanc juice (49), then subsequently as the glutathione conjugate of 3-MH **30**, **31** in Sauvignon blanc juice (50) and most recently the glutathione conjugate of 4-MMP **32** (51) (Figure 6). The non-volatile cysteine precursors are released as the corresponding volatile thiol by yeasts during fermentation (49), by putative sulfur lyases (52). No direct precursor has been identified for 3-MHA **19** in grapes and

grape must but it has been shown that 3-MH **18** is converted into 3-MHA by *Saccharomyces cerevisiae*, probably via the ATF1 enzyme (53).



**Figure 6.** Chemical structures of the diastereoisomers of various known thiol precursors found in grape juice.

Investigations of grape berry constituents found that the S-cysteine conjugates of 4-MMP **32** and 4-MMPOH precursors (Cys-4MMP **33** and Cys-4MMPOH) were found in equal amounts in the grape juice and skin, whereas the amount of 3-mercaptohexan-1-ol precursor (Cys-3MH **28**, **29**) was present in almost eight times higher concentrations in the skin (54). It was also reported that minimal skin contact could increase the concentration of cys-4MMP **33** and cys-4MMPOH and increase to an even greater extent the Cys-3MH **28**, **29** in the must (45). The Cys-3MH **28**, **29**

concentration continued to increase with extended juice-skin contact in the grape musts, and this was more apparent at higher temperatures (45).

It has been shown that the glutathione present in grapes rapidly decreases after crushing, because of the start of redox and enzymatic processes (55). It has been reported that S-cysteinyl conjugates are found along the metabolic pathway of the degradation of the relevant S-glutathionyl conjugates, and for this reason the glutathione derivatives are considered the real precursors to a variety of important wine aroma compounds (51). This was also implied from the work on the biogenesis of 3-MH **28, 29** in Sauvignon blanc wines (56).

Semi-quantitative methods for measuring the cysteine and glutathione conjugates in must have been used to assess the impact on the aromatic potential of these compounds in Merlot and Cabernet Sauvignon grape varieties used to produce rosé wines in Bordeaux (31). Various winemaking parameters, including the preparation of the juice, the yeast strain selected and fermentation temperature are all factors that can affect the concentrations of the thiols produced in the wine (55).

[Updated literature for this section can be found in the Introductions of Chapters 5, 6, 7, 8 & 9].

### **Important fermentation-derived flavour compounds**

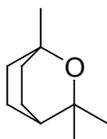
During yeast fermentation many volatile compounds are formed but many of these compounds do not greatly impact on the overall wine flavour. The concentrations of compounds formed are often present below their aroma detection thresholds. The compound types that are usually produced during fermentation are:

- Ethanol (produced from catabolism of hexoses – glucose and fructose),
- Higher chain length alcohols, also known as *fusel alcohols* which are formed from decarboxylation and deamination of amino acids, in addition to sugar catabolism (57),
- Volatile acids,
- Acetate esters and the ethyl esters of fatty acids and
- Low molecular weight sulfur compounds.

Fermentation usually occurs in two stages; firstly a primary yeast alcoholic fermentation (AF), followed by a secondary microbial, or malolactic fermentation (MLF). The main purpose of the secondary fermentation is to convert malic acid to lactic acid, which decreases the acidity in the wine. Of the volatiles produced through fermentation, the esters are reported to be of greatest importance, primarily impacting on young wine aroma (6). Esters are formed via enzymatic reactions within the yeast cell (58). The enzymatic reactions for ester synthesis take place between higher alcohols and acetyl-CoA (acetate esters) and ethanol and short-chain fatty acyl-CoA compounds (fatty acid ethyl esters) during alcoholic fermentation (58-60). Similarly to the thiols, the amount of esters found in wine is dependent on the yeast species and strains selected (61). The characteristic aromas of the esters are 'fruity' and described as 'apple', 'banana', 'perfume' and 'rose' (58). It has been shown that esters are present in all wines and they are considered to impart a positive influence on wine aroma (58-60). Other important compounds released during fermentation are the varietal thiols mentioned earlier.

### Flavour compounds of inconclusive origin – 1,8-cineole

The characteristic aroma of 1,8-cineole **34**, commonly known as eucalyptol, is 'eucalyptus', 'fresh', 'cool', 'medicinal' and 'camphoraceous'. A study by Herve et al. has shown that 1,8-cineole **34** plays an important role in the occurrence of 'eucalyptus' character in wine (62). They also determined the detection and recognition thresholds of 1,8-cineole **34** in a California Merlot to be 1.1  $\mu\text{g/L}$  and 3.2  $\mu\text{g/L}$  respectively (62).



1,8-Cineole  
**34**

**Figure 7.** Chemical structure of 1,8-cineole.

The origin of 1,8-cineole **34** in wine is unclear, but several theories have been reported. Herve et al. (62) proposed that the 'eucalyptus' character in wines occurs when vineyards are surrounded by *Eucalyptus* trees, whereas Farina et al. (63)

proposed that terpene compounds such as  $\alpha$ -terpineol **5** and limonene are possible precursors of 1,8-cineole **34**. Farina et al. postulated that the pathway to the formation of 1,8-cineole is by the hydration of limonene, forming  $\alpha$ -terpineol **5**, which is then followed by the cyclisation of *trans*-1,8-terpine, but not *cis*-1,8-terpine to 1,8-cineole **34**. They also suggest other pathways involving epoxidation of the double bonds of limonene and  $\alpha$ -terpineol **5**, which may have lead to a complex mixture of minor compounds ( $\alpha$ -terpineol **5**, 1,4-cineole, 1-terpineol) (63). Their studies with model wine showed that 1,8-cineole can be produced from limonene and  $\alpha$ -terpineol under conditions related to those of red wine aging but did not give quantitative data for the products (63). Finally, they found that 1,8-cineole **34** concentrations in grapes at the beginning of the ripening were very low, then showed an important increase throughout the ripeness.

