Polyfunctional Thiols in Wine

Chirality, Precursor Stereochemistry, Winemaking Impacts, and Fate

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A thesis submitted for the degree of Doctor of Philosophy

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Adelaide, Australia

September 2019

To the past, present, and future.

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Thesis Summary

Polyfunctional thiols (also known as varietal thiols) with odour detection thresholds (ODTs) in the nanogram per litre range are one of the most potent volatiles present in wine and are regarded as odour-active compounds affording significant sensory contributions. These compounds have been intensively investigated for the past few decades, along with their non-volatile grape-derived precursors in more recent years, but knowledge of the analysis, biogenesis, and fate of thiols, as well as the influences of environmental and winemaking factors, is still incomplete. This thesis therefore begins by reviewing the current knowledge of polyfunctional thiols in wine and precursors in grapes (**Chapter 1**), and then covers the analytical approaches that have been developed to identify and quantify thiols in foods and beverages, with a particular focus on wine (**Publication** in **Chapter 2**). A number of original research studies (**Publications** in **Chapters 3** to **6**) are then presented to address the knowledge gaps related to characteristic thiols in wine and their precursors in grapes.

Polyfunctional thiols 3-sulfanylhexan-1ol (3-SH) and 3-sulfanylhexyl acetate (3-SHA) are two of the most evaluated thiols in wine. 3-SH and 3-SHA are chiral molecules, which give rise to pairs of enantiomers that differ in aroma quality and ODT. However, chiral analytical methods required to study 3-SH and 3-SHA enantiomers in wine were essentially non-existent. Addressing this gap, a novel stable isotope dilution assay (SIDA) with chiral high-performance liquid chromatography with tandem mass spectrometry (HPLC–MS/MS) using a polysaccharide-based column has been developed and validated for analysing the enantiomers of 3-SH and 3-SHA, after extraction from wine as their 4-thiopyridine derivatives. Authentic derivatives were synthesised to enable chiral column screening, and method validation encompassed calibration range, linearity, accuracy, precision, limit of detection, and matrix effects. The validated

method demonstrated excellent analytical performance and offers opportunities for further research around chirality of polyfunctional thiols. By applying this method, the distribution pattern of enantiomers of 3-SH and 3-SHA in a set of commercial wines has been reported (**Publication** in **Chapter 3**).

The formation of 3-SH and 3-SHA during fermentation involves conjugated precursors that are present as pairs of diastereomers in grapes. However, there was no literature on thiol precursor stereochemistry in grapes in relation to thiol chirality in wine. Employing the newly developed method for analysing thiol enantiomers, the relationship between precursor diastereomers in grapes and thiol enantiomers in wine was examined for the first time through a fermentation trial using five co-located clones of Sauvignon blanc grapes (**Publication** in **Chapter 4**). No correlation was observed between precursor diastereomers in grapes analysed by SIDA HPLC–MS/MS and 3-SH or 3-SHA enantiomers in wines measured by SIDA chiral HPLC–MS/MS, but the results have provided insight that can be further explored to understand the implications of thiol biogenesis on thiol precursor stereochemistry.

Tackling thiols and their precursors from a viticultural and oenological perspective, the impacts of sub-region within one geographic indication, grape clone, grape amino acids, yeast strain, commercial enzyme, fermentation nutrient, and pre-fermentation freezing have been assessed in controlled fermentation trials with Sauvignon blanc (**Publications** in **Chapter 4** and **5**). Substantial intraregional variations existed among thiol precursors and thiols in the examined grapes and wines, respectively, and clonal differences were noted at the diastereomeric and enantiomeric levels for precursors and thiols, respectively. In terms of the impact of grape metabolites on thiols and precursors, grape amino acids were revealed for the first time to have stronger correlations to 3-SH precursors in grapes (e.g., $|r| > 0.73$ and 0.62 for glutamic

acid and glycine) than thiols in wines ($|r| < 0.42$), highlighting the potential interaction between grape amino acids and thiol precursor metabolism in grapes. With regard to thiol management during winemaking, significant elevations of polyfunctional thiols in wine occurred with the use of a commercial enzyme in juices (up to an approximate two-fold increase in a clone-dependent manner) or pre-fermentation freezing treatment on fresh grapes (up to an approximate 10-fold enhancement regardless of clone). These practical approaches and novel results are of potential interest for winemakers who seek to be one step closer to thiol management during winemaking.

The fate of polyfunctional thiols in wine requires continued investigation to comprehend the impacts on varietal aroma profiles of wine. Based on the co-existence of 3-SH and acetaldehyde in wine, the presence of a new volatile sulfur compound (VSC) with an oxathiane structure was theorised. After the synthesis of a deuterated standard and the development of a SIDA headspace solid-phase microextraction (HS–SPME) with gas chromatography and mass spectrometry (GC–MS) method, 2-methyl-4-propyl-1,3-oxathiane was identified and quantitated in wine for the first time. Only detectable as the *cis*-isomer, this compound strongly correlated ($r = 0.72$) with the concentration of 3-SH determined by HPLC–MS/MS analysis. The ODT of this newly discovered wine volatile was determined in a neutral white wine. Concentrations of *cis*-2-methyl-4-propyl-1,3-oxathiane (up to 460 ng/L) determined in a range of surveyed commercial wines were below the measured ODT of 7.1 µg/L. Nonetheless, the presence of this new wine volatile still has potential implications for wine aroma, due to its direct link with the fate of important wine aroma compound 3-SH. On one hand, this may help account for 3-SH that is lost during fermentation and ageing, and on the other hand, a sizeable proportion of 3-SH could be masked as the oxathiane, thus diminishing the impact of 3-SH on wine aroma (**Publication** in **Chapter 6**).

Thesis summary

In summary, this PhD thesis has combined modern analytical methods, chemistry synthesis, fermentation trials, and sensory testing to shed light on aspects of wine aroma related to important polyfunctional thiols and their precursors. The outcomes not only contribute to a better scientific understanding of thiol chemistry but also offer opportunities for potential industrial applications.

Declaration

I certify that this work contains no material which has been accepted for the award of any other degree or diploma in my name, in any university or other tertiary institution and, to the best of my knowledge and belief, contains no material previously published or written by another person, except where due reference has been made in the text. In addition, I certify that no part of this work will, in the future, be used in a submission in my name, for any other degree or diploma in any university or other tertiary institution without the prior approval of the University of Adelaide and where applicable, any partner institution responsible for the joint-award of this degree.

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Liang Chen

Signature: Date: 26 Nov 2019

Publications

This doctoral thesis is submitted as a collection of papers that were published in peer-reviewed scientific journals during candidature according to the "*PhD by Publication Guidelines*" of the University of Adelaide.

1 **Chen L.**, Capone D. L., Jeffery D. W. Analysis of potent odour-active volatile thiols in foods and beverages with a focus on wine. *Molecules* **2019**, 24, 2472. DOI.

2 **Chen L.**, Capone D. L., Jeffery D. W. Chiral analysis of 3-sulfanylhexan-1-ol and 3-sulfanylhexyl acetate in wine by high-performance liquid chromatography–tandem mass spectrometry. *Analytica Chimica Acta* **2018**, 998, 83–92. DOI.

3 **Chen L.**, Capone D. L., Tondini A. F., Jeffery D. W. Chiral polyfunctional thiols and their conjugated precursors upon winemaking with five *Vitis vinifera* Sauvignon blanc clones. *Journal of Agricultural and Food Chemistry* **2018**, 66, 4674–4682. DOI.

4 **Chen L.**, Capone D. L., Nicholson E. L., Jeffery D. W. Investigation of intraregional variation, grape amino acids, and pre-fermentation freezing on varietal thiols and their precursors for *Vitis vinifera* Sauvignon blanc. *Food Chemistry* **2019**, 295, 637–645. DOI.

5 **Chen L.**, Capone D. L., Jeffery D. W. Identification and quantitative analysis of 2-methyl-4-propyl-1,3-oxathiane in wine. *Journal of Agricultural and Food Chemistry* **2018**, 66, 10808–10815. DOI.

The journals in which these papers were published are closely related to the research of the field of this work. The publications are listed in the order of chapters. A statement of authorship, signed by all of the authors listing individual contributions to the work is included at the beginning of **Chapters 2** to **6**.

Conferences

The list of **oral presentations** at international and national conferences participated as student presenter:

1. Identification of 2-methyl-4-propyl-1,3-oxathiane as a new volatile sulfur compound in wine.
   Oeno IVAS – In Vino Analytica Scientia, Bordeaux, France, 25–28 June 2019

2. Polyfunctional thiols in wine: Analytical evaluation and exploration.
   School of Agriculture, Food & Wine Research Day, Adelaide, SA, Australia, 30 November 2018

3. Polyfunctional thiols in Sauvignon blanc wine: precursor stereochemistry, clone, and winemaking.
   Crush – The grape and wine science symposium, Adelaide, SA, Australia, 13 and 14 November 2018 (Best presentation award)

4. Polyfunctional thiols in wine: chirality, precursor stereochemistry, and Sauvignon blanc clone type.
   69th American Society for Enology and Viticulture (ASEV) National conference, Monterey, CA, USA, 18–21 June 2018

5. Flavour profiles of leaves from different varieties of Gearldton Wax.
   2nd International Flavor and Fragrance (IFF) conference, Wuxi, Jiangsu, China, 28–31 May 2018

6. Chiral analysis of 3-SH and 3-SHA in wine.
   Crush – The Grape and Wine Science Symposium, Adelaide, SA, Australia, 13 and 14 November 2017

Conferences

Other co-authored conference items:

7 Fate of tropical odorants in wine: Identification and stability of 2-methyl-4-propyl-1,3-oxathiane.
Poster at Australian Wine Industry Technical Conference, Adelaide, SA, Australia, 21–24 July 2019

8 The effects of pre-fermentative additions on yeast volatile aromas and thiols in Sauvignon blanc and Chardonnay.
Poster at Australian Wine Industry Technical Conference, Adelaide, SA, Australia, 21–24 July 2019

9 Distribution of enantiomers of 3-sulfanylhexan-1-ol and its acetate in wine determined by HPLC–MS/MS.
Short talk at MARCOWINE 2018, Zaragoza, Spain, 28–31 May 2018

10 Quantitation of potent polyfunctional thiols and their enantiomers in wine using HPLC–MS/MS after derivatization.
Talk at 254th National Meeting and Exposition of the American Chemical Society (ACS) on Chemistry's Impact on the Global Economy, Washington, DC, USA, 20–24 August, 2017

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Acknowledgements

Like a festival event. Time well spent.
Like a wine ferment. Scents nicely present.
This special journey is blessingly content.
To all of you, my sincerest acknowledgement sent.

To Associate Professor Dr. David Jeffery – Thank you, my dear mentor and friend. Four years in the blink of an eye, every moment in my PhD is just enjoyable and rewarding. There were no unrealistic expectations, empty talks, or unanswered emails, never; just everyday guidance, constant encouragement, and true understanding from the beginning to the end. Never asking for any credit, you put endless efforts into this project. Thank you for being so generous with your time, always listening to my dreams when needed, holding a torch for me when it's dark, and raising a glass when celebrating. You are always there for me. Thank you Dave, for letting me work on my PhD with you, for letting me learn from your knowledge, heart, mind, and soul. I consider myself truly fortunate to have you as my mentor and friend.

To my co-supervisor Dr. Dimitra Capone – thank you. I am sincerely grateful to have you – an extremely knowledgeable researcher – as my co-supervisor on this project. Thank you for your enormous inputs and encouragement throughout my study.

Acknowledgements

Thanks to Prof. Kerry Wilkinson, Associate Prof. Chris Ford, Associate Prof. Sue Bastian, Dr. Richard Muhlack, Dr. Phil Clements, Dr. Carolyn Puglisi, Dr. Sijing Li, Dr. Chen Liang, Dr. Laura Wilkinson, Dr. Ana Hranilović, Dr. Yongqiang Zhao, Dr. Renata Restic, Dr. Michelle Walker, Dr. Tommaso Watson, Dr. Patrick Setford, Wenyu Kang, Suzanne Balacey, Ross Sanders, Mandy Lin, and Jo Hards, at the University of Adelaide; Prof. Philippe Darriet, Dr. Alexandre Pons, Delphine Winstel, and Alicia Jouin at the University of Bordeaux; Dr. Yoji Hayasaka and Dr. Marlize Bekker at The Australian Wine Research Institute (AWRI); Sue Maffei, Dr. Christine Böttcher, and Dr. Paul Boss at CSIRO; and my friend Chen, Zi, Laura, Alicia, Delphine, and Rod. You all have made this journey a little bit easier and more fun. In particularly, thanks to Zi, my best childhood friend, for encouraging and believing in me, since day one we met at primary school; thanks to Laura for your kindness and generosity; thank you to Carolyn, for being a great supportive and caring friend; thank you to Rod, for taking care of me when I was vulnerable. You are all very special. Life brought us together for a shorter or longer period of time, and I hope you have enjoyed our time together as much as I enjoyed your accompany. Every little kindness will make a big difference, and I will always treasure your kindness.

Special acknowledgements to the University of Adelaide (UA), China Scholarship Council (CSC), French Embassy of Australia, and University of Bordeaux (ISVV) for scholarships; Wine Australia for funding and scholarship; and Australian wine industry partners, including David Coleman of Adelaide Hills Vine Improvement Inc. (AHVII), Grapeworks, Casella Family Brands, Shaw+Smith, Spring Dale, Australian Vintage Ltd., and Nova Vita Wines, for their generous in-kind contributions to the project.

I am forever grateful for the love and support from my parents.

Life can only be understood backwards; but it must be lived forwards.

Søren Aabye Kierkegaard (1813–1855)

Chapter 1

Literature Review & Research Questions & Aims

The literature review in this chapter covers the literature up to December 2015. The literature beyond this date has been included in the publication in Chapter 2 and introductions of the publications in Chapters 3 to 6.

1.1. Polyfunctional thiols in wine

Aroma is one of the most complicated and important criteria for wine quality [1]. Of >800 volatile components reported in wines [2], polyfunctional thiols (or varietal thiols) are a category of volatile sulfur compounds (VSCs) that are of great importance to the aroma quality of wine of many varieties [3–5], especially for Sauvignon blanc, the variety known for its distinctive tropical fruit aroma characteristics [6].

From 2000 to 2010, Sauvignon blanc has significantly increased in its cultivation area in all major wine countries worldwide (**Table 1**) and become one of the top white grape varieties [7], which appears to reflect the modern wine consumers' preference of immediate fruit aromas over subtlety and ageing ability [8].

Table 1.

Cultivation area (hectare), share (%) in national wine grape area, and decadal increase (DI) for Sauvignon blanc in key wine-producing countries [7].