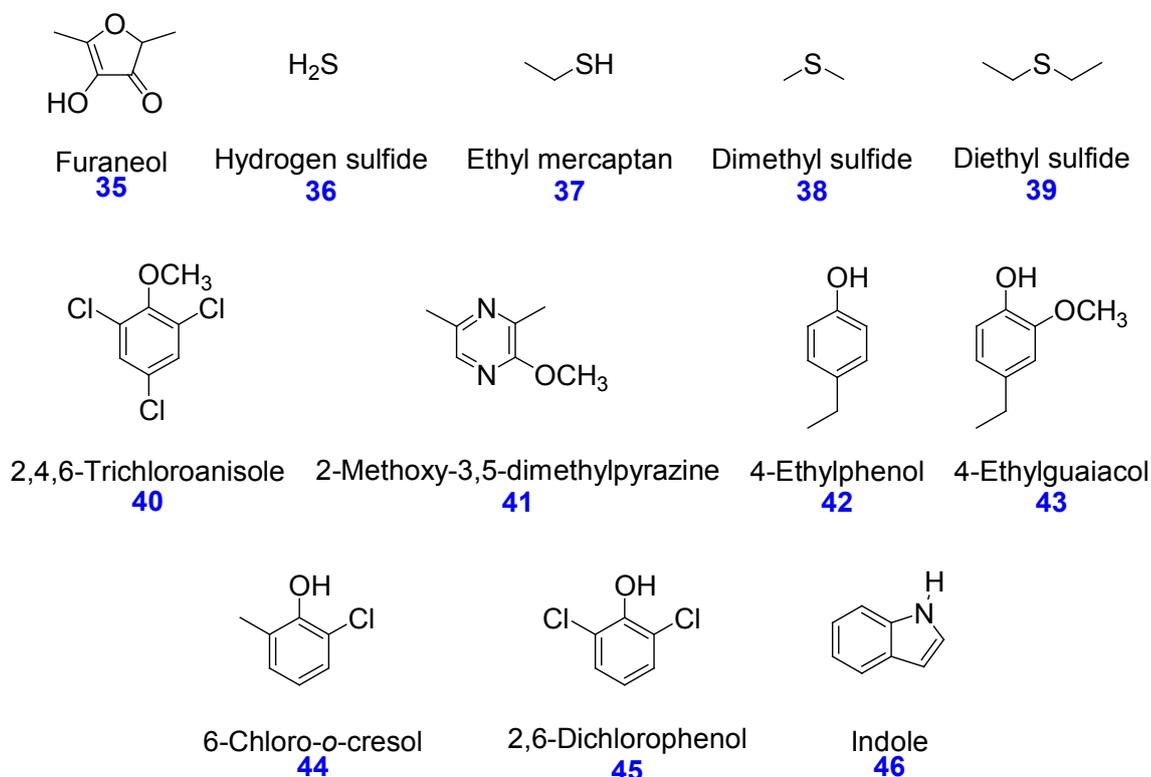
Another study by Kalua and Boss (64) suggested that Cabernet Sauvignon grapes have the ability to form 1,8-cineole **34**, which was the major monoterpene found early in berry development but decreased during ripening. This is in direct contrast to the observations of Farina et al. (63). Kalua and Boss also found that 1,8-cineole **34** was detected at similar levels in berries situated immediately next to *Eucalyptus* trees or at some distance from the trees (64) which is in contrast to the suggestion of Herve et al. (62). Kalua and Boss also suggested that the existence of 1,8-cineole **34** in berries may be attributable to the persistence of the compound from floral tissues or alternatively, the production of 1,8-cineole **34** may be promoted by herbivore predation, as reported for other plant species (64 and ref. therein).

It would be advantageous to clarify how this character arises in wine. If it is due to the presence of *Eucalyptus* trees, as proposed by Herve et al. (62), it would give winemakers and viticulturalists a choice of retaining or removing these trees within their vineyards, depending on whether they wish to maintain or reduce this character in their wines.

### **Off flavours in wine**

It has been suggested that any flavour compound present at very high concentrations might convey an undesirable aroma in a wine. Many compounds have caused off-flavours in wine and these have resulted from different grape varieties, wine processing and wine storage conditions. Compounds such as furaneol **35**

(undesirable 'strawberry-like' flavour), sulfur-containing compounds like hydrogen sulphide **36**, ethyl mercaptan **37**, dimethyl sulfide **38**, diethyl sulfide **39** ('rotten egg', 'garlic', 'cooked vegetable' and 'onion-like' aromas), have been found as undesirable compounds depending on the grape variety and wine processing. Compounds such as 2,4,6-trichloroanisole (TCA) **40**, known as 'cork taint', has a 'musty', 'earthy' or 'mouldy' aroma (2) and 2-methoxy-3,5-dimethylpyrazine **41**, which also has a 'musty' aroma is too derived from wine corks (65) and is associated with wine storage in wine bottles. Other compounds such as, 4-ethylphenol **42** and 4-ethylguaiacol **43**, which have 'barnyard', 'horse' and 'wet leather' aromas, are associated with spoilage by *Brettanomyces* (66). More recently other compounds such as 6-chloro-*ortho*-cresol **44**, 2,6-dichlorophenol **45** and indole **46** have been identified in wine as causing 'plastic' or 'chlorine' aromas, the first two have been shown to occur from contaminated processing aids, whereas indole was linked to stuck or sluggish fermentations (67).



**Figure 8.** Chemical structures of various off flavours that have been found in wine.

### **Techniques for wine analysis**

Early wine analytical methods only focussed on measuring ethanol, organic acids and sugars which are the major components of wine. Chromatographic techniques such as gas chromatography (GC), enabled a new era in scientific discovery for analytical chemistry (2).

The same general techniques for analysing volatile compounds are used by both wine chemists and flavour chemists in general. The main difference and disadvantage for wine chemists is that they have to deal with the presence of ethanol which causes complications in compound extractions and GC chromatograms. The other challenge is the numerous compound interferences such as sugars, acids and polyphenols which can contaminate GC columns (2).

Wine chemists have access to many isolation techniques but need to be aware that they can cause biases in flavour profiles. Therefore, it is crucial for the chemist to understand the advantages and disadvantages of each specific isolation technique and how the choice of technique could affect the results (68-70).

Different isolations techniques include, but are not limited to, the following:

- Distillation: common for ethanol and fusel alcohols in wine (9).
- Extractions:
  - Solvent extractions were the most common technique used because it has the ability to remove 'contaminants', isolate specific volatiles and in some cases be able to concentrate the analytes (71). Solvents with a low affinity for ethanol are most often chosen e.g. Freon (less common now) or pentane/ethyl acetate (2:1) (17). The recent decrease in the use of solvent extracts is mainly due to the high costs of disposal of solvents, environmental and safety concerns.
  - Supercritical fluid extraction (SFE), "supercritical fluid is a substance that is above its critical temperature and pressure and has diffusivity properties that are gas-like and solvating properties that are liquid-like. Carbon dioxide is usually the extracting solvent used as it is available in pure form, is non-toxic and has a convenient critical temperature" (72). The main advantage of SFE is that it requires minimal use of organic

solvents but is better suited for the extraction of solid materials such as corks or grapes.

- Solid phase extraction (SPE) involves the absorption of analytes on a solid phase support. It is commonly used for sample preparation (concentrating) and cleanup. C<sub>18</sub> columns and XAD-2 resins have been the most common for isolation of glycosidically bound aroma precursors from grapes and wines (71). LiChrolut EN resins have been used for a cleanup step for the removal of fatty acids and various interfering compounds prior to performing an extraction with *p*-hydroxymercurybenzoate for the analysis of potent thiols (73).
- Headspace analysis:
  - Static (equilibrium) headspace sampling (HS) is most frequently used for volatiles in solid and liquid samples. A sample of the headspace is injected into the GC and this technique relies on the analyte partitioning between the sample and headspace. The main disadvantage of this type of method is that the detection limits of the higher molecular weight, higher boiling analytes and polar analytes in aqueous samples are reduced (74).
  - Dynamic headspace/purge and trap (DHS-PT) was developed to overcome the sample size limitation that is found with static headspace. With dynamic headspace, an unrestricted amount of gas can be purged over the sample surface. Although the concentration of analytes in the gas is low the samples can be concentrated on cold traps, solvent traps or solid adsorbents (74). The most common trapping material used for the analysis of wine samples is Tenax. Tenax is chosen due to its low affinity for water and ethanol. Volatiles are first trapped onto the Tenax and then thermally desorbed into a GC injector. This technique has now become automated with the use of equipment such as a Gerstel automatic sampler with the dynamic headspace attachment.
  - Solid phase microextraction (SPME) is a solventless sampling technique; the sample is extracted from a liquid or gas on to a fibre coated with a stationary phase. This stationary phase is then inserted into an inlet and then thermally desorbed (75). This technique was developed by Arthur and Pawliszyn in 1990 where they used it in

headspace mode (HS-SPME) and coupled with (GC) (75). SPME is now easily automated and combines both sample extraction and concentration into one step. There are many advantages of SPME including the speed and that it is a lot easier than solvent extractions and distillation procedures. Sampling and analysis times usually vary from 10 to 60 minutes. Many applications utilising this technique for wine analysis have been developed e.g. (27,76,77). Since a single fibre type will not extract all classes of compounds identically, caution must be taken when using this technique for quantification of compounds (77,78). Method optimisations using SPME should include examinations of varying sample temperature and varying matrix effects such as ethanol, salt concentration and pH (79).

- Solid-phase dynamic extraction (SPDE), is a relatively new technique recently used to analyse fermentation profiles in a fermenting grape must (80). It comprises an internally coated steel needle (81,82) which is used for the extraction and preconcentration from the solution headspace. A dynamic extraction occurs by repeated aspiration and dispersion of the syringe volume and as a result the analytes present in the sample are adsorbed onto the sorbent inside the needle, and then thermally desorbed into a GC injector port followed by MS analysis. SPDE is generally not as widely applied as SPME, and reference to the use of this technique for wine is limited (80 and ref. therein).

### **Quantitation and identification**

Important odorants of foods and beverages have been identified and quantified at low levels due to the development of numerous analytical techniques which include: fourier transform nuclear magnetic resonance (FT-NMR), infra-red spectroscopy (IR) and most importantly gas chromatography (GC), mass spectrometry (MS) and the so-called hyphenated techniques such as GC-MS and GC/Olfactometry (GC/O). GC-MS with the use of SPME has enabled the quantification of compounds at levels from parts per billion ( $\mu\text{g/L}$ ) to parts per trillion ( $\text{ng/L}$ ) in wine (72).

For quantification of analytes, internal standards are most often used in order to check analyte recoveries and to reduce variability that can occur during sample

preparation and injection. The choice of standard is important as it must not interfere with the chromatographic separation and ultimately needs to respond in a similar way to the analyte. The best type of analytical standards are stable isotope labelled analogues of analytes as they have near-identical and physical properties to the analyte of interest. In theory, no matter what happens during sample preparation and analysis, the ratio of the isotopically-labelled standard to the non-labelled analyte remains the same. Ideally a stable, isotopically-labelled standard is needed for each analyte, but these standards are not commonly commercially available and need to be synthesised (83). Isotopically-labelled standards do not usually resolve from the analyte during GC separation (although the greater the extent of labelling the greater the separation), but by using MS, individual mass ions of the analyte and its standard can be monitored and quantified separately. There are now many examples in the literature that employ labelled standards for quantification of many different flavour and taint compounds such as monoterpenes (17), norisoprenoids (27), sesquiterpenes (84), cork taint (65,85), thiols (86) and fermentation products (76).