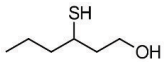
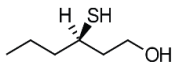
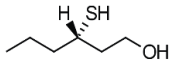
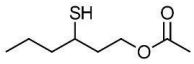
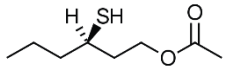
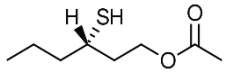
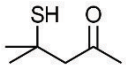
Country	Cultivation area			Share		
	2000	2010	DI	2000	2010	DI
France	20933	26839	28	2.4	3.2	24
NZ[1]	2423	16205	569	24.4	50.7	52
Chile	6662	12159	83	5.9	10.9	46
South Africa	5436	9551	76	5.8	9.5	39
US[2]	4191	6584	57	2.2	2.9	17
Australia	2602	6467	149	2.0	4.3	53

[1] NZ: New Zealand. [2] United States.

The typical tropical fruit aromas in Sauvignon blanc wine are mostly from three polyfunctional thiols: 3-sulfanylhexan-1-ol (3-SH), 3-sulfanylhexyl acetate (3-SHA) and 4-methyl-4-sulfanyl-pentan-2-one (4-MSP) (**Table 2**). Due to their aroma qualities and extremely low odour detection thresholds (ODTs), 3-SH, 3-SHA, and 4-MSP have been intensively investigated during the past two decades.

Table 2.

Structures, odour descriptions, odour detection thresholds (ODTs), and reported odour activity values (OAVs) of three potent polyfunctional thiols in wine.

Name	Structure [1]	Odour description	ODT [2]	OAV
3-SH		Grape fruit [9]	60 [9]	12–214 [6]
(*R*)-3-SH		Grape fruit, citrus peel [10]	50 [10]	
(*S*)-3-SH		Passion fruit [10]	60 [10]	
3-SHA		Box tree, grape fruit, passion fruit [3]	4 [3]	53–194 [6]
(*R*)-3-SHA		Passion fruit [10]	9 [10]	
(*S*)-3-SHA		Boxwood [10]	2.5 [10]	
4-MSP		Box tree, cat urine [4]	0.8 [4]	5–28 [6]

[1] Thiols bearing a chiral centre are most often studied as their racemic mixtures although they are present as pairs of enantiomers. Where available, the properties of both the racemate and individual enantiomers are given. [2] ng/L, measured in a 12% (*v*/*v*) aqueous alcohol solution.

1.1.1. Aroma contribution of polyfunctional thiols

Generally, certain polyfunctional thiols are recognised as the aroma compounds responsible for the typical 'tropical fruit' and 'citrus fruit' aromas of wines. In the very first instance, the profound sensory contribution of polyfunctuional thiols to wine was highlighted for 4-MSP exhibiting a 'guava-like' aroma through a sensory evaluation on white wines [11]. Later by gas chromatography–olfactometry (GC–O), 4-MSP was tentatively identified in Sauvignon blanc wine with aroma described as 'powerful box tree' [12]. Afterwards, 3-SHA was identified in Sauvignon wines by gas chromatography with flame photometric detector (GC–FPD) and found to have a 'box tree' odour with 'passion fruit' and 'grapefruit' aromas [3]. 3-SH with a 'grapefruit' aroma was also identified in Sauvignon blanc wine [9]. Although polyfunctional thiols are normally associated with pleasant fruity aroma, their sensorial properties are concentration-dependent. It has been shown that polyfunctional thiol 4-MSP at moderate concentration enhanced the 'overall fruit aroma' whereas negative aromas such as 'cat urine' and 'sweaty' could arise when at high concentrations [13].

Additionally, 3-SH and 3-SHA are chiral molecules both bearing a pair of enantiomers that differ in aroma quality. The aroma of (*R*)-3-SH was reminiscent of 'grapefruit', while the aroma of the (*S*)-3-SH was like 'passion fruit'. The (*R*)-form of 3-SHA showed more 'herbaceous' aroma, with a smell like 4-MSP, while (*S*)-3-SHA displayed 'boxwood' note [10]. The changes of ratios of the enantiomers of 3-SH and 3-SHA is reported to result in different wine aroma profiles [13].

The measured ODTs of 4-MSP, 3-SH and 3-SHA were extremely low, at 0.8, 60 and 4 ng/L (**Table 2**), respectively, which made them one of the most potent volatiles found in nature. These three polyfunctional thiols are readily able to impart significant sensory contributions to wine aroma. With respect to enantiomers of 3-SH and 3-SHA, the detected ODTs of 3-SH enantiomers were similar (50 ng/L for the (*R*)-form and 60 ng/L for (*S*)-form), but ODTs of (*S*)-3-SHA (2.5 ng/L) and (*R*)-3-SHA (9 ng/L) were very different [10].

Odour activity value (OAV) is the ratio of the measured concentration over the ODT of the compound, which is also referred as the aromatic index (*I*). It can be calculated to evaluate the aroma contribution of volatile compounds: an OAV above 1.0 indicates a likely contribution of the compound to the overall aroma [14]. The OAV data of polyfunctional thiols (**Table 2**) demonstrated their undeniable sensory impact on wine aroma [6]. In addition to OAV, aroma extract dilution analysis (AEDA) technique has also been applied to screen potent volatile odourants, and its result is expressed as flavour dilution (FD) factor which means the dilution degree that a substance can still be smelled [15]. AEDA studies on wine aroma have found that polyfunctional thiols had high FD, frequently higher in white [16] and rosé wine [17], but to a lesser extent in red wine [18]. However, as a tool to detect odour-active compounds, AEDA has some downsides, such as odourant losses during isolation [19]. Therefore, sensory reconstruction study was proposed to elucidate the specific role of certain volatile compounds to the overall aroma profiles [19]. The omission of 4-MSP in Scheurebe model wine resulted in a completely different aroma profile to the original wine, revealing that 4-MSP was one of the most important volatile compounds responsible for aroma of the Scheurebe wine [19]. The contribution of 3-SH to aroma of Grenache rosé wines has also been demonstrated using AEDA [17].

1.1.2. Analysis of polyfunctional thiols

1.1.2.1. Extraction

Extraction is the first step for chemical analysis of polyfunctional thiols. However, extraction of thiols is a challenging task for wine researchers due to two main impediments: the ultra-trace concentrations and the unstable chemistry nature of polyfunctional thiols [20]. To overcome analytical challenges, liquid-liquid extraction (LLE), headspace solid-phase microextraction (HS–SPME), and solid phase extraction (SPE) have been used for thiol analysis in wine.

1.1.2.1.1. LLE and *p*HMB

As the early methods for polyfunctional thiol extraction, LLE required the use of organic solvents (e.g. diethyl ether and pentane) to obtain volatile fractions from wine followed by specific polyfunctional thiol extraction procedures. In the particular selective extraction steps, polyfunctional thiols were bound by *p*-hydroxymercuribenzoate (*p*HMB), converted to, and preserved as the stable thiol-*p*HMB. Next, the bounded polyfunctional thiols were treated with glutathione (GSH) to release polyfunctional thiols by replacing thiols with GSH [4]. This extraction method demonstrated good extraction selectivity by using mercury to attack thiols. An improved method based on this approach added an additional basic anion exchange chromatography step to eliminate impurities [6], and its modified versions have been subsequently developed [21–24]. LLE combined with covalent chromatography procedure for isolation of polyfunctional thiols has also been proposed too [25]. Afterwards, a modified method using mercuric bounded agarose gel was suggested [26]. LLE in combination with *p*HMB-related extraction strategy provided exclusive separations of polyfunctional thiols from wine, but had several major drawbacks, such as the requirement of large volumes of sample and solvent, tedious sample preparation procedures, and the use of hazardous chemical (e.g., mercury).

1.1.2.1.2. Derivatisation

Although there were only limited derivatisation reagents available for thiol derivatisation, derivatisation overcame some of the disadvantages of the traditional *p*HMB extraction methods. The first reported derivatisation method [27] applied 2,3,4,5,6-pentafluorobenzyl bromide (PFBBr) as a derivatisation reagent together with a secondary reagent (tribuytilamine) to react with the benzene extract of polyfunctional thiols, to obtain stable pentafluorobenzyl derivatives. In the following studies, PFBBr derivatisation was used for more polyfunctional thiols (3-SH and 3-SHA) [28] and upgraded by introducing a methoximation procedure (for 4-MSP) [29, 30]. Modified versions were employed to extract polyfunctional thiols from wines [31, 32]. Ethyl propiolate (ETP) was presented a low molecular weight derivatisation reagent [33]; however, this extraction method was unable to analyse 4-MSP because the derivatisation was influenced by wine matrix. Later, *o*-methylhydroxylamine hydrochloride (*o*-CH_3ONH_2·HCl) was suggested as a new derivatisation reagent for 4-MSP [34], and the sensitivity of quantitation of 4-MSP was below its ODT (0.8 ng/L). With regard to the derivatisation of 3-SH and 3-SHA, *o*-phthaldialdehyde (OPA) was evaluated [35]. More recently, a novel thiol derivatisation procedure using 4,4'-dithiodipyridine (DTDP) for thiols (including 4-MSP, 3-SH, and 3-SHA) was reported [20].

1.1.2.1.3. HS–SPME and SPE

HS–SPME was employed for 4-MSP extraction, in which the concentrations of 4-MSP in all samples were considerably high (at µg/L) [36]. SPME offered a simpler experiment procedure than LLE and derivatisation. Direct HS–SPME extraction was proposed for 3-SH and 3-SHA [37]. Apart from directly extracting thiols from wine, SPME has also been combined with derivatisation. For instance, five polyfunctional thiols were extracted by HS–SPME with on-fibre

derivatisation [27]. SPE has also been used for polyfunctional thiol extraction [37, 38]. Other than direct extraction through SPE cartridges [37], thiols can be extracted by on-cartridge derivatisation [29] or derivatised prior to SPE [20].

1.1.2.2. Identification and quantitation

Modern gas or liquid chromatography (GC, LC) is a useful and efficient tool for polyfunctional thiols analysis due to its tremendous separation power. The initial 'identification' of polyfunctional thiols was accomplished through GC–O, by comparing the retention time and sensory intensities between wine samples and reference polyfunctional thiols [4]. This procedure narrowed down the odorous zones of interest. Later, retention indices (RIs) of polyfunctional thiols were calculated and mass spectrometry (MS) was used for identifications [9, 16, 39]. As a common technique normally coupled with GC and LC for aroma analysis, MS gives accurate identification by providing the unique mass spectra of the targets. To enhance identifications, selected ion monitoring (SIM) and multiple reaction monitoring (MRM) modes are commonly applied for GC–MS [28, 33, 36, 37, 40] and LC–MS [20, 32, 34, 41], respectively.

For quantitation, several unlabelled internal standards (ISs) have been used, such as 4-methoxy-2-methyl-2-mercaptobutane [6, 42, 43], propyl thioacetate [36], and 6-mercaptohexan-1-ol [37, 40, 44]. The behaviour of different ISs for polyfunctional thiol quantitation was evaluated [28, 29]. Currently, the most effective and precise approach for identification and quantitation is stable isotope dilution assay (SIDA). SIDA uses deuterium labelled analogous as IS, which have almost identical physicochemical properties and chromatographic

behaviour as unlabelled analytes [45]. This offers superior identification and quantitation accuracy. Many labelled polyfunctional thiol analogues have been applied in quantitation, such as d_1-3-SHA [35, 43, 46], d_2-3-SH [29, 46, 47], d_5-3-SHA [29, 32, 48], d_{10}-4-MSP [20, 32, 48, 49], d_{10}-3-SH [20, 32, 49, 50].

1.1.2.3. Chiral analysis

Very limited scientific attentions have been paid to the chiral analysis of 3-SH and 3-SHA in wine. In one chiral GC–MS method developed to analyse 3-SH and 3-SHA enantiomers in wines [10], the separation of (*R*)- and (*S*)-form of 3-SH on the tested chiral GC column was achieved, but the resolution for the enantiomers of 3-SHA was unsatisfactory. Therefore, 3-SHA enantiomers were tentatively quantitated [10]. Another downside of this method is that per GC–MS run was more than two hours. A more efficient chiral separation method (only for 3-SH, *resolution* = 1.3 for 3-SH enantiomers) with half of the run time was demonstrated later [50]. The investigations on the distribution (**Table 3**) and evolution patterns of 3-SH and 3-SHA enantiomers in wines have been limited in a small number of wine samples using previously developed chiral methods [10, 50–52]. The distribution of (*R*)- and (*S*)- enantiomers of 3-SH in wines was almost even [10], but the enantiomers of 3-SHA were unequally present, which indicated different acetylation abilities from 3-SH enantiomers to 3-SHA enantiomers. Vintages and grape varieties were reported to have no influences on the ratios of enantiomers of 3-SH and 3-SHA, although the total amounts of 3-SH or 3-SHA varied in different grape varieties. However, *Botrytis cinerea* significantly affected the enantiomers of 3-SH (**Table 3**) [10].

Table 3.

Distribution of enantiomers of 3-SH and 3-SHA in wines [10].

Variety	Wine style	3-SH (*R*) : (*S*)	3-SHA (*R*) : (*S*)
Sauvignon	dry [1] (n = 8)	≈ 50:50	≈ 30:70
	sweet [2] (n = 1)	34:66	N.D.
Semillon	dry [1] (n = 7)	≈ 50:50	≈ 30:70
	sweet [2] (n = 6)	≈ 30:70	N.D.

[1] Made from healthy grape. [2] Made from grape affected by *Botrytis cinerea*.

N.D.: not detected.

1.1.3. Occurrence of polyfunctional thiols

With the analytical methods available, polyfunctional thiols have been assessed in a range of wines, including white, red, and rosé wines of many varieties. Generally, the presence of 3-SH and 3-SHA is more ubiquitous than 4-MSP; 3-SH is at the highest concentration and 4-MSP is normally present at the lowest amounts. The varietal differences are obvious too, with Sauvignon blanc wine containing relatively larger amounts of polyfunctional thiols than other varieties (**Table 4**).

Table 4.

Concentrations (ng/L) of polyfunctional thiols in wines of selected varieties.

Variety	3-SH	3-SHA	4-MSP
Sauvignon blanc [21]	3570±118 – 170±1	N.D. – 1012±167	9.6±1.5 – 24.8±1.3
Sauvignon blanc [35]	718 – 2262	19–1029	N.A.
Riesling [32]	172 – 1060	N.A.	N.A.
Pinot gris [32]	108 – 1021	N.A.	N.A.
Gewurztraminer [32]	96 – 1237	N.A.	N.A.
Chardonnay [53]	508 – 776	79.2 – 121	N.A.

N.D.: not detected. N.A.: not analysed.