GC/O is the most common technique used for the identification of compounds that exhibit a particular aroma. It involves an individual sniffing the effluent eluting off the end of a GC column and recording the aroma descriptors and timing of detecting the odorants. In 1997 (19) and 1998 (87) Guth conducted comprehensive studies using GC/O and static headspace in wine to identify and quantify using SIDA, important odour impact compounds in Scheurebe and Gewurtztraminer wine. This study led to the first identification of compounds **42** (19) and **43** (87) in wine. In more recent studies, GC/O was used to identify TPB **13** (26), rotundone (21), and a cork taint compound (2-methoxy-3,5-dimethylpyrazine **41**) (65) in wine for the first time.

#### **Additional considerations with thiol analysis:**

The analytical determination of thiol compounds at near-threshold concentrations in wine is difficult, since very low levels need to be reliably measured (low ng/L to  $\mu\text{g/L}$  range). A number of difficulties encountered with 3-MH analysis included poor sensitivity and poor chromatography and intense tailing of peaks which was most likely due to the thiol functionality on the stationary phase or residual active sites which can occur in some chromatographic systems (73). Additional problems encountered with the analysis of these compounds is due to their instability and capacity to react with oxygen and other oxidants, and thiols can form complexes and

precipitates with many different metal ions (88). Therefore there are not many analytical methods described for the quantitative analysis of these compounds at trace level.

The most common method applied to the varietal thiol compounds uses *p*-hydroxymercurybenzoate (*p*-HMB) solution to promote thiol extraction (34). *p*-HMB is highly toxic, and the methods employed are expensive, use large volumes of wine and involve tedious or complicated extractions (34,36,38). Despite these limitations, further modifications of this method have been published (40,73). These methods improve the quality of the extracts containing the thiols, but still did not solve the difficulties with sensitivity and compound instability. Such analytical methods have been enhanced by using stable isotope dilution analysis (86). Unfortunately, these methods are still time consuming, the procedures are complicated and the use of mercury complexes yet to be eliminated.

Researchers have investigated the use of derivatising agents such as 2,3,4,5,6-pentafluorobenzyl bromide (PFBBBr), with on-fibre derivatisation (89) or in-cartridge (SPE) derivatisation (90). These alternative methods have not been taken up, possibly due to limitations with the linear range, along with reactivity, repeatability and sensitivity issues. These various methods also use negative chemical ionisation mass spectrometry which is not available in many laboratories. A new method for analysing these compounds is required so that they can be done in most laboratories using conventional electronic impact ionisation (EI) mass spectrometry and eliminating the use of mercury complexes.

## Summary of research aims

The aim of this work was to develop novel analytical methods using stable isotope dilution analysis (SIDA) for two important wine aroma compounds and to then apply these in studies to improve understanding of the formation and fate of these compounds. Such novel analytical methods will advance understanding of the effects of viticultural practices, winemaking practices, storage and ageing conditions on overall wine flavour. This will in turn improve decision making capabilities of winemakers and viticulturalists to control these aromas in wine.

### Specific objectives

Develop SIDA methods using GC-MS for:

- 1,8-Cineole **34** and
- Varietal thiol 3-MH **18**

Develop a SIDA method using HPLC-MS/MS for:

- Cysteine (**28, 29**) and glutathione conjugates (**30, 31**) of 3-MH and
- Apply these new analytical methods to research and commercial scale vineyard and winery studies to elucidate aspects of the compounds origin, formation and fate in wine.

### 1,8-cineole (eucalyptol)

A method was required for measuring 1,8-cineole in grapes and wine. With a suitable method in hand it was necessary to verify how the 'eucalyptus' character is transferred / formed in wine, as there have been conflicting views reported. It was necessary to determine the prevalence of 1,8-cineole in Australian wine and this would be achieved by carrying out a survey of commercial red and white wines.

Following this it would be advantageous to investigate factors affecting 1,8-cineole evolution during wine making or whether 1,8-cineole can be easily formed from precursors or degraded. It would be especially useful to determine the influence of *Eucalyptus* trees in the vineyard on 1,8-cineole concentrations in wine. Determination of its stability in wine along with investigations of various closures types affecting 1,8-cineole concentration in wine would also provide useful insight to this research area. Detailed results of these studies can be found in Chapters 2 and 3.

### 3-MH

Sensitive and specialised methods are paramount to understanding thiol compounds due to their reactivity and low concentrations. A new method for measuring 3-MH would enable grapes and juices to be assessed, which was something that has not been achieved up to now. The current, routine method for selective isolation of thiols from wine employs mercury complexes in the extraction, which are extremely toxic.

The important thiol to be targeted for development of a new method which eliminates the use of mercury was 3-mercaptophexanol **18**. To enable the ultimate in accuracy, deuterium labelled standards will be employed for the analysis. An optimised method would then be used in various applications such as examination of 3-MH during ripening, the effect of freezing and its prevalence in other grape varieties. A GC-MS method for measuring 3-MH will be used in conjunction with a 3-MH precursor method to gain a greater insight into 3-MH formation. Details of these studies can be found in Chapters 4 and 5.

### 3-MH Precursors

Knowledge of thiol precursors is important to understanding the formation of the free thiols. Some thiols arise from grape components and others from yeast biosynthesis. A HPLC-MS/MS method for both diastereoisomers of the thiol precursors Cys-3-MH (**27, 28**) and Glut-3-MH (**29, 30**) needed to be developed. Various factors that may affect 3-MH precursor concentration would be studied and these include: freezing grapes and wines, grape ripeness, grape processing operations and transportation. These studies will be essential in continuing to improve understanding of 3-MH formation and how this compound might be manipulated in wine. Further details of thiol precursor studies can be found in Chapters 4 to 9.

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## Chapter 2

### **Evolution and Occurrence of 1,8-cineole (Eucalyptol) in Australian Wine**

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*Journal of Agricultural Food Chemistry* – **2011**, 59, 953-959

NOTE:

Statements of authorship appear in the print copy of the thesis held in the University of Adelaide Library.

Capone, D.L., Van Leeuwen, K., Taylor, D.K., Jeffery, D.W., Pardon, K.H., Elsey, G.M. & Sefton, M.A. (2011). Evolution and occurrence of 1,8-cineole (eucalyptol) in Australian wine. *Journal of Agricultural and Food Chemistry*, v. 59 (3), pp. 953-959.

NOTE:

This publication is included on pages 34-40 in the print copy of the thesis held in the University of Adelaide Library.

It is also available online to authorised users at:

<http://dx.doi.org/10.1021/jf1038212>



## **Chapter 3**

### **Vineyard and Fermentation Studies to Elucidate the Origin of 1,8-Cineole in Australian Red Wine**

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*Journal of Agricultural Food Chemistry* – **2012**, 60: 2281-2287

NOTE:

Statements of authorship appear in the print copy of the thesis held in the University of Adelaide Library.

Capone, D.L., Jeffery, D.W. & Sefton, M.A. (2012). Vineyard and fermentation studies to elucidate the origin of 1,8-cineole in Australian red wine.

*Journal of Agricultural and Food Chemistry*, v. 60 (9), pp. 2281-2287.

NOTE:

This publication is included on pages 43-49 in the print copy of the thesis held in the University of Adelaide Library.

It is also available online to authorised users at:

<http://dx.doi.org/10.1021/jf204499h>



## Chapter 4

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

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*Journal of Agricultural Food Chemistry* – 2011, 59, 4649-4658.

NOTE:

Statements of authorship appear in the print copy of the thesis held in the University of Adelaide Library.

Capone, D.L., Sefton, M.A. & Jeffery, D.W. (2011) 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.

*Journal of Agricultural and Food Chemistry*, v. 59 (9), pp. 4649-4658.

NOTE:

This publication is included on pages 52-61 in the print copy of the thesis held in the University of Adelaide Library.

It is also available online to authorised users at:

<http://dx.doi.org/10.1021/jf200116q>

## Chapter 5

### **Analysis of Precursors to Wine Odorant 3-Mercaptohexan-1-ol using HPLC-MS/MS – Resolution and Quantitation of Diastereomers of 3-S-Cysteinyhexan-1-ol and 3-S-Glutathionylhexan-1-ol**

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*Journal of Agricultural Food Chemistry* – **2010**, *58*, 1390-1395

NOTE:

Statements of authorship appear in the print copy of the thesis held in the University of Adelaide Library.

Capone, D.L., Sefton, M.A., Hayasaka, Y. & Jeffery, D.W. (2010) Analysis of precursors to wine odorant 3-mercaptohexan-1-ol using HPLC-MS/MS - resolution and quantitation of diastereomers of 3-S-cysteinyhexan-1-ol and 3-S-glutathionylhexan-1-ol. *Journal of Agricultural and Food Chemistry*, v. 58 (3), pp. 1390-1395.

NOTE:

This publication is included on pages 64-69 in the print copy of the thesis held in the University of Adelaide Library.

It is also available online to authorised users at:

<http://dx.doi.org/10.1021/jf903720w>

## Chapter 6

### **Effects of Transporting and Processing Sauvignon Blanc Grapes on 3-Mercaptohexan-1-ol Precursor Concentrations**

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*Journal of Agricultural Food Chemistry* – **2011**, *59*, 4659-4667

NOTE:

Statements of authorship appear in the print copy of the thesis held in the University of Adelaide Library.

Capone, D.L. & Jeffery, D.W. (2011) Effects of transporting and processing Sauvignon blanc grapes on 3-mercaptophexan-1-ol precursor concentrations.  
*Journal of Agricultural and Food Chemistry*, v. 59 (9), pp. 4659-4667.

NOTE:

This publication is included on pages 72-80 in the print copy of the thesis held in the University of Adelaide Library.

It is also available online to authorised users at:

<http://dx.doi.org/10.1021/jf200119z>



## Chapter 7

### **Identification and Quantitation of 3-S-Cysteinyglycinehexan-1-ol (Cysgly-3-MH) in Sauvignon Blanc Grape Juice by HPLC-MS/MS**

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*Journal of Agricultural Food Chemistry* – **2011**, 59, 11204-11210

NOTE:

Statements of authorship appear in the print copy of the thesis held in the University of Adelaide Library.

Capone, D.L., Pardon, K.H., Cordente, A.G. & Jeffery, D.W. (2011) Identification and quantitation of 3-S-cysteinylglycinehexan-1-ol (cysgly-3-MH) in Sauvignon blanc grape juice by HPLC-MS/MS. *Journal of Agricultural and Food Chemistry*, v. 59 (20), pp. 11204-11210.

NOTE:

This publication is included on pages 83-89 in the print copy of the thesis held in the University of Adelaide Library.

It is also available online to authorised users at:

<http://dx.doi.org/10.1021/jf202543z>



## **Chapter 8**

### **Analytical Investigations of Wine Odorant 3-Mercaptohexan-1-ol and its Precursors**

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In "Flavor Chemistry of Wine and Other Alcoholic Beverages", American Chemical  
Society: Washington, DC, **2011**; *Accepted*

NOTE:

Statements of authorship appear in the print copy of the thesis held in the University of Adelaide Library.

Capone, D.L., Sefton, M.A. & Jeffery, D.W. (2012), 'Analytical investigations of wine odorant 3-mercaptohexan-1-ol and its precursors', in M. Qian & T. Shellhammer (eds), *Flavor Chemistry of Wine and Other Alcoholic Beverages*. ACS Symposium Series, American Chemical Society, Washington, DC, pp. 15-35.

NOTE:

This publication is included on pages 92-126 in the print copy of the thesis held in the University of Adelaide Library.

It is also available online to authorised users at:

<http://dx.doi.org/10.1021/bk-2012-1104.ch002>

## Chapter 9

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

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*Journal of Agricultural Food Chemistry* – **2012**, 60: 3515-3523

NOTE:

Statements of authorship appear in the print copy of the thesis held in the University of Adelaide Library.