1.2. Biogenesis of polyfunctional thiols

1.2.1. Thiol precursors

Polyfunctional thiols are essentially absent in grapes (only around 100 ng/L found in grapes) or juices [32], but become available after fermentation [20]. This enrichment phenomenon of polyfunctional thiols from grape to wine suggested the presence of precursors of thiols in grapes. Until now, the identified cysteinylated and glutathionylated conjugates, and unsaturated carbonyl compounds have been nominated as thiol precursors (**Figure 1**).

1.2.1.1. Cysteinylated precursors

Cysteinylated precursors are the first type of polyfunctional thiol precursors identified in grapes [54]. When treating a crude grape must extract with a cysteine *β*-lyase preparation (*E1* in **Figure 1**), significant elevations of concentrations of polyfunctional thiols were observed by GC–FPD, which led to the hypothesis that *S*-cysteine conjugates could possibly act as thiol precursors [54]. The *in vitro* release of polyfunctional thiols in a model medium supplemented with synthesised *S*-cysteine conjugates (Cys-3-SH, Cys-4-MSP) was demonstrated under the same enzymatic condition. Finally, *S*-cysteine conjugates of polyfunctional thiols have been identified in grapes by GC–MS in the form of their trimethylsilylated derivatives which confirmed their presences [54].

1.2.1.2. Glutathionylated precursors

Further study revealed glutathionylated conjugates as another type of polyfunctional thiol precursors [52]. The concentrations of Cys-3-SH in grape must increasing by 49–537% after being loaded through an immobilised γ-glutamyltranspeptidase (γ-GGT, *E2* in **Figure 1**) column [55], so the presence of glutathione conjugated 3-SH in the must was suggested. Furthermore, *S*-3-(hexan-1-ol)-glutathione (Glut-3-SH) in Sauvignon blanc must was tentatively

Cys-4-MSP: *S*-4-(methylpentan-2-one)-L-cysteine;
Cys-3SH: *S*-3-(hexan-1-ol)-L-cysteine;
Glut-4-MSP: *S*-4-(methylpentan-2-one)-L-glutathione;
Glut-3-SH-al: *S*-3-(hexanal)-L-glutathione;
Glut-3-SH: *S*-3-(hexan-1-ol)-L-glutathione;
CysGly-3-SH: *S*-3-(hexan-1-ol)-L-cysteine-glycine;
GluCys-3-SH: *S*-3-(hexan-1-ol)-L-glutamic acid-cysteine.
Glut-3-SH-SO_3: *S*-3-(1-hydroxyhexane-1-sulfonate)- L-glutathione;
E1: β-lyase;
E2: γ-glutamyltranspeptidase;
E3: carboxypeptidase;
E4: alcohol dehydrogenase (suggested);
E5: acetyltransferase.

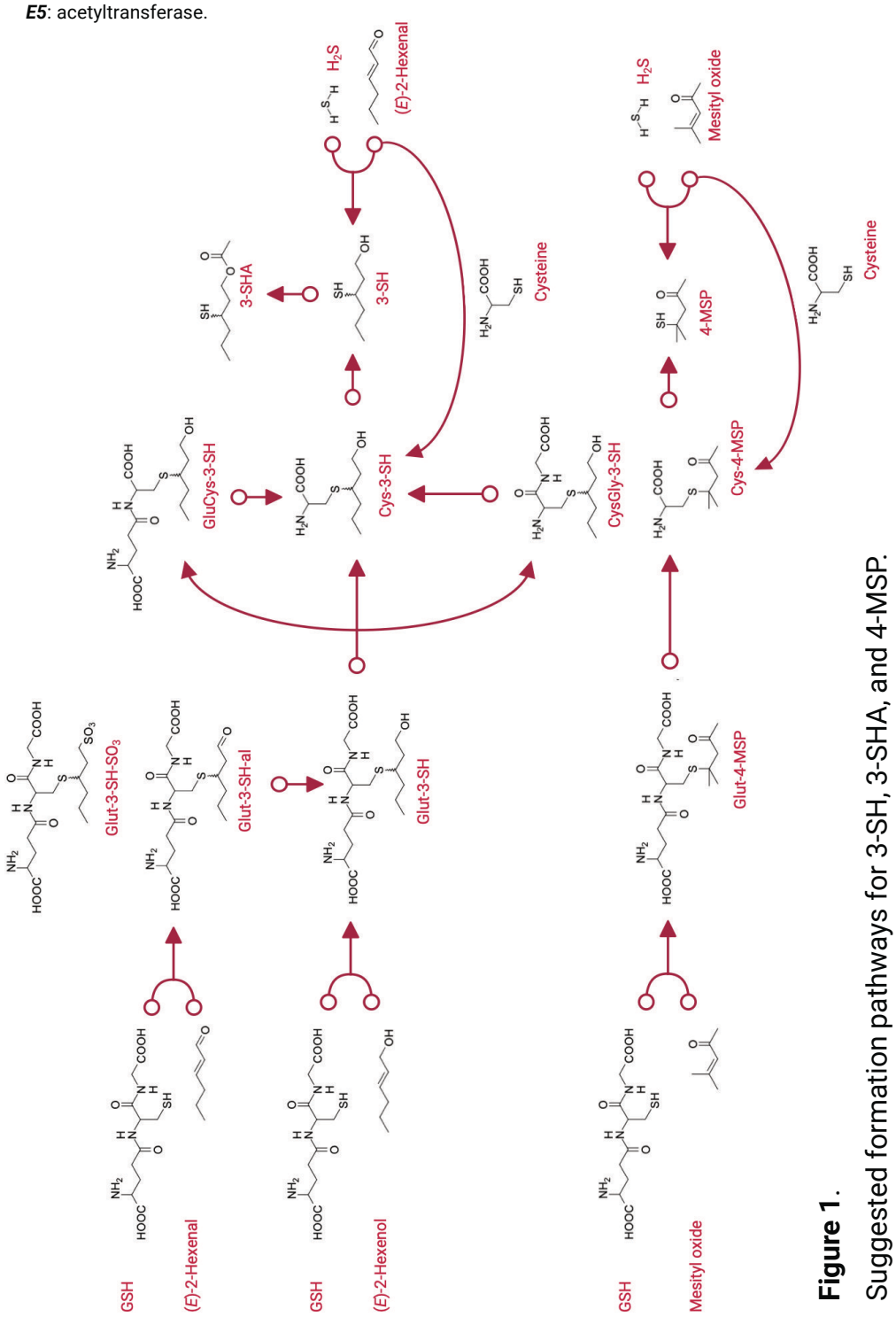


Figure 1.
Suggested formation pathways for 3-SH, 3-SHA, and 4-MSP.

identified by high resolution liquid secondary ion mass spectrometry (HR–LSIMS). The evidence of 4-*S*-glutathionyl-4-methylpentan-2-one (Glut-4-MSP) as another glutathionylated thiol precursor in Sauvignon blanc juice was discovered later [56].The identification of Glut-4-MSP was conducted comparing synthesised Glut-4-MSP as standard and Sauvignon blanc juice spiked with Glut-4-MSP by HPLC–MS/MS [56].

The intermediates from Glut-3-SH to Cys-3-SH, dipeptides CysGly-3-SH and GluCys-3-SH, have been more recently identified in Sauvignon blanc juice [57] and fermented grape juice model medium respectively, while the identification of GluCys-3-SH was tentatively [4] and still needs to be confirmed.

1.2.1.3. Carbonyl precursors

Carbonyl compounds constitute another type of thiol precursors. Based on structure similarity to 3-SH and 4-MSP, (*E*)-2-hexenal and mesityl oxide were suggested as the putative precursors of 3-SH and 4-MSP [58]. This hypothesis was tested by adding synthesised d_8-(*E*)-2-hexenal and d_{10}-mesityl oxide as deuterated analogues of (*E*)-2-hexenal and mesityl oxide to a Melon B. must. After fermentation, d_8-3-SH and d_{10}-4-MSP were detected in final wine by GC ion trap mass spectrometry (GC–ITMS), so (*E*)-2-hexenal and mesityl oxide were concluded as another two new thiol precursors, possibly forming 3-SH or 4-MSP indirectly through 1,4-addition of cysteine, or in a direct way through 1,4-addition of H_2S [58]. The corresponding alcohol of (*E*)-2-hexenal, (*E*)-2-hexenol, could react with H_2S to form 3-SH in the early stage of fermentation [24]. These studies have shown that the formation of polyfunctional thiols related to the unsaturated carbonyl compounds. Later, Sauvignon blanc grapes were spiked with deuterium-labelled aldehyde d_8-(*E*)-2-hexenal prior crushing, and d_8-Glut-3-SH-al and d_8-Glut-3-SH were identified in the juices by HPLC–MS/MS [59], which suggested that

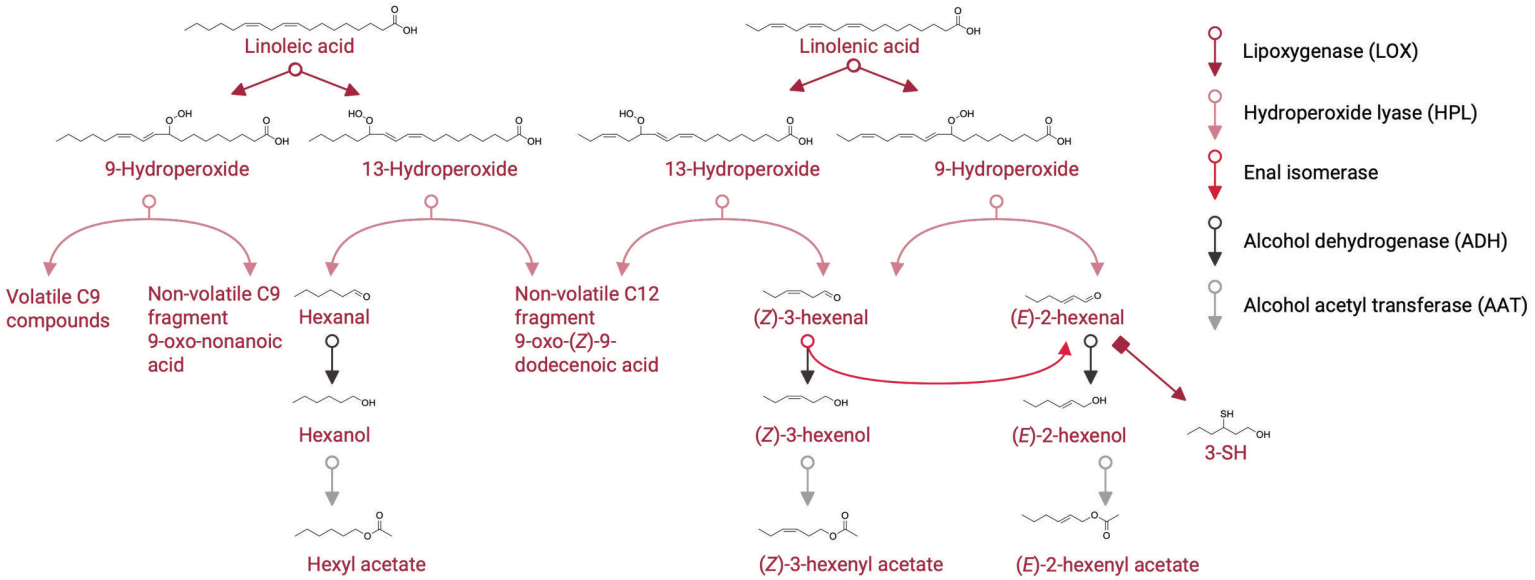


Figure 2.

Potential link between degradation pathway of fatty acids and formation pathway of varietal thiols.

(*E*)-2-hexenal involved with polyfunctional thiol formation by incorporating with GSH to generate Glut-3-SH-al and then to Glut-3-SH [59]. The confirmed identification of Glut-3-SH-al in Sauvignon blanc juice was recently reported [60], through spiking fermentation experiment and ultra HPLC–Fourier transform mass spectrometry (UHPLC–FTMS). Glut-3-SH-SO_3 has been proposed as a new polyfunctional thiol precursor in the same study [60]. Also, the formation of Glut-3-SH has been rationalised as the result of glutathione *S*-transferases induced by (*E*)-2-hexenal [61]. These studies pointed out the importance of unsaturated C6 in thiol biogenesis. As for the origin of unsaturated C6 in grapes, they are degraded from the unsaturated fatty acids through lipoxygenase/hydroperoxide lyase (LOX/HPL) pathway (**Figure 2**). Regarding the connective roles of (*E*)-2-hexenal and (*E*)-2-hexenol both in LOX/HPL pathway and 3-SH formation (**Figure 2**), it is likely that there are some other missing carbonyl components from certain branched chain unsaturated fatty acids responsible for 4-MSP formation, but this hypothesis has never been verified.

1.2.2. Analysis of thiol precursors

1.2.2.1. Extraction

For extracting polyfunctional thiol precursors from grape, must, juice or wine matrix, various methods using several techniques, such as LLE, affinity chromatography (AC), or SPE have been developed.

1.2.2.1.1. Traditional column chromatography

Column purification was used for the extraction of cysteinylated precursors. In the early proposed method, Sauvignon blanc must (45 L) was firstly cleaned by partition chromatography (C_{18} silica). The purified fraction was loaded on a basic anion column and the un-retained fraction was collected, which was subjected to a chelating Sepharose 4B column (containing Cu^{2+}, capable of fixing tryptophane and cysteine) and washed by hydrochloric acid to obtain polyfunctional thiol precursors. Before analysis, thiol precursors were derivatised with a mixture of *N*,*O*-bis(trimethylsilyl)trifluoroacetamide, trimethylchlorosilane, and pyridine (3:1:3) to obtain Cys-4-MSP and Cys-3-SH derivatives [54]. Later, a simplified method was developed [62]. This method involved adding a relatively smaller amount of Sauvignon blanc must (20 mL) into a reaction medium and loaded them on a DEAE column [62]. This method did not require column purification or derivatisation. Another extraction method only used 500 µL of sample and a single Chelating Sepharose 4B column for extraction [63]. Based on this published method [63], diastereomers of Cys-3-SH in grape must were analysed with modifications (column pH adjustment and derivatisation) [51]. In addition to cysteinylated precursors, column chromatography has also been used to extract glutathionylated precursors, for instance, the reported Glut-4-MSP

extraction from Sauvignon blanc juice through C18 sorbent and C18 column [64].

1.2.2.1.2. SPE

SPE methods, with advantageous sample preparation procedures than column chromatography, have also been applied for the extraction of thiol precursors. For instance, for Cys-3-SH extraction, only a small amount of sample (8 mL) was required when using Supelclean Envi-18 SPE cartridge [65]. Another procedure used Strata SDB-L SPE cartridge for the extraction of diastereomers of both Cys-3-SH and Glut-3-SH [41], CysGly-3-SH [57] and GluCys-3-SH [48]. For Glut-3-SH, the use of Dowex 50WX4-100 cation exchange resin followed by C18 cartridge was proposed [26], which only needed 1200 µL centrifuged sample for extracting 4-MSP and 3-SH precursors.

1.2.2.1.3. Other extraction methods

LLE has been demonstrated for the extraction of Cys-3-SH and Glut-3-SH from grape leaf, stem, skin, juice, and seed [66]. Briefly, the pulverized sample was macerated in methanol water (10:90) buffer containing 0.1 % formic acid for 16 hours. After this, the supernatant was filtered ready for instrument analysis.

1.2.2.1.4. No extraction required

Recently developed methods for thiol precursor analysis did not need any thiol precursor extraction procedures [44, 67, 68].

1.2.2.2. Identification and quantitation

To identify potential polyfunctional thiol precursors, the authentic reference standards are required, but these compounds were often commercially unavailable at the stage of discovering new compounds, so chemical synthesis is necessary. Apart from the identification and quantitation purpose, those synthesised precursors can also be used as substrate tracers in fermentation trials to investigate of the conversion of precursors to free thiols, as well as to evaluate the effects of winemaking practices. To date, several studies have reported the synthesis of polyfunctional thiol precursors and their labelled analogues (reviewed in references [5, 69, 70]). Generally, polyfunctional thiol precursors were synthesised through Michael addition of Boc-protected cysteine or glutathione to the corresponding α,β-unsaturated carbonyl compounds [51, 52]. The identification and quantitation of polyfunctional thiol precursors are usually achieved by GC–detectors or HPLC–MS. Polyfunctional thiol precursors are non-volatile, therefore derivatisation (perfluoroacylation [51], silylation [54]) is required prior to GC. In comparison, HPLC–MS is more suitable for polyfunctional thiol precursor analysis. Meanwhile, the sample preparation protocol for HPLC–MS analysis is simpler and easier, being achieved by SPE purification [41] or by simple centrifugation and filtration [67, 71].

1.2.3. Occurrence of thiol precursors

Once the identification and quantitation of polyfunctional thiol precursors have been achieved with the synthesised reference standards and instrument analysis, polyfunctional thiol precursor concentrations have been largely assessed in grapes, juices, musts, and wines (**Table 5**, **6**).

Table 5.

Concentrations (μg/L) of polyfunctional thiol precursors in grape skin, juice, and whole berry of various varieties.

Variety	Cys-4-MSP	Cys-3-SH	Glut-4-MSP	Glut-3-SH
	skin : juice			
Sauvignon blanc	20:80 [72]	50:50 [72], 78:28 [73]	81:19 [73]	57:43 [73]
Koshu	N.A.	N.A.	N.A.	50:50 [66]
Cabernet Sauvignon	N.A.	»60:40 [63]	N.A.	N.A.
Merlot	N.A.	»60:40 [63]	N.A.	N.A.
Melon B.	N.A.	skin only [73]	skin only [73]	28:72 [73]
	concentration in whole berry [67]			
Sauvignon blanc	12.6±1.4	174±7.1	7.7±1.3	1557±86
Gewürztraminer	8.0±1.5	89.2±6.3	6.6±0.8	1154±56
Muscat	N.D.	157±7.6	8.3±0.9	1673±71

N.A.: not analysed. N.D.: not detected.

1.2.3.1. In grape

Thiol precursors varied in quantity in grapes. In Sauvignon blanc grapes, the seeds only contained negligible amount of thiol precursors, but predominant levels of precursors were found in skin and juice [72]. About 80% Cys-4-MSP was found in juice, while Cys-3-SH equally distributed in juice and skin. The concentrations of Glut-3-SH and Cys-3-SH were also studied in leaf, stem, seed, skin, and juice of *Vistis vinifera* L. cv Koshu during grape ripening [66]. Similarly, Glut-3-SH and Cys-3-SH were barely present in grape seed and stem in the whole period, but found in skin, juice and leaf, with the highest amounts in leaf.

Skin and juice contained similar levels of Cys-3-SH regardless ripening stage, but higher concentrations of Glut-3-SH were observed in skin than juice [66]. In Cabernet Sauvignon and Merlot, around 60% of Cys-3-SH presented in skin [63]. In Melon B., Cys-3-SH and Glut-4-MSP were found to be in the skin and Glut-3-SH occurred in both juice (72 %) and skin (28 %) [73] (**Table 5**).

1.2.3.2. In juice and wine

In contrast to concentrations of polyfunctional thiols in wine in ng/L range (**Table 4**), thiol precursors present in much larger quantities (around µg/L range). **Table 6** lists the quantitative data of Cys-4-MSP, Cys-3-SH, Glut-4-MSP, Glut-3-SH, and CysGly-3-SH. Sauvignon blanc juice and wine contained higher concentrations of polyfunctional thiol precursors than other varieties. However, dramatically high concentrations of Glut-3-SH (1200 – 9855 µg/L) was recently reported in Spanish Merlot must [71]. Generally, cysteinylated and glutathionylated conjugates were the two major types of precursors, and 3-SH precursors were higher than 4-MSP precursors which was in accord with the distributions of 3-SH and 4-MSP (**Table 4**). Glutathionylated precursors were presented in larger amounts than cystinylated precursors in juice and wine from Australia [41] and New Zealand [21]. However, other studies found that cysteinylated precursors were more abundant [26, 66, 74–76]. Stereochemically, (*S*)-diastereomers of polyfunctional thiol precursors dominated over (*R*)-diastereomers [41]. The concentrations of polyfunctional thiol precursors in juice and wine depended on many variables, and the effects of terroir and winemaking on polyfunctional thiol precursors were discussed in the following context.

Table 6.

Concentrations (µg/L) of polyfunctional thiol precursors in grape, juice, must, and wine of various verities.

	Variety	Cys-4-MSP	Glut-4-MSP	Cys-3-SH[1]	Glut-3-SH[1]	CysGly-3-SH[1]
Juice	Sauvignon blanc (NZ)			7.3–111 [21]	22–541 [21]	0–180[2] [74]
	Sauvignon blanc (AU)			*S* 14–41 *R* 7–14 [41]	*S* 210–556 *R* 35–140 [41]	10–28.5 [57]
	Sauvignon blanc (FR)	2.61–6.23 [75]	0.03–4.30 [75]	15.53–30.69 [75]	1.35–7.54 [75]	
	Chardonnay			*S* 4–22 *R* 3–16 [41]	*S* 77–342 *R* 34–175 [41]	
	Riesling			*S* 2–8 *R* 4–10 [41]	*S* 75–219 *R* 22–56 [41]	
	Pinot Grigio			*S* 13–16 *R* 10–11 [41]	*S* 266–392 *R* 72–83 [41]	
	Melon B.	1.07–3.83 [75]	N.D. [75]	<1.05 [75]	< 0.20 [75]	
	Gewürztraminer	0.54–0.82 [75]	0.10–0.18 [75]	58.25–58.28 [75]	2.29–2.90 [75]	
Must	Sauvignon blanc			7.9–35.5 [26]	1.3–7.5 [26]	
	Riesling			15.1–30.8 [26]	0.7–2.0 [26]	
	Gewürztraminer			52.9–65.2 [26]	5.6–7.1 [26]	
	Petite Arvine			31–85 [65]		
	Lugana			36.1–363.0 [44]	7.1–173.7 [44]	
	Merlot	LOQ–0.58±0.1 [71]	LOQ–0.25±5.9 [71]	3.10±0.9–558±7.1 [71]	1200±0.9–9855±9.0 [71]	
Wine	Sauvignon blanc			*S* 26–35 *R* 13–15 [41]	*S* 295–392 *R* 79–94 [41]	
	Riesling			*S* 4–16 *R* 1–12 [32]	*S* 18–315 *R* 13–99 [32]	
	Pinot Grigio			*S* 19 *R* 11 [41]	*S* 241 *R* 82 [41]	
	Chardonnay			*S* 8 *R* 7 [41]	*S* 52 R 37 [41]	
	Muscat			*S* 3 – 77 *R* 2–30 [32]	*S* 19–405 *R* 12–98 [32]	
	Pinot gris			*S* LOQ–53 *R* 1–26 [32]	*S* 57–194 *R* 21–55 [32]	
	Gewurztraminer			*S* LOQ–43 *R* LOQ–17 [32]	*S* 18–315 *R* 13–99 [32]	

NZ: New Zealand. AU: Australia. FR: France. [1] Data represent the concentrations of combined diastereomers, unless indicated as *S*: (*S*)-form; *R*: (*R*)-form. LOQ: limit of quantitation. [2] Estimated data adapted from figure.

The correlation between polyfunctional thiol precursors and free thiols in resulting wines has been investigated on 3-SH, and no correlation being noticed either in small-lot fermentation [21] or commercial scale fermentation [32]. Another study on 55 Sauvignon blanc juices fermented under controlled conditions reported no total or individual correlation between 3-SH precursors in the juices and 3-SH in the wines [74]. In contrast, positive correlations between 3-SH precursors in juices and 3-SH in wines were noted for Koshu grape fermentation [66]. 3-SHA is formed through the acetylation of 3-SH. In a fermentation study, Cys-3-SH or Glut-3-SH was spiked into synthetic media and 3-SH and 3-SHA were quantitated after fermentation [42]. The concentrations of 3-SHA using two substances (Cys-3-SH, Glut-3-SH) showed no significant differences, but the concentrations of 3-SH varied, which indicated 3-SHA was not regulated by 3-SH. Also, production of 3-SHA could be related to the forms of precursors [60]. However, another fermentation trial using yeast deletant mutants observed a strong correlation between 3-SH and 3-SHA across all fermentations [48].

1.2.4. From precursors to thiols

Polyfunctional thiols are formed from their precursors during fermentation. Many studies have investigated the “real contribution” of proposed polyfunctional thiol precursors to free thiols. The conversion yields of thiol precursors to polyfunctional thiols have been measured in both model medium and real grape juice conditions. To accurately monitor the changes of thiol precursors and free thiols during fermentation, synthesised thiol precursors or labelled precursors (deuterated isotopes) were spiked to model medium or juice at known amounts. The supplemented labelled substances acted as tracers to track the formation of

Table 7.

Conversion yields (%) from putative precursors to free thiol 3-SH and 4-MSP.

Thiol	Precursor	Fermentation condition			Conversion yield[1]	Ref
		Tracer	Medium	Yeast strain		
3-SH	Cys-3-SH	N.S.	juice	VL3C	< 10	[63]
		(*R*), (*S*)-Cys-3-SH	SCD liquid media	AWRI 1655	1.0 (*R*), 1.1 (*S*)	[50]
		d_8-Cys-3-SH	synthetic medium	ES1, ES2	< 1	[77]
		Cys-3-SH	synthetic medium	VIN13 (*CSL*1)	14	[52]
		Cys-3-SH	synthetic medium	VL3	0.5	[42]
	Glut-3-SH	d_2/d_3-Glut-3-SH	juice	VIN13	4.4	[26]
		(*R*)-Glut-3-SH	synthetic medium	VIN13 (*CSL*1)	3	[52]
		Glut-3-SH	synthetic medium	VL3	0.5	[42]
		Glut-3-SH	synthetic medium	BY4742 and its mutants	1.05[3]	[48]
	Glut-3-SH-Al	Glut-3-SH-Al	juice & synthetic medium	X5	0.40–0.45	[60]
	Glut-3-SH-SO_3	Glut-3-SH-SO_3	juice & synthetic medium	X5	0.38–0.42	[60]
	(*E*)-2-hexenal	d_8-(*E*)-2-hexenal	must	KD yeast	10	[58]
		d_8-(*E*)-2-hexenal	must	ES1, ES2	N.P.	[77]
		(*E*)-2-hexenal	synthetic medium (MS 300)	VIN13, X5, V1116	58.8	[24]
	(*E*)-2-hexenol	(*E*)-2-hexenol	synthetic medium (MS 300)	VIN13, X5, V1116	9.4	[24]
	C6 compounds[2]	C6 compounds[2]	juice & synthetic medium	X5	0.004–0.01	[60]
4-MSP	Cys-4-MSP	Cys-4-MSP	synthetic medium	EC1118, X5, F15^{OX}	0.2, 1, <67	[79]
	Glut-4-MSP	d_{10}-Glut-4-MSP	must	VIN13	0.3	[78]
		Glut-4-MSP	synthetic medium	BY4742 and its mutants	0.5[3]	[48]

^{ox}: overexpression of full-length *IRC7*. SCD: synthetic complete dextrose. YPS: yeast peptone sucrose. N.S.: no spiking. [1] molar conversion (%). [2] (*E*)-2-hexenal + (*E*)-2-hexenol. [3] conversion yield on wild-type yeast. N.P.: not provided.

polyfunctional thiols from their precursors. For 3-SH production, 3-SH precursors (d_8-Cys-3-SH and d_8-(*E*)-2-hexenal) were spiked into Sauvignon must [77], and through fermentation, only less than 1% of Cys-3-SH was converted to 3-SH, accounting for less 10% of total 3-SH production. Meanwhile, 5 ng/L of 3-SH was formed from d_8-(*E*)-2-hexenal, representing 0.1% of 3-SH final concentration. For 4-MSP production, synthesised d_{10}-Glut-4-MSP [78] was added into Sauvignon blanc and synthetic media, and after fermentation, about 0.3% of Glut-4-MSP contributed to the final amounts of 4-MSP, explaining only 20% of 4-MSP in Sauvignon blanc wines. During yeast fermentation, glutathionylated precursors can be degraded to cystinylated precursors. For instance, about 20% of Glut-3-SH was degraded to Cys-3-SH within the 24 h of inoculation of wild-type BY4742 yeast, as well as 0.25% of GSH-3-SH to GlutCys-3-SH, and 0.5% yield rate of Glut-4-MSP to Cys-4-MSP [48]. The reported conversion rates of precursors to polyfunctional thiols are listed in **Table 7**.

As seen, despite the variations of fermentation conditions, the overall conversion rates from polyfunctional thiol precursors to free thiols are around 10%, with the exception for the data obtained using genetically modified yeasts [38, 79]. Clearly, large amounts of thiol precursors in the juice remain non-converted during fermentation [42, 77].

During alcoholic fermentation, the availabilities of polyfunctional thiol precursors utilised by the yeast were different. Cys-3-SH was reported to be more easily metabolised at an earlier stage than Glut-3-SH [42, 66]. In synthetic medium, four times higher Cys-3-SH than Glut-3-SH was converted to 3-SH during alcoholic fermentation [66]. The difference in the availability of polyfunctional thiol precursor uptake by the yeast may be related to their different conversion pathways (**Figure 3**).

GAP1 deletion yeast strain liberating significantly lower concentrations of 3-SH and 3-SHA in synthetic fermentation condition demonstrated GAP1p was the transporter responsible for Cys-3-SH uptake on synthetic medium [80]. However, *GAP1* was suggested not associated with the uptake of Cys-4-MSP [79]. Opt1 was the main transporter for Glut-4-MSP and Glut-3-SH into the yeast [48]. The important role of Ecm38 involving with 3-SH release from Glut-3-SH was also identified in the same study [48]. *IRC7* was believed to be accountable for converting Cys-3-SH and Cys-4-MSP to free thiols [81].