Capone, D.L., Black, C.A. & Jeffery, D.W. (2012) Effects of 3-mercaptohexan-1-ol precursor concentrations from prolonged storage of Sauvignon blanc grapes prior to crushing and pressing. *Journal of Agricultural and Food Chemistry*, v. 60 (13), pp. 3515-3523.

NOTE:

This publication is included on pages 129-137 in the print copy of the thesis held in the University of Adelaide Library.

It is also available online to authorised users at:

<http://dx.doi.org/10.1021/jf300054h>



## Chapter 10

### Concluding remarks and future perspectives





## Concluding remarks and future perspectives

The research discussed in this thesis was undertaken to provide a greater understanding of two wine aroma compounds. Aroma compounds are important contributors to wine quality and they can impact consumer impressions and level of enjoyment of different wine styles. This greater awareness of wine aroma composition can be achieved by the development of novel analytical methods for volatiles and precursor compounds. These methods can be used to embark on advancing our knowledge of the formation and fate of a diverse range of chosen compounds. The analytical methods developed in the scope of this research used stable isotope dilution analysis (SIDA). SIDA was chosen as stable isotope labelled standards have near-identical chemical and physical properties to the analyte of interest.

The aroma compound 1,8-cineole, commonly known as eucalyptol, was chosen as its origin in wine was unresolved and *Eucalyptus* trees are found throughout Australia. There are many species of *Eucalyptus* trees and most contain volatile essential oils in their leaves, with up to 60 – 90% of the composition of the oil being 1,8-cineole. Several theories had been reported prior to this work including that the ‘eucalypt’ character in wines occurs when vineyards are surrounded by *Eucalyptus* trees, whereas other research pointed to terpene compounds such as  $\alpha$ -terpineol and limonene acting as precursors to 1,8-cineole. A SIDA/SPME/GC-MS method was developed for accurately determining the concentration of 1,8-cineole in wine (Chapter 2). This method was applied to an extensive survey of 190 commercially available Australian wines of mixed varieties revealing 1,8-cineole in significant concentrations in red wines only (Chapter 2). Of more than 150 commercially available Australian red wines which were analysed, 40% were found to contain 1,8-cineole above its reported aroma detection threshold.

Following this survey, it was shown that a continuous increase in the concentration of 1,8-cineole occurred during red wine fermentation but ceased at pressing off the skins. This indicated that the compound was extracted from the grape skins and/or matter other than grapes (MOG) (Chapter 2). This result also explained why 1,8-cineole was not found in white wines, as white wines are made with limited skin contact. Hydrolysis studies of limonene and  $\alpha$ -terpineol at wine pH demonstrated that these compounds cannot be significant precursors to 1,8-cineole, since very low

molar conversions (<0.6%) were achieved over a two year period. The results of these studies are described in detail in Chapter 2.

1,8-Cineole was found to be stable in wine and barely affected by closures (Chapter 2) so it was desirable to determine how this character arose in wine to enable the adaptation of 1,8-cineole concentrations to meet consumer demands. Therefore further studies including vineyard and fermentation treatments were carried out to provide evidence that it was vineyard position and winemaking conditions that had the most noticeable influence on the 1,8-cineole concentration in red wine (Chapter 3). The levels of 1,8-cineole in grape berries, leaves and stems were also quantified at set distances from the *Eucalyptus* trees over multiple vintages and showed that the greatest concentrations of 1,8-cineole in each sample type was found closer to the trees.

The concluding experiment in Chapter 3 demonstrated the profound effects that matter other than grapes (MOG, e.g. *Eucalyptus* or grape leaves) had on 1,8-cineole concentration in wine. It revealed that the presence of *Eucalyptus* leaves and to a lesser extent grape vine leaves and stems in the harvested grapes could be the main contributor to 1,8-cineole concentrations in wine. An additional and fortuitous outcome from the fermentation study was a demonstration for the first time that the concentration of rotundone in red wine can be strongly influenced by grapevine leaves and stems in the ferment. Measurements of grape stem and grape leaf soaks showed that the concentration of rotundone was three times greater in the stems compared to the leaves. This was an important finding since it gives wine producers unprecedented control over the 'peppery' characteristic of their wine.

Polyfunctional thiols are other important flavour compounds found in wine that require further investigation. Sensitive and specialised methods are vital to understanding thiol compounds due to their reactivity and low abundance in grapes and wine. The most widely used method for quantitating these compounds was developed some time ago and employs toxic mercury complexes. Therefore a novel method was developed for the analysis of 3-MH which eliminated the use of mercury and employed conventional electron ionisation GC-MS. The development of this sensitive method and its application to wines and grape juices is detailed in Chapter 4. This new method was applied to a number of studies including the assessment of 3-MH evolution during ripening of five Sauvignon blanc clones (Chapters 4 and 8). This was

the first time that natural 3-MH was measured during grape ripening; 3-MH was barely detectable at veraison and increased to approximately 100 ng/L at mid-ripening, before remaining relatively static until harvest. The concentrations measured were above the aroma detection threshold of 3-MH and the tropical fruit characters associated with varietal thiols were clearly evident around the mid-ripening time point when tasting these berries in the vineyard. 3-MH was also determined in the finished wines and as expected, after fermentation the concentration of 3-MH significantly increased.

Complementing the studies of 3-MH, a SIDA method was developed for the analysis of individual diastereomers of known 3-MH precursors: 3-S-cysteinylhexan-1-ol (Cys-3-MH) and 3-S-glutathionylhexan-1-ol (Glut-3-MH). HPLC-MS/MS was chosen in this case as the most appropriate method to analyse these water soluble, non-volatile compounds as opposed to derivatisation and GC-MS as previously employed for Cys-3-MH. This provided the first method for the analysis of both diastereomers of Cys- and Glut-3-MH in one analysis (Chapter 5).