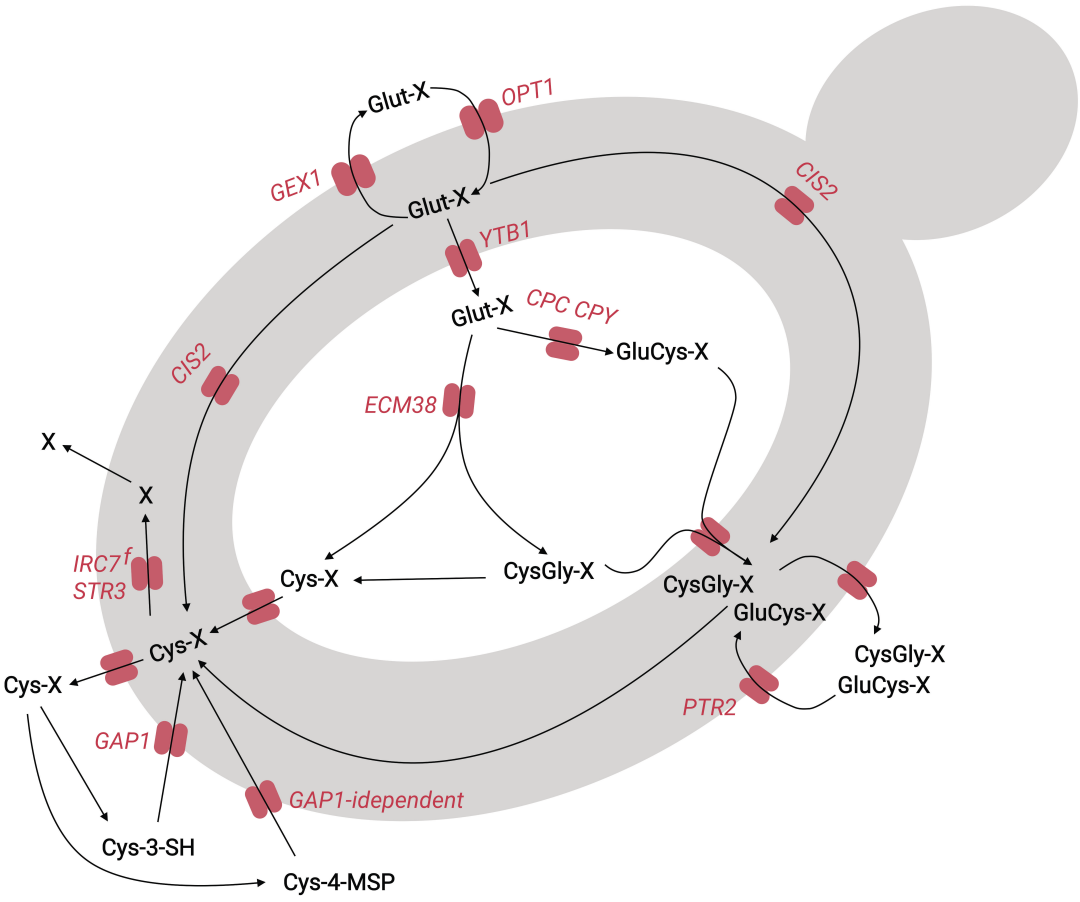


Figure. 3. Polyfunctional thiol precursor metabolism pathways in yeast [48,79].

1.3. Impacts of viticulture and oenology

1.3.1. Terroir

Polyfunctional thiol precursors are influenced by grape cultivation regions and the related environmental conditions. The evolutions of Glut-3-SH and Cys-3-SH of Koshu grapes from different vineyards with similar soil compositions and cultivation practices, were reported to be influenced by cultivation locations. Water [82, 83], nitrogen status of vine [82], ultraviolet irradiation [83], and biological stimulation during grape ripening [83] all up-regulated polyfunctional thiol precursors. Another study compared the concentrations and distributions of thiol precursors of Sauvignon blanc grape sourced from different vineyards in France and their results demonstrated that the productions of Cys-3-SH was enhanced in grapes cultivated under the Mediterranean dry climate [73]. Although moderate water status seemed to improve the formation of cysteinylated precursors [73], severe water deficit was proven to limit their production [82]. No correlation between nitrogen status and cysteinylated precursors was observed [82]. It was suggested that either nitrogen deficiency or high vine nitrogen should be avoided [82]. Botrytisation considerably elevated the Cys-3-SH in Sauvignon and Semillon grapes [84]. Another factor, clone type played a role on polyfunctional thiol precursors [57]. The diastereomers of Cys-3-SH, CysGly-3-SH and Glut-3-SH in five Sauvignon blanc clones grown in a single vineyard were different [57]. Clone has also been depicted to influence 3-SH and 3-SHA concentrations in the Sauvignon blanc wines by a recent study [85].

1.3.2. Grape ripening and harvest date

Polyfunctional thiol precursors in grape berries accumulated during ripening. In a study on Sauvignon blanc ripening, the evolutions of cysteine conjugates (Cys-3-SH and Cys-4-MSP) differed across different vintages and types of

precursors [82]. For Koshu grape, Cys-3-SH and Glut-3-SH accumulated from 8 post flowering weeks (PFWs), peaked at 16 PFWs in both berries and skins, and then decreased afterwards [66]. From veraison to harvest, the variations of Cys-3-SH changed slightly, but the fluctuation on Cys-4-MSP was observed [82]. Apparent increases of Cys-3-SH and Glut-3-SH have been noticed in five Sauvignon blanc clones ripening period [32]. Trace amounts of 3-SH (100 ng/L) in Sauvignon blanc juice has been detected for the first time (around 100 ng/L) [32]. In another study, the same authors investigated 3-SH precursors (Cys-3-SH, Glut-3-SH, and CysGly-3-SH) in Sauvignon blanc from veraison to post commercial maturity point, and they observed precursors accumulated to maturation and declined after sugar level reaching 24° Brix [86]. Generally, grapes with higher total soluble solid (TSS) contained higher concentrations of thiol precursors, especially for cysteinylated precursors [68].

Grapes of different harvest dates had different ripening degrees. In Melon B. and Sauvignon blanc grapes harvested on three different dates with one week interval between every two consecutive dates , concentrations of Glut-3-SH and Cys-3-SH were higher in late harvest grapes, while the levels of Glut-4-MSP and Cys-4-MSP fluctuated [76]. In Melon B., only Cys-4-MSP varied at different harvest dates. These evidence indicated the variety-dependent pattern of harvest date impact on the polyfunctional thiol precursors. Harvesting timing also influenced 3-SH precursors [53]. 3-SH precursors accumulated at early morning and declined during the day in grapes, and wines produced from early morning harvested grapes contained higher levels of 3-SH and 3-SHA [53].

1.3.3. Harvest operations

Grape harvesting process could affect polyfunctional thiol precursors.

Comparing hand and machine harvesting techniques, machine harvesting enhanced the polyfunctional thiol precursors (about 70% and 65% increases of Glut-3-SH and Cys-3-SH [59] in Australian Sauvignon blanc grapes). Berry damage by machine harvesting was believed to boost the formation of polyfunctional thiol precursors [59]. Similar results were shown by another study in which 3 out of 5 studied juices from machine-harvested grapes were found to contain higher Cys-3-SH and Glut-3-SH [21]. Regarding to free thiols, higher levels of 3-SH and 3-SHA were found in wine fermented with machine-harvested grapes [21, 87]. As another harvest-related operation, transportation can be viewed as extended harvesting simulation [22]. The effect of transporting of machine-harvested Sauvignon blanc on 3-SH has been investigated at commercial scale [59]. After transportation for 12 h, the concentrations of Cys-3-SH and Glut-3-SH saw dramatic increases (around 10-fold and 2-fold, respectively) [59], and CysGly-3-SH went up by 20-fold compared with non-transported samples [57]. Both of harvest and transport operations influenced the composition of juices. Berry damage happened at these stages (which potentially triggered the production of C6 aldehydes from fatty acids (**Figure 2**)) affected the formation of polyfunctional thiol precursors and the production of polyfunctional thiols (**Figure 1**).

1.3.4. Pre-fermentation

After harvested from vineyards and transported to winemaking facilities, grapes are ready for fermentation. Including pressing and maceration, many pre-fermentative winemaking variables could influence the polyfunctional thiol precursors and free thiols.

1.3.4.1. Pressing

Pressing affected the extraction of polyfunctional thiol precursors from the berry to the juice. The effect of pressing on Cys-3-SH was evaluated in three Sauvignon blanc juices during winey press cycles, and 3.8 to 4.4 times higher of Cys-3-SH was found in pressed juices than in free run juices [88]. Concentrations of Cys-3-SH and Glut-3-SH of Sauvignon juices increased when higher pressure applied (1 bar) [21]. The higher concentrations of thiol precursors in heavier pressed operation was theorised because of higher extraction of polyfunctional thiol precursors [32, 73]. The extraction depended on grape origins and Cys-3-SH was more inclined to be extracted than Glut-3-SH in Sauvignon blanc and Melon B. [73]. This was expected because precursors were located in the grape skin and juice (**Table 5**), which were more easily extracted to juice under high pressures.

Although pressed juices held higher polyfunctional thiol precursors, the wines made from them somehow exhibited lower amounts of polyfunctional thiols [21, 89]. However, contrary results were reported by other study [73], but different pressing protocols were used in these studies (winery pressing [73, 89], 80 L water bag press [21]). More research is needed to explain the disagreement. For each polyfunctional thiol, 3-SH and 3-SHA were more likely to be affected by pressing, and instead, 4-MSP was more stable during and pressing [21]. This was more likely related to the distribution and concentration of their precursors in berries, but this is still unclear.

1.3.4.2. Maceration

Maceration affected both polyfunctional thiol precursors and free polyfunctional thiols. The concentration of Cys-3-SH continuously increased (62%) during skin-contact maceration in Merlot must, and the same trends were observed for Cabernet Sauvignon and Cabernet franc [72]. The increase of cysteinylated precursors (30% for Cys-4-MSP and 50% for Cys-3-SH) was observed after a 19 h skin contact [72]. Moreover, longer maceration duration increased the content of Cys-3-SH [88]. As could be expected, maceration temperature played an important role, with higher temperature promoting the concentration of Cys-3-SH during maceration. Thiol precursors in must macerated at 20 °C increased 59% than that at 10 °C [63]. The better extract ability of polyfunctional thiol precursors was expected to be achieved at higher temperature and longer time, as they favoured the diffusion of precursors from grape solids to juice [63, 88]. However, Cys-3-SH of musts from 20 and 25 °C skin contact conditions showed no difference and other compounds were also affected during maceration [63]. Although lower maceration temperature limited the content of Cys-3-SH in juice [72], cryogenic maceration significantly increased 3-SH and 3-SHA contents in wine [87]. These above results indicate the importance of a balanced maceration condition on thiol-related quality of wine.

1.3.4.3. Oxidation

From harvest, grape handling, crush, to maceration, grapes and juices experienced various chemical or enzymatic changes. Since oxidation status of grapes or juices is of great importance to such changes, the effects of oxidation status of grapes and juices on polyfunctional thiol precursors and free thiols are presented.

Once grape bunches were picked from the vines into a picking bin, the influence of oxidation status began. The oxidative status of grapes and juices has been investigated by many studies, and the oxidative status were controlled through the addition of O_2 or SO_2. O_2 addition experiment on Sauvignon blanc and Melon B. juices improved the production of Glut-3-SH but Cys-3-SH, Cys-4-MSP, and Glut-4-MSP were not much affected by oxygen content [76]. The elevation of Glut-3-SH was likely caused by the Michael addition between GSH and (*E*)-2-hexenal derived from fatty acids [76]. Effects of adding SO_2 of different doses into grapes and juices showed that higher SO_2 reserved more 3-SH and 3-SHA, with optimal SO_2 dose at 120 mg/kg, and the acetylation of 3-SH to 3-SHA was limited when SO_2 was at 300 mg/kg [22]. One explanation of this was that the added SO_2 interacted with polyphenol quinones, therefore reserved the polyfunctional thiols consumed by quinones, and this was in accord with the investigation on kinetics between polyfunctional thiols and *o*-quinones [90]. Moreover, the addition of SO_2 protecting the participation of GSH in polyphenol oxidation [91], and GSH can form Glut-3-SH-al and Glut-3-SH [59, 76], therefore it was expected that more polyfunctional thiol precursors would be produced with the presence of SO_2. However, SO_2 at 500 mg/L (highest dose treatment in this study) lowered Cys-3-SH [59] as SO_2 on one hand, inhibited the formation of (*E*)-2-hexenal from oxidation of fatty acids (**Figure 2**) or bound the (*E*)-2-hexenal compounds, and on the other hand inhibited the activities of enzymes involving with degradation of Glut-3-SH. Therefore the excessive SO_2 (500 mg/L) also led to lower levels of Glut-3-SH, which was justified by the low availability of (*E*)-2-hexenal and enzymes (i.e. enzymes responsible for production of Glut-3-SH).

The inhibition of grape enzymes responsible for formation of Glut-3-SH in berries during post-harvest period was investigated through snap-freezing and protein-precipitating [86].

The effect of oxidative maceration has also been examined. Higher oxidative maceration status increased the concentrations of Glut-3-SH in Muller-Thurgau and Sauvignon blanc juice, and at the same time GSH diminished in oxidative juices [92]. Again, the increase of Glut-3-SH in oxidative status was explained as appropriate amounts of oxygen were required for the formation of (*E*)-2-hexenal from fatty acid degradation pathways, whose availability affected the production of Glut-3-SH [92]. In this study, the trend of precursors was consistent with previous studies [92], but polyfunctional thiols contents were lower in wines made from hyper-reductive winemaking. Also, the loading and pressing protocols with different oxidative status influenced polyfunctional thiols and their precursors [44].

1.3.5. Fermentation

1.3.5.1. Yeast

During alcoholic fermentation, yeast liberates polyfunctional thiols from their precursors, and such thiol-releasing abilities of yeasts have been largely investigated [36, 93–97]. Selected examples of yeasts (*Saccharomyces cerevisiae*) affecting polyfunctional thiol production are presented in **Table 8**.

Example 1 was conducted in a synthetic medium, in which CWY8 yeast strain elevated 4-MSP concentration up to 138 times higher than that in control medium in which CWY 1-6 yeast strains only liberated minor amounts of 4-MSP [36]. The effects of yeast on 4-MSP production were seen for other polyfunctional thiols. For instance, 3-SH and 3-SHA were also modulated by commercial yeasts in Sauvignon blanc juice fermentation process [97]. With regard to 4-MSP and 3-SH, the thiol-releasing ability from the yeast seemed more important.

Table 8.

Selected examples of studies on the effects of yeasts on polyfunctional thiols.

Thiols	Yeast strains	Medium	Thiol releasing ability
4-MSP	CWY1-CWY8	Synthetic [36]	CWY8 (138-fold increase) > CWY1-7
3-SH 3-SHA 4-MSP	NT116, VIN7, VL3, L2056, QA23, VL3, X5	Sauvignon blanc Juice [97]	VIN7 showed highest increase on 4-MSP; VIN13 elevated 3-SH; QA23 rated higher on conversion 3-SH to 3-SHA.

In terms of 3-SHA, its production was related to the thiol-converting (3-SH to 3-SHA conversion) capability, and this appeared to be irrelevant to 3-SH releasing potential [97]. In real winemaking, the effects of yeasts on thiol aromas of wine were less apparent than in media fermentation, which possibly resulted from the masking effect by other wine volatiles such as the ester aromas [97]. Based on the observation that different thiol releasing abilities from specific strains, co-inoculation with yeast strains of high thiol-releasing and thiol-converting

abilities was suggested as one possible solution for thiol enhancement [97]. Higher concentrations of 3-SH and 3-SHA in wine fermented with a set of three yeasts co-inoculation were observed [98]. The sensory scores of co-inoculated wine rated higher in passion fruit note, well in accord with the thiol concentrations [98]. However, the mechanism of elevation of polyfunctional thiols (3-SH and 3-SHA) from certain yeast strains or co-fermentation is still unclear. It should be noted that the characteristic sensory attributes of polyfunctional thiols were not only influenced by yeast strains but also might be affected by other aroma compounds, for instance, esters and methoxypyrazines [97,99].

Yeast performance on releasing polyfunctional thiols during fermentation relates to several aspects of the yeast, such as enzymes and transporters involving with the uptake of polyfunctional thiol precursors and the release of thiols. As shown in **Figure 1** and **2**, the biogenesis of polyfunctional thiols from their precursors required specific enzymes and transporters. The uptake of extracellular polyfunctional thiol precursors is expected to affect the polyfunctional thiol production. Using Σ1278b *ura3* as a reference strain, 3-SH released from Cys-3-SH was compared to strain Σ1278b *ura3* deletion mutant *gap1Δ* [80], which demonstrated *GAP1* as an uptake transporter of Cys-3-SH, but this was not validated in grape must fermentation and some cysteine transporters (including *GAP1*) were reported that not associated with the uptake of Cys-4-MSP) [79]. Opt1p was suggested to be necessary for the uptake of Glut-4-MSP and Glut-3-SH. The transpeptidase *CIS2* also contributed to the uptake of Glut-3-SH. With gene deletion experiments, the role of yeast carbon-sulfur lyases on 4-MSP releasing from Cys-4-MSP was suggested [100].

Moreover, the cystathionine *β*-lyase Irc7p has been shown to be the principal enzyme for the liberation of polyfunctional thiols (4-MSP and 3-SH) from cysteinylated precursors [101], which was confirmed by subsequent research [102]. The substrate preference of *IRC7* protein for Cys-4-MSP than Cys-3-SH was also observed by comparing the cleavage abilities of crude protein extracts on different precursors [102]. However, the clear mechanism behind the uptake of polyfunctional thiol precursors and release of polyfunctional thiols by yeast is still need to be elucidated to better understand the fate of polyfunctional thiols during fermentation.

In addition to *S. cerevisiae*, the effects of yeast on polyfunctional thiols were also investigated on other species, including *S. bayanus* var. *uvarum*, hybrids *S. cerevisiae* × *bayanus* var. *uvarum* strains [94], and other non-*S. cerevisiae* species [96]. Strain-dependent variations are also found among non-*S. cerevisiae* yeasts, and certain yeast strains or hybrids demonstrated higher proficiency in releasing polyfunctional thiols [96].

1.3.5.2. Yeast nutrition

The nutrition of yeast has been identified as an influential factor for polyfunctional thiol precursor degradation and polyfunctional thiol production. Using urea and diammonium phosphate (DAP) in a synthetic medium supplemented with Cys-3-SH, the impacts of nitrogen source on Cys-3-SH consumption was investigated [80]. Cys-3-SH was depleted in medium with urea at high concentration, but was largely left in DAP treatment, which suggested that the degradation of polyfunctional thiol precursors was regulated by nitrogen catabolite repression (NCR). Moreover, the general amino acid permease (GAP1p) was a limited factor for thiol production in synthetic medium, but the repetitive results were not observed with actual grape must. Nonetheless, DAP addition into grape musts prevented the production of polyfunctional thiols.

A following study [103] showed that 17 genes of yeast involved in nitrogen metabolism related to 3-SH production and highlighted the importance of nitrogen. However, it has been suggested that NCR regulated the production of 4-MSP from Cys-4-MSP, and GAP1 seemed to be irrelevant [79]. Although the thiol production from their precursors is still unclear, the reported evidence demonstrates the critical relevance between yeast nutrition status and polyfunctional thiols.

1.3.5.3. Fermentation temperature

Fermentation temperature also affected polyfunctional thiol production. Comparing fermentation conducted at 18 and 28 °C in synthetic medium, the lower fermentation temperature favoured 4-MSP production [36]. Fermentation temperature was investigated further in model medium (13, 20 and 24 °C) and grape juice (13 °C and 20 °C) [95]. In model medium, the concentrations of 4-MSP and 3-SH decreased in lower temperature conditions for VL3c and VIN13 strains. In grape juice, greater amounts of 4-MSP, 3-SH and 3-SHA were produced at 20 °C than 13 °C, irrespective of the yeast strains and the origin of the must.

1.4. Research questions

Polyfunctional thiols in wine have been and will continously be a topic of interest for wine chemists due to the significant aroma contributions by these potent volatiles. Given studies and knowledge presented in this literature review, the following research questions have been proposed as the focus of this PhD thesis, aiming to expand our knowledge of thiols in the field of wine chemistry.

Question 1: How can a suitable chiral analytical method be developed for analysing enantiomers of polyfunctional thiol 3-SH and 3-SHA wine?

Most of the research has focused on 3-SH and 3-SHA in wine as their racemic mixture and little attention has been paid to the chirality of 3-SH and 3-SHA, despite their enantiomers differing in aroma quality and ODT. The quantitative data of enantiomers of 3-SH and 3-SHA are also extremely limited. This gap is largely due to the fact that an analytical method which can fully resolve 3-SH and 3-SHA enantiomers is still missing. A robust chiral analytical method for analysing 3-SH and 3-SHA enantiomers in wine is required.

Question 2: What is the potential stereochemical relation between precursor diastereomers in grape and thiol enantiomers in wine?

Some thiol precursors (Glut-3-SH, CysGly-3SH, and Cys-3-SH) exist in grapes as pairs of diastereomers which are metabolised to 3-SH enantiomers by yeast *Saccharomyces cerevisiae*. This stereochemical relation from precursors in grapes to thiols in wines in this biological process is unknown. With a new method for analysing 3-SH and 3-SHA enantiomers (**Question 1**) to be developed, the puzzle of thiol precursor stereochemistry is expected to be solved through winemaking trials.

Question 3: How do the regionality, grape composition, and winemaking factors impact thiol precursors in grapes and/or thiols in wines?

Yet fully understood, thiol metabolism is a complex biochemical process that involves the formation and degradation of both precursors and thiols, as well as their interactions with other grape metabolites. Various geographic, grape, and winemaking factors will be tested for their impacts on thiols and/or precursors, with the hope to propose new strategies for effective thiol management.

Question 4: How to discover new volatile compounds that can potentially explain the fate of 3-SH in wine?

Focusing on the most important polyfunctional thiol 3-SH in wine, this research will also attempt to discover new volatile compound candidate(s) related to 3-SH.

1.5. Summary of research aims

In view of the current state of knowledge of polyfunctional thiols in wine that has been detailed in the literature review chapter, this project aims to evaluate and explore polyfunctional thiol phenomena, specifically through investigation of their chirality, precursor stereochemistry, formation through winemaking, and potential fate through reactions in the wine matrix.

Objective 1: Evaluation of chirality of 3-SH and 3-SHA in wine by developing a novel analytical method

The chemical analysis of polyfunctional thiols in wine is a challenging task and current approaches use various forms of sample preparation combined with sensitive instrument analysis techniques, despite that the majority of analytical methods have been developed only for racemic polyfunctional thiols. Such information on thiol analysis has been reviewed, with a focus on wines, and presented in the publication in **Chapter 2.**

Chirality of volatile compounds in wine is an important topic in aroma chemistry because enantiomers of chiral volatiles often have different aroma quality and ODT. Two polyfunctional thiols, 3-SH and 3-SHA, are chiral molecules that exist as pairs of enantiomers with different sensory properties. However, analytical methods focusing on 3-SH and 3-SHA enantiomers in wine were extremely limited, thereby hampering research on the chirality of 3-SH and 3-SHA. To fill this gap, a SIDA coupled to HPLC–MS/MS has been developed and applied to evaluate the chirality pattern of 3-SH and 3-SHA in a selection of commercial wines. Full details of this study, including the method development, validation, and application are presented in the publication in **Chapter 3**.

Objective 2: Investigating the link between thiol and precursor stereochemistry under controlled winemaking conditions

Polyfunctional thiol 3-SH in wine is formed from its precursors in grape during fermentation. Likely 3-SH precursors, termed Glut-3-SH and Cys-3-SH, are present as diastereomers in grape and are converted to 3-SH enantiomers through yeast metabolism. This stereochemical link between 3-SH precursor diastereomers from grapes and 3-SH enantiomers in wine is important for understanding thiol formation, but has remained relatively untested.

Applying the novel SIDA chiral HPLC–MS/MS method for 3-SH and 3-SHA (**Objective 1**), the stereochemical relationship between Glut-3-SH and Cys-3-SH diastereomers to 3-SH and 3-SHA enantiomers has been investigated through controlled laboratory-scale winemaking trials, employing fruit from five Sauvignon blanc clones grown in the same location. The detailed results of the winemaking trials on thiol/precursor stereochemistry are presented in the publication in **Chapter 4**.

Objective 3: Polyfunctional thiol production and management during winemaking

The biogenesis of polyfunctional thiols is a complicated and dynamic biochemical process that can be influenced by grape, geographical, regional, viticultural, and oenological factors, yet the impacts of these factors on thiol metabolism are still unclear.

Aiming to provide new insight into thiol production and potentially offering new strategies for thiol management, multiple factors, including grape clone, grape amino acids, subregion, yeast, nutrient addition, the use of commercial enzyme, and pre-fermentation freezing treatment have been evaluated for their impact on polyfunctional thiols and/or thiol precursors during winemaking. The outcomes are presented in the publication in **Chapter 4** (yeast, nutrient addition, and commercial enzyme) and in the publication in **Chapter 5** (subregion, grape amino acids, pre-fermentation freezing treatment).

Objective 4: Identification of a new volatile compound associated with the fate of 3-SH

3-SH is the most studied polyfunctional thiol in wine, but research focusing on the fate of 3-SH is very limited. Indeed, other than the disulfide (oxidised) form, volatile compounds that are closely linked to 3-SH have barely been suggested. To achieve a better understanding of the fate of 3-SH, identification of a new volatile compound has been completed by critically theorising the chemical structure of a proposed volatile candidate (an oxathiane formed by reaction of 3-SH with acetaldehyde). A deuterium labelled standard was synthesised followed by the development of a targeted analytical method. Results and details of the newly identified volatile compound, its occurrence, ODT, and potential chemical/sensorial implications with respect to 3-SH, are presented and discussed in the publication in **Chapter 6**.

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Chapter 2

Analysis of potent odour-active volatile thiols in foods and beverages with a focus on wine.

Molecules 2019, 24, 2472. DOI.

Chen L., Capone D. L., Jeffery D. W.

Statement of Authorship

Title of Paper	Analysis of potent odour-active volatile Thiols in foods and beverages with a focus on Wine
Publication Status	☑ Published ☐ Accepted for Publication ☐ Submitted for Publication ☐ Unpublished and Unsubmitted work written in manuscript style
Publication Details	Chen L., Capone D. L., Jeffery D. W. Analysis of potent odour-active volatile Thiols in foods and beverages with a focus on Wine. Molecules **2019**, 24, 2472

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Contribution to the Paper	Conceptualisation, writing—original draft preparation and corrections to revised manuscript.		
Overall percentage (%)	80%		
Certification:	This paper reports on original research I conducted during the period of my Higher Degree by Research candidature and is not subject to any obligations or contractual agreements with a third party that would constrain its inclusion in this thesis. I am the primary author of this paper.		
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iii. the sum of all co-author contributions is equal to 100% less the candidate's stated contribution.

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Review

Analysis of Potent Odour-Active Volatile Thiols in Foods and Beverages with a Focus on Wine

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Academic Editors: Michael C. Qian and Yanping L. Qian
Received: 7 June 2019; Accepted: 3 July 2019; Published: 5 July 2019

Abstract: Certain volatile thiols are some of the most potent odour-active molecules that are found in nature. Thiols play significant roles in the aroma qualities of a range of foods and beverages, including wine, with extremely low odour detection thresholds (nanogram per litre range). A fundamental understanding of their formation, fate, and impact essentially depends on the development of suitable analytical methods. The analysis of volatile thiols in foods and beverages is a challenging task when considering (1) the complexity of food and beverage matrices and (2) that thiols are highly reactive, low molecular-weight volatiles that are generally present at trace to ultra-trace concentrations. For the past three decades, the analytical evaluation of volatile thiols has been intensively performed in various foods and beverages, and many novel techniques related to derivatisation, isolation, separation, and detection have been developed, particularly by wine researchers. This review aims to provide an up-to-date overview of the major analytical methodologies that are proposed for potent volatile thiol analysis in wine, foods, and other beverages. The analytical challenges for thiol analysis in foods and beverages are outlined, and the main analytical methods and recent advances in methodology are summarised and evaluated for their strengths and limitations. The key analytical aspects reviewed include derivatisation and sample preparation techniques, chromatographic separation, mass spectrometric detection, matrix effects, and quantitative analysis. In addition, future perspectives on volatile thiol research are also suggested.

Keywords: derivatisation; sample preparation; gas chromatography; high performance liquid chromatography; mass spectrometry; untargeted identification; targeted quantitation; matrix effect; stable isotope dilution assay

1. Introduction—Importance of Thiols to the Aroma of Foods and Beverages

Aroma is inarguably one of the most important quality aspects for any food or beverage product, with unique and characteristic aromas being attributed to a large range of volatile compounds with various physico-chemical properties. At the time of writing this review, a commercial database, Volatile Compounds in Food, had compiled a total of 9514 volatile components that were identified in natural and processed food products from published literature data [1], and the list continues to grow. Amongst the vast numbers of volatiles in the database, volatile sulfur compounds (VSCs, sulfur-containing volatiles) are the second largest category just after volatile esters, and they represent around 13% of total volatiles (Figure 1a) [1]. VSCs play an important role in the aromas of foods and beverages, not only because of their broad presence, but also for their significant sensory contributions due to concentrations that are well above their low odour detection thresholds (ODT) [2,3].