The concentrations of both 3-MH and its precursors were measured in a survey of commercial white wine varieties, revealing that, while Sauvignon blanc contained the greatest concentration of these compounds, other varieties also contained significant quantities (Chapters 4 and 8). This indicated that 3-MH may be an important aroma compound in a range of grape varieties, especially when present well above its aroma threshold. Interesting results were also obtained in another study where precursor concentrations were measured during ripening; stark increases in precursor levels were found in the latter stages of ripening (Chapter 5). In another ripening study (Chapter 8) the 3-MH precursor concentrations generally increased during ripening in accord with the initial observations, up to a sugar level of approximately 24 °Brix (around ordinary commercial maturity), and then declined beyond this time. The increase in the lead up to harvest can most likely be explained in terms of loss of membrane integrity combined with an increase in available precursor constituents.

Applying the precursor method to an array of precursor studies has provided some new insights into the relationship between Cys- and Glut-3-MH. Freezing studies highlighted the difference between frozen storage of juice and grape samples, where large amounts of Glut-3-MH were only formed when grapes were frozen, yet Cys-3-

MH levels were not similarly affected. This indicated that Glut-3-MH formation could be attributed to loss of berry integrity and provided results that showed that juices could be frozen for later precursor analysis but not grapes (Chapter 4). Fining a juice with gelatin had minimal effect on precursor concentrations, while the effects of machine-harvesting and transportation of fruit were quite significant (Chapter 8).

Fruit transported after being commercially harvested had up to 10 times as much Cys-3-MH and around twice as much Glut-3-MH compared to the non-transported samples (Chapter 6), revealing the ability to form precursors during processing. This was particularly important since Cys-3-MH is more easily utilised by yeast during fermentation, and the levels found in this experiment were the highest we have found for healthy fruit. These results confirmed that Glut-3-MH could be further formed post-harvest, but it was most surprising to find it could be converted to substantial amounts of Cys-3-MH given an adequate amount of time. The formation and degradation of Glut-3-MH in this case, either partially or totally under the control of various enzymes, highlights the dynamic nature of these precursors indicating that processing methodology can play an important role (Chapter 6). Similar results to the transport study were achieved by holding fruit bins in a cold room prior to crushing, demonstrating that higher precursor levels could be achieved without the use of transportation (Chapter 9).

An experiment conducted to inhibit the enzymes usually active during berry crushing showed that concentrations of Glut-3-MH could be significantly reduced while Cys-3-MH levels remained unaffected. This result led to the assumption that the Cys-3-MH, arising from the breakdown of Glut-3-MH, is present in the grape berry and relatively resistant to short term grape processing effects while the larger proportion of Glut-3-MH forms post-harvest so processing conditions can have major influence on concentrations.

As a follow on from these experiments, additional objectives were completed with the identification of a new conjugated aldehyde which was the obvious missing link between the reaction of (*E*)-2-hexenal and glutathione in the formation of Glut-3-MH. This identification is detailed in Chapter 6 and was the first evidence of the presence of such an intermediate aldehyde. Also success was achieved with the identification and quantitation of 3-*S*-cysteinylglycinehexan-1-ol (Cysgly-3-MH), which is the

dipeptide intermediate between cysteine and glutathione precursors of 3-MH (Chapter 7). The results from these studies (Chapters 5, 6, 7, 8 and 9) have provided an understanding into the formation and fate of precursors to 3-MH and emphasised their dynamic nature, which continue beyond berry development and into the winemaking process.

Although a much greater understanding of the varietal thiol compound 3-MH was achieved, future work should focus on extending the new 3-MH method to include 3-mercaptohexyl acetate (3-MHA) and 4-mercapto-4-methylpentan-2-one (4-MMP). These thiols, along with 3-MH are important impact odorants in wine and are especially potent, having some of the lowest aroma thresholds of any volatile food compound. Due to these extremely low thresholds and the concentrations of a few ng/L to low  $\mu\text{g/L}$  found in wine, the analytical methods developed will need to continue to be extremely sensitive. It would also be beneficial to develop chiral methods for the analysis of both 3-MH and 3-MHA to determine their importance to wine sensory characteristics. The ratios of these compounds have been shown to vary in wines, and they are directly related to the precursor diastereomers of 3-MH which can now be measured. Thiol precursors should continue to be studied with the addition of other precursors such as Glut and Cys-4-MMP in particular, as they are also important for understanding the formation of the corresponding varietal aroma compounds.

Additional outcomes from the fermentation studies with varying MOG showed not only that 1,8-cineole concentration could be strongly influenced by grapevine leaves and stems in the ferment but surprisingly so too could the 'peppery' impact compound rotundone (Chapter 3). This result paves the way for future research to determine if any additional compounds may be influenced by MOG and to what extent. These results clearly demonstrate that there is more to consider than just grape composition and yeast biochemistry when investigating wine aroma.



## **Appendices**

These are additional peer reviewed papers generated during candidature, which I have co-authored.

They were not a central part of my thesis work but some of the publications have included applications of the analytical methods developed as described in this thesis (Appendix 1 to 4).

The additional manuscripts included applications of other analytical methods developed (Appendix 6 to 12) or supervised (Appendix 13 and 14) by the candidate during candidature.