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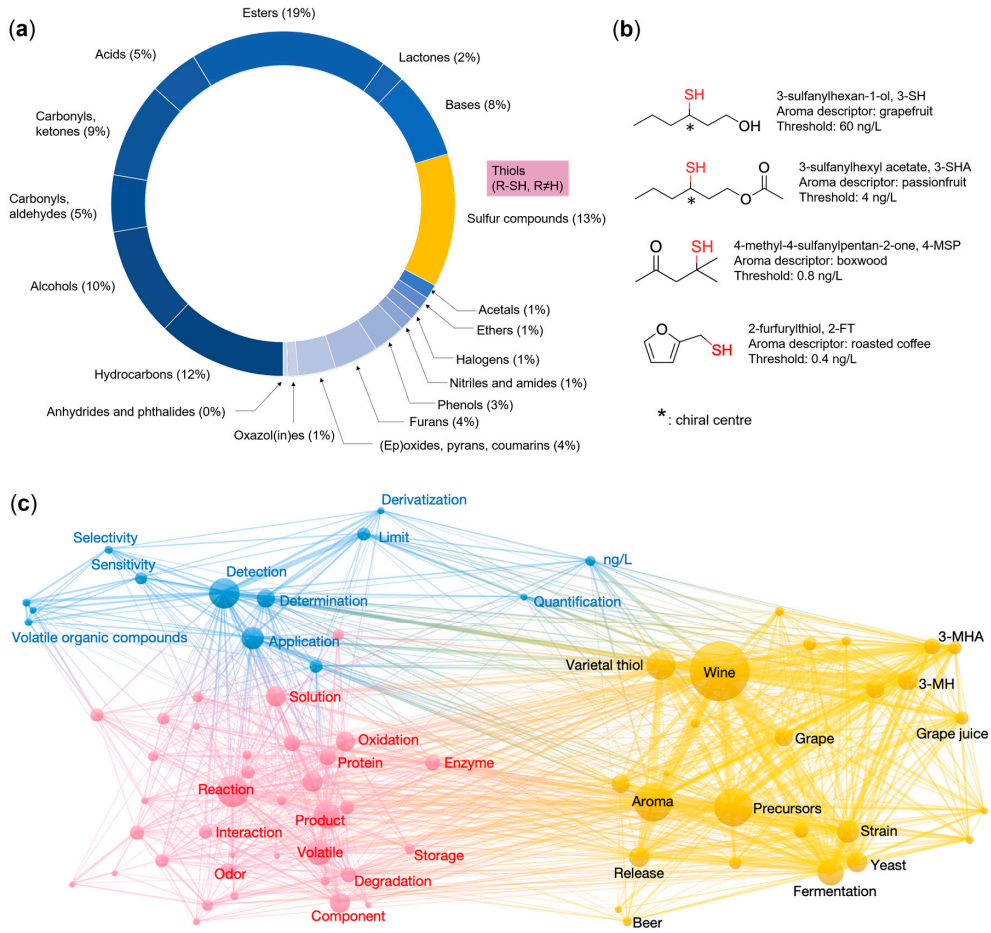

Figure 1. (**a**) Doughnut chart showing the relative percentages of volatile sulfur compounds identified in foods according to Volatile Compounds in Foods database [1], where each segment represents one chemical category of volatiles; (**b**) Examples of chemical structure, aroma descriptor, and ODT of some of the most studied volatile thiols in wine [4]; and, (**c**) Bibliometric map of volatile thiol research visualised from a total of 395 publications (from 1990–2019) retrieved from Web of Science Core Collection using "Volatile Thiols" as keyword. Literature analysis and graph construction by VOSviewer [5]. Note the abbreviations 3-MH and 3-MHA used in panel (**c**) are in keeping with much of the earlier literature; however, the IUPAC names (i.e., sulfanyl prefix instead of mercapto) are used in this review and abbreviated as 3-SH and 3-SHA.

Volatile thiols, historically known as mercaptans and consisting of the structure R–SH, are a sub-category of VSCs that are of particular interest, because they have some of the lowest ODTs (ng/L and lower) of any volatile compound identified in nature [6]. Such potent volatile thiols have been in the spotlight of aroma research and they are frequently regarded as "potent" [7], "key aroma" [8], "aroma-active" [9,10], or "aroma-impacting" [11] odorants. Some of the most famous examples in foods and beverages include 3-sulfanylhexan-1-ol (aroma descriptor: grapefruit, ODT: 60 ng/L) and 3-sufanylhexyl acetate (aroma descriptor: passionfruit, ODT: 4 ng/L) in wine [6], 4-methyl-4-sulfanylpentan-2-one (aroma descriptor: boxwood, ODT: 0.8 ng/L) in beer [12] and wine [6], and 2-furfurylthiol (aroma descriptor: roasted coffee aroma, ODT: 0.4 ng/L) in coffee [13] (Figure 1b).

From a total of 395 publications (1990 to 2019, using "Volatile Thiol" as the search keyword) retrieved from Web of Science Core Collection and visualised by a network approach, it was obvious that research on volatile thiols had been focusing on "detection" and "reaction", and the majority

of publications had emerged from the field of "wine" research (Figure 1c). Indeed, much progress on the development of analytical methods for untargeted identification and targeted quantitation for volatile thiols has been achieved in wine [4]. As such, this review emphasises the field of wine research and particularly covers literature that is dedicated to developing analytical methods. The analytical challenges and requirement for volatile thiol analysis in the context of wine, foods, and other beverages are first presented, followed by strategies that were developed to address those analytical challenges. Sample preparation techniques (selective extraction with metal ions, derivatisation), chromatographic separation, mass spectrometric detection, quantitative analysis with stable isotope dilution assay (SIDA), and matrix effects have been reviewed. Finally, future trends and directions for volatile thiol research have been proposed. However, exhaustive occurrences, sensory interactions, chemical synthesis and biogenesis, and other unmentioned aspects of thiols in foods and beverages are considered as beyond the scope of this review.

2. Analytical Challenges and Requirements

The analysis (either untargeted identification or targeted quantitation) of volatile thiols in foods and beverages has always been a challenging task due to two reasons:

- matrix complexity;
- properties of thiols.

Volatile thiols can be found in many foods and beverages, including wine, beer, cheese, olive oil, coffee, fruit, meat, and vegetable [14]. On one hand, these foods and beverages possess drastically different matrices (animal or vegetal, fermented or unfermented, liquid or solid, aqueous or lipid, etc.) and, on the other, the matrices are compositionally complex (containing many other volatile and non-volatile metabolites). Such complexity and diversity in matrices pose analytical challenges for developing suitable and efficient analytical methods [15]. Potent volatile thiols are highly unstable small molecules that are present at extremely low abundances with diverse chemical structures. The sulfhydryl (–SH) group in thiols is one of the most reactive functional groups found in natural organic matter [16]. As such, thiols are prone to oxidation, isomerisation, and rearrangement [17]. The highly active –SH group can cause chromatographic separation difficulties even when thiols are well preserved throughout extraction, such as peak tailing during analysis by gas chromatography (GC) [18]. Apart from the instability, volatile thiols differ in chemical structure, with the majority containing either acid (–COOH), alcohol (–OH), aldehyde (–CHO), ester (–OC(O)–), ether (–O–), and/or aromatic ring functional group(s), with only a small portion belonging to aliphatic thiols [14]. Those additional functional groups should be taken into consideration at an early stage of method development in order to minimise their modification. For instance, the analysis of thiol acetates should avoid the occurrence of acetate hydrolysis during sample preparation and analysis [19]. Besides structural diversity, some thiols are also characterised with chirality, owing to the carbon bearing the sulfur atom (Figure 1b), which gives a pair of thiol enantiomers. As with any enantiomer pairs, enantiomeric thiols are of almost identical physical and chemical properties, but they often differ in aroma quality and ODT [4]. The separation of enantiomers has always been a complex task as there is no golden rule to predict chiral separation. Lastly, thiols are generally found at trace to ultra-trace concentrations and, in many cases, at part per trillion levels. Such extremely low abundances require the careful consideration of effective sample isolation and enrichment steps, and sensitive detection techniques.

An ideal analytical method for thiol analysis should be fast, simple, reliable, robust, green, sensitive, and cost-effective. For analytical methods that are dedicated to screening/discovering new volatile thiols, analytical information that is provided by the methods should be sufficient for identification. As for quantitative methods, limit of detection (LOD, ideally below ODT), matrix effects, repeatability, precision, and accuracy are among the important factors.

3. Thiol Isolation—Extraction and Derivatisation

Due to the analytical challenges previously mentioned, particularly the instability of volatile thiols, but also their low abundance, efficient isolation using the routine extraction techniques employed for aroma analysis is hard to achieve. This reflects on the fact that many of the established thiol isolation methods have combined rather sophisticated forms of sample pre-treatment, specific extraction, derivatisation, clean-up, and enrichment (concentration). From the methods that are summarised in Table 1, it is evident that the isolation of volatile thiols from wine (and similar applications in foods and other beverages) is an evolving process that has advanced from traditional, time-consuming methods to better-sequenced and much simplified procedures. Although being proposed in various formats, the currently available thiol preparation methods can generally be categorised into three main groups:

- non-specific extraction, e.g., straightforward application of headspace solid-phase microextraction (HS–SPME), solid phase extraction (SPE), liquid-liquid extraction (LLE), purge and trap (P+T), and vacuum distillation;
- selective extraction with metal ions (e.g., Hg^+ or Ag^+);
- derivatisation (coupled with LLE, HS–SPME, SPE, or gas purge microsyringe extraction (GP–MSE)).

As the non-specific extraction techniques for thiol isolation are principally similar to those that are commonly applied for other volatile compounds, selective extraction and derivatisation-based methods have been selected as the focus in this review.

3.1. Selective Extraction with Metal Ions

Thiol-specific extraction methods are based on the strong affinity between thiols and metal ions, such as mercury (Hg^+) and silver (Ag^+). In fact, the word mercaptan, which is the historical synonym for thiol, arose from the Latin term *cercurium captans*, which means mercury-seizing [20]. Organomercurial compounds have long been applied for thiol-specific extraction (Table 1, Entries 1–4) [21,22] by reversibly binding with thiols and forming stable Hg-thiol complexes (mercaptides). The thiol moiety can then be replaced from Hg-thiol complexes by an excess of other thiol (e.g., glutathione [21], cysteine [22], or D,L-dithiothreitol [23]) during an elution step, which releases the thiols of interest. Specific organomercurial compounds that have been used in this manner include 4-hydroxymercuribenzoate (usually referred to as *p*-hydroxymercuribenzoate and abbreviated *p*-HMB) [21,22,24], phenylmercuric chloride [25], and 4-aminophenylmercuric acetate [23]. These agents efficiently and selectively bind thiols; for instance, the reaction between *p*-HMB, the most popular organomercurial reagent, and thiols in wine requires less than 90 s, and, more importantly, *p*-HMB does not react with thioesters, sulfides, or disulfides [21].

Pioneered for wine with the identification of varietal aroma compounds that are associated with box tree odour, the early developed *p*-HMB extraction method (Table 1, Entry 1) requires pH adjustment on a large volume of sample (1000 mL), followed by LLE with organic solvent prior to thiol extraction with aqueous *p*-HMB solution [21]. While using this extraction method, 4-MSP was identified for the first time in the plant kingdom (a Sauvignon wine). For quantitation purposes that require cleaner thiol extracts, a strong anion exchange column was introduced as a clean-up step after the extraction of thiols from the organic phase with *p*-HMB solution, prior to eluting with cysteine solution to release the thiols (Table 1, Entry 2) [22]. This approach has been modified and used for thiol extractions in beer [12] and cheese [26]. One limitation of applying this extraction method is that it requires pH adjustment at several points (in the raw sample, during extraction, and in pooled extracts [22]), and the high pH conditions could induce thiol oxidation or unwanted changes in the sample matrix, such as the formation of quinones that can react with thiols. Therefore, the *p*-HMB extraction method has been modified to avoid tedious pH adjustment of wine by using a Tris buffer solution (pH > 7) [24].

Table 1. Isolation methods developed for analysis of potent volatile thiols in wines, foods, and other beverages.

Entry No.	Reference	No. of Analytes [1]	Matrix	Sample Amount	Isolation Overview [2]	Major Methodological Parameters [3]	Comments [4]
1	1995 [21]	1 [ID]	Wine	1000 mL	LLE ⇒ selective extraction [*p*-HMB]	• LLE × 3 using *p*-HMB solution • Glutathione added at 20-fold the *p*-HMB amount	+ Reversible tagging allows thiols to be analysed in native form by GC–O + Suitable for thiol screening with GC based methods – Large sample volume needed – High demand for organic solvents – *p*-HMB is highly toxic – Very time consuming ♦ cheese [26]
2	1998 [22]	5 [QT]	Wine	500 mL	LLE ⇒ selective extraction [*p*-HMB] ⇒ strong anion exchange column ⇒ LLE	• 4-Methoxy-2-methyl-2-butane as IS • pH adjustments • Dowex 1X2-100 column • >45 min for column step	
3	2003 [25]	3 [QT]	Wine	500 mL	LLE ⇒ selective extraction [*p*-HMB]	• SIDA • Wine protected in ice bath under N_2 • LLE for 15 min • Affi-Gel 501 • 1,4-dithio-DL-threitol (DTT) elution	
4	2017 [27]	1 [QT]	Hops	350 g	LLE ⇒ SAFE ⇒ selective extraction [*p*-HMB] ⇒ SAFE	• SIDA • Mercurated agarose gel prepared from Affi-Gel 10 • LLE duration > 3 h • SAFE at 40 °C • DTT elution • SAFE to remove DTT	
5	2017 [28]	6 [QT]	Beer, hops	20 mL, 2 g	LLE ⇒ Ag^+ resin based SPE	For beer: • LLE for 15 min • Centrifugation for 15 min • Meta-Sep IC-Ag SPE cartridge • Cartridge reversed • Salted eluate shaken for 15 min • Centrifugation for 15 min	+ Novel SPE concept – Multiple extraction steps