- Appendix 1** **148**  
Formation of varietal thiol aroma compounds in wine: synthetic and analytical studies of grape and wine thiol conjugates.  
Dimitra L. Capone, Markus J. Herderich, Kevin H. Pardon, Yoji Hayasaka, Antonio G. Cordente, Paris A. Grant-Preece, Mark A. Sefton, Gordon M. Elsey, David W. Jeffery.  
In *Proceedings of the 9th Wartburg Symposium on Flavor Chemistry and Biology*, Eisenach, Germany, 13-16 April, 2010; Hofmann, T.; Meyerhof, W.; Schieberle, P., Eds. Deutsche Forschungsanstalt für Lebensmittelchemie: Freising, Germany, **2011**; pp 242-248.
- Appendix 2** **155**  
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.  
Paris A. Grant-Preece, Kevin H. Pardon, Dimitra L. Capone, Antonio G. Cordente, Mark A. Sefton, David W. Jeffery and Gordon M. Elsey.  
*J. Agric. Food Chem.* **2010**, 58: 1383-1389.
- Appendix 3** **162**  
Engineering yeast for improved wine flavor: release of 3-mercaptohexan-1-ol during fermentation through overexpression of a yeast gene, STR3.  
Sylvester Holt, Antonio G. Cordente, Simon J. Williams, Dimitra L. Capone, Wanphen Jitjaroen, Ian R. Menz, Chris Curtin, and Peter A. Anderson.  
*Appl. Environ. Microbiol.*, Vol. 77, No. 11, **June 2011**, p. 3626–3632.
- Appendix 4** **169**  
Evolution of 3-mercaptohexanol, hydrogen sulfide, and methyl mercaptan during bottle storage of Sauvignon blanc wines. effect of glutathione, copper, oxygen exposure and closure-derived oxygen.  
Maurizio Ugliano, Mariola Kwiatkowski, Stephane Vidal, Dimitra Capone, Tracey Siebert, Jean-Baptiste Dieval, Olav Aagaard, and Elizabeth J. Waters.  
*J. Agric. Food Chem.* **2011**, 59: 2564-2572.

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Spice up your life: analysis of key aroma compounds in Shiraz	
Markus J. Herderich, Tracey E. Siebert, Mango Parker, <u>Dimitra L. Capone</u> , David W. Jeffery, Patricia Osidacz and Ian L. Francis.	
In "Flavor Chemistry of Wine and Other Alcoholic Beverages".	
American Chemical Society: Washington, DC; <b>2011</b> , (Accepted).	
<b>Appendix 6</b>	<b>191</b>
Formation of damascenone under both commercial and model fermentation conditions.	
Natoiya D. R. Lloyd, <u>Dimitra L. Capone</u> , Maurizio Ugliano, Dennis K. Taylor, George K. Skouroumounis, Mark A. Sefton, and Gordon M. Elsey.	
<i>J. Agric. Food Chem.</i> <b>2011</b> , 59: 1338-1343.	
<b>Appendix 7</b>	<b>197</b>
The role of yeast in the generation of the odorant damascenone in wine.	
Natoiya D. R. Lloyd, <u>Dimitra L. Capone</u> , Maurizio Ugliano, Dennis K. Taylor, Mark A. Sefton, and Gordon M. Elsey	
In <i>Proceedings of the 9th Wartburg Symposium on Flavor Chemistry and Biology</i> , Eisenach, Germany, 13-16 April, 2010; Hofmann, T.; Meyerhof, W.; Schieberle, P., Eds. Deutsche Forschungsanstalt für Lebensmittelchemie: Freising, Germany, <b>2011</b> ; pp 447-451.	
<b>Appendix 8</b>	<b>202</b>
The formation of wine lactone from grape-derived secondary metabolites.	
Joanne Giaccio, <u>Dimitra L. Capone</u> , Anders E. Hakansson, Heather E. Smyth, Gordon M. Elsey, Mark A. Sefton, and Dennis K. Taylor.	
<i>J. Agric. Food Chem.</i> <b>2011</b> , 59: 660-664.	
<b>Appendix 9</b>	<b>207</b>
Identification and analysis of 2-chloro-6-methylphenol, 2,6-dichlorophenol and indole: causes of taints and off-flavours in wines.	
<u>Dimitra L. Capone</u> , Katryna A. Van Leeuwen, Kevin H. Pardon, Merran A. Daniel, Gordon M. Elsey, Adrian D. Coulter and Mark A. Sefton.	
<i>Aust. J. Grape Wine Res.</i> <b>2010</b> , 16: 210-217.	

- Appendix 10** **215**  
Production of indole by wine associated microorganisms under oenological conditions.  
Maria Arevalo-Villena, Eveline J. Bartowsky, Dimitra L. Capone and Mark A. Sefton. *Food Microbiol.* **2010**, 27, 685-690.
- Appendix 11** **221**  
Molecular fingerprinting by PCR-denaturing gradient gel electrophoresis reveals differences in the levels of microbial diversity for musty-earthly tainted corks.  
Chantal Prat, Olaya Ruiz-Rueda, Rosalia Trias, Enriqueta Antico, Dimitra Capone, Mark Sefton and Lluís Banera.  
*Appl. Environ. Microbiol.* **2009**, 75(7), 1922-1931.
- Appendix 12** **231**  
Riesling acetal is a precursor to 1,1,6-trimethyl-1,2-dihydronaphthalene (TDN) in wine.  
Merran A. Daniel, Dimitra L. Capone, Mark A. Sefton and Gordon M. Elsey.  
*Aust. J. Grape Wine Res.* **2009**, 15(1), 93-96.
- Appendix 13** **235**  
Odor detection thresholds and enantiomeric distributions of several 4-alkyl substituted  $\gamma$ -lactones in Australian red wine.  
Rachel C. Cooke, Katryna A. van Leeuwen, Dimitra L. Capone, Richard Gawel, Gordon M. Elsey and Mark A. Sefton.  
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- Appendix 14** **241**  
Quantification of several 4-alkyl substituted  $\gamma$ -lactones in Australian wines.  
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NOTE:

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NOTE:

This publication is included on pages 162-168 in the print copy of the thesis held in the University of Adelaide Library.

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NOTE:

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NOTE:

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NOTE:

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It is also available online to authorised users at:

<http://dx.doi.org/10.1021/jf103741n>

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NOTE:

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NOTE:

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NOTE:

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NOTE:

This publication is included on pages 241-245 in the print copy  
of the thesis held in the University of Adelaide Library.

It is also available online to authorised users at:

<http://dx.doi.org/10.1021/jf8026974>