Table 1. *Cont.*

Entry No.	Reference	No. of Analytes [1]	Matrix	Sample Amount	Isolation Overview [2]	Major Methodological Parameters [3]	Comments [4]
6	2006 [18]	2 [QT]	Wine	10 mL	HS–SPME with automated on-fibre derivatisation [PFBBr]	• PDMS/DVB SPME fibre • Wine bubbled with N_2 at 4 °C	+ Moderate amounts of sample required + Less solvents needed − Multiple steps for some − Hazardous PFBBr
7	2007 [29]	4 [QT]	Wine	6 mL	LLE ⇒ derivatisation [PFBBr]	• Four IS • LLE with benzene • Sample bubbled with N_2 • 35 min LLE and centrifugation • PFBBr reaction at 4 °C for 40 min	
8	2008 [30]	5 [QT]	Wine	10 mL	Derivatisation [*o*-methylhydroxylamine for carbonyl of 4-MSP] ⇒ SPE with in-cartridge derivatisation [PFBBr]	• SIDA • Sample purged with N_2 • *o*-methylhydroxylamine reaction at 55 °C for 45 min • SPE with Bond Elut-ENV	
9	2009 [31]	3 [QT]	Wine	100 mL	Derivatisation [*o*-methylhydroxylamine for carbonyl of 4-MSP] ⇒ SPE with derivatisation [PFBBr] ⇒ HS–SPME	• Similar to [30] • SIDA • DVB/CAR/PDMS for 30 min at 100 °C	
10	2011 [32]	1 [QT]	Wine	200 mL	LLE ⇒ derivatisation [PFBBr] ⇒ HS–SPME	• SIDA • LLE and back extraction with ice-cold aqueous NaOH • PFBBr reaction at room temperature for 20 min • pH adjustment • PDMS/DVB fibre • SPME for 30 min at 80 °C	
11	2015 [33]	3 [QT]	Wine	40 mL	Simultaneous LLE extraction and derivatisation [PFBBr] ⇒ HS–SPME	• SIDA • pH adjustment • PFBBr reaction and LLE for 10 min at room temperature • PDMS/DVB fibre • SPME for 60 min at 70 °C	
12	2013 [19]	3 [QT]	Wine	50 mL	Derivatisation [ETP] ⇒ SPE	• SIDA • pH adjustment • ETP reaction for 10 min under stirring • SPE with ENVI-18	− Poor reaction efficiency with 4-MSP

Table 1. *Cont.*

Entry No.	Reference	No. of Analytes [1]	Matrix	Sample Amount	Isolation Overview [2]	Major Methodological Parameters [3]	Comments [4]
13	2015 [34]	3 [QT]	Beer, wort, hops	20 mL	Derivatisation [ETP] and SBSE	• SIDA • pH adjustment • PDMS stir bar • ETP reaction for 10 min at 25 °C • NaOH addition • SBSE for 180 min at 1500 rpm	− Long extraction time
14	2014 [35]	1 [QT]	Wine	3 mL	Automated derivatisation of 4-MSP carbonyl [*o*-methylhydroxylamine] and HS–SPME	• SIDA • DVB/CAR/PDMS fibre • SPME for 45 min at 55 °C	+ Easy automated extraction approach − Only one analyte assessed
15	2015 [36]	2 [QT]	Wine	180 mL	LLE ⇒ derivatisation [OPA]	• SIDA • Add potassium metabisulfite and PVPP, stir for 10 min • Centrifugation for 10 min • pH adjustment and sodium borohydride addition • LLE for 20 min • Reaction for 5 min at room temperature	− Large sample volume − Complicated protocol
16	2013 [37]	7 [QT]	Olive oil	2 g	Single step derivatisation [ebselen]	• 4-Methoxy-α-toluenethiol • Reaction maintained under N_2 • 1 min reaction	+ Simple and fast extraction − Requires high resolution MS ♦ wine [38], beer [38], brewed coffee [39], roasted coffee [40]
17	2015 [7]	5 [QT]	Wine	20 mL	Derivatisation [DTDP] ⇒ SPE	• SIDA • Reaction for 30 min at room temperature • Bond Elut C18 SPE cartridge	+ Simple extraction + Suitable for multiple thiols + Chiral analysis possible ♦ wines [41,42]
18	2014 [43]	1 [ID]	Beer	100 µL	Single step stable isotope labelled chemical derivatisation [d_0/d_7-BQB]	• BQB dried under N_2 • Gly-HCl buffer • Reaction for 1 h at 60 °C	+ Stable isotope derivatisation applied + Precursor ion scan − Synthesis of reagents required
19	2017 [44]	6 [QT]	Wine	100 mL	Selective extraction [*p*-HMB] & SPE ⇒ LLE ⇒ Stable isotope labelled chemical derivatisation [d_0/d_4-AENM]	• LiChrolut-EN SPE mercurated with *p*-HMB • Reaction at 40 °C for 10 min	
20	2018 [45]	4 [QT]	Coffee bean, cookies, fried nuts, biscuit	2 g	GP–MSE ⇒ derivatisation [PIPD]	• Gas purge with N_2 • 1.0 mL of syringe loaded with 0.5 mL of MeOH as extraction solvent • Sample heated for 30 min at 190 °C • Derivatisation for 10 min	+ No pre-enrichment step − Customised extraction apparatus required − Sample subjected to high temp. − Synthesis of reagent required

[1] ID: identification; QT: quantitation. [2] Only major steps are presented. See text and Figure 2 for reagent abbreviations; GP–MSE: gas purge microsyringe extraction. [3] IS: internal standard; SAFE: solvent-assisted flavour evaporation; PDMS: polydimethylsiloxane; DVB: divinylbenzene; CAR: carboxen; SIDA: stable isotope dilution assay; PVPP: polyvinylpolypyrrolidone; MeOH: methanol. [4] +: advantage; −: disadvantage; ♦: application of similar extraction approaches reported in foods and beverages; GC–O: gas chromatography–olfactory; MS: mass spectrometry.

p-HMB extraction procedures are quite time consuming due to the need for pH adjustment and clean-up, and, more importantly, thiols are prone to oxidation during the laborious sample preparation steps, which could influence their quantification. Affinity chromatography with Affi-Gel 501 (formed from Affi-Gel 10 by treatment with 4-aminophenylmercuric acetate) was developed for thiol extraction from extracts prepared by LLE to simplify the selective extraction step (Table 1, Entry 3) [25]. Affinity chromatography with Affi-Gel appears to be less time-consuming when compared to the previously developed *p*-HMB protocol, but it demonstrates lower recovery rates (e.g., for 4-MSP, 38% vs. 75%–80%). This approach is possibly more suited to thiol discovery, with a similar affinity chromatography approach having been used to screen for volatile thiols in various fruits and wines [23]. Another slight limitation is that Affi-Gel 501 needs to be prepared in-house, and these approaches still involve intense extraction and concentration steps (more problematic for routine quantitation than for thiol screening).

Another selective extraction method is based on the high affinity between thiols and Ag^+ (Table 1, Entry 5) [28]. Using commercially available Ag^+ based SPE cartridges (Meta-Sep IC-Ag), volatile thiols in the organic extracts of beer and hops were retained and then eluted with thioglycerol in CH_2Cl_2 [28]. Ag^+ extraction is advantageous when compared to Hg^+, because it avoids the use of toxic mercury and the SPE cartridges can be commercially obtained. However, the Ag^+ extraction procedure is still somewhat complicated, with the LLE extraction of volatiles, and multiple clean-up and concentrating steps after SPE. However, from purely an extraction viewpoint, selective extraction methods have high reaction efficiency, selectivity, and permit the recovery of thiols for analysis in their unmodified form. This is particularly useful for GC–Olfactometry (GC–O) screening for new thiol odorants, and it has enabled the discovery of many important volatile thiols in wine [21,22,24], tea [46], hop extracts [12], and beer [12,47]. On the other hand, the drawbacks of these extraction approaches are obvious: large amounts of sample and solvent are required for the preparation of volatile extracts; procedures are lengthy and time-consuming; final concentrated thiol distillate/extracts are in their original sulfhydryl form, which can cause reaction, separation, and detection issues; in the case of *p*-HMB, handling highly toxic organomercurial compounds poses significant health and environmental risks.

3.2. Derivatisation Approaches

The adaptation of derivatisation for more selective, efficient, and simplified isolation procedures and/or stabilisation of thiols has been the major development in thiol isolation. These approaches are designed to improve the sensitivity of instrumental analyses, because, after derivatisation, volatile thiols are easier to be extracted, chromatographed, and detected. On one hand, derivatisation intends to block the sulfhydryl group (or mask a carbonyl group in the case of 4-MSP, for example) and the formed thiol derivatives are chemically stable for isolation, as well as thermally stable for GC analysis. On the other hand, introducing a substituent means that thiol derivatives exhibit greater hydrophobicity, less polarity, and/or stronger proton affinity, which leads to better liquid chromatography (LC) separation and signal enhancement for mass spectrometry (MS)-based detection [48]. When selecting suitable derivatisation reagents, the factors to consider include reaction specificity and efficiency, matrix compatibility, required sample manipulation, introduction of interferences, and whether it occurs before or after the extraction of analytes. Figure 2 shows common derivatisation reagents and related reaction conditions that are proposed for volatile thiols analysis in wine, foods, and other beverages, and categorised into those for GC analysis (Figure 2a), or LC analysis with conventional (Figure 2b) or stable isotope labelled (Figure 2c) derivatisation reagents.

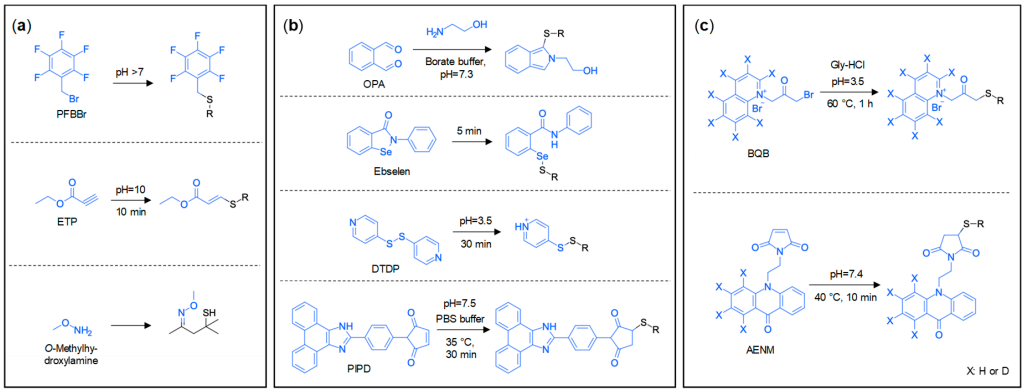

Figure 2. Derivatisation reagents and reactions of volatile thiols in wine, foods, and other beverages for (**a**) gas chromatography (GC) analysis, (**b**) liquid chromatography (LC) analysis, and (**c**) LC with stable isotope labelled derivatisation reagents. PFBBr: 2,3,4,5,6-pentafluorobenzyl bromide; ETP: ethyl propiolate; OPA: *o*-phthaldialdehyde; ebselen: 2-phenyl-1,2-benzisoselenazol-3(2H)-one; DTDP: 4,4′-dithiodipyridine; PIPD: 1-(4-(1*H*-phenanthro[9,10-d]imidazol-2-yl)phenyl)-1*H*-pyrrole-5-dione; BQB: ω-bromoacetonylquinolinium bromide; AENM: acridone-10-ethyl-*N*-maleimide.

3.2.1. Derivatisations for GC Analysis of Thiols

As seen in Table 1 (Entries 6–14), 2,3,4,5,6-pentafluorobenzyl bromide (PFBBr), ethyl propiolate (ETP), and *o*-methylhydroxylamine have been used as derivatisation reagents for GC-based thiol analysis. PFBBr and ETP both react with the sulfhydryl group, whereas *o*-methylhydroxylamine derivatises the carbonyl group in 4-MSP (forming a methoxime). After derivatisation(s), extractions can be conducted in combination with modern extraction techniques such as HS–SPME, SPE, or stir bar sorptive extraction (SBSE), in contrast to the traditional LLE or affinity chromatography normally practised in selective extractions with Hg^+ or Ag^+ (although LLE, in particular, may still feature along with derivatisation).

PFBBr (Table 1, Entries 6–11) is frequently used as a derivatisation reagent for thiols, due to the bromide atom being particularly susceptible to nucleophilic substitution by thiols in the presence of base, and the obtained PFBBr thiol derivatives offer desired properties, not only by stabilising the thiol, but also with regard to electron-capturing abilities and MS detection [18]. The derivatisation of volatile thiols in wine with PFBBr has been evaluated in various formats: automated headspace on-fibre derivatisation [18], derivatisation in organic solvent system [29] or aqueous phases [32] followed by HS–SPME, in-cartridge SPE derivatisation [30], and HS–SPME coupled with SPE [31]. The SPME on-fibre derivatisation (Table 1, Entry 6) is fast, automated, and solventless. A polydimethylsiloxane/divinylbenzene (PDMS/DVB) SPME fibre is exposed in sequence to the vapours of tributylamine (5 min), PFBBr solution (5 min), and then pre-incubated wine sample (containing ethylenediaminetetraacetic acid, salt, and internal standard (IS)) for extraction for 10 min at 55 °C [18]. This approach provided convenience and less potential interferences by using an autosampler and HS-SPME [18], but the linear ranges for the studied thiols were not very wide (and extremely narrow for 2-methyl-3-furanthiol, 2-MFT) and only two thiols (2-FT and 3-SHA) out of five were able to be analysed with this method. To improve the procedure, three conditions (two-phase liquid-liquid system, two-phase liquid-liquid system with a phase transfer catalyst, and two-phase liquid-solid system) were evaluated for wine and PFBBr derivatisation was finally conducted in a homogeneous organic solvent (benzene) system, based on relatively higher derivatisation yields and lower extraction of polar compounds from wine (Table 1, Entry 7) [29]. Apart from switching from on-fibre derivatisation to a homogeneous organic solvent system, 1,8-diazabicyclo[5.4.0]undec-7-ene (DBU) was used as a non-nucleophilic base to better deprotonate thiols (enhancing reactivity) and enable a decrease in the amount of PFBBr, which lessened the chance of PFBBr carryover. After comprehensive optimisation,

the method provided larger linear ranges than SPME with on-fibre derivatisation [18], but it was still unable to achieve consistent results for 2-MFT and required the use of the carcinogenic solvent benzene [29]. Subsequently, PFBBr derivatisation (after carbonyl derivatisation) in an SPE cartridge was suggested for volatile thiols in wine (Table 1, Entry 8) [30]. This protocol involved multi-step washings, derivatisation reaction, and the elution of PFBBr derivatives, but it still suffered from interferences regardless of the improved clean-up with SPE. This was ultimately compensated for by adapting to a method involving SIDA [30]. A method with methoximation and PFBBr derivatisation in combination with both SPE and HS–SPME has been developed, also using SIDA, to further eliminate matrix effects (Table 1, Entry 9) [31]. After methoximation (for 4-MSP) [30], the wine sample was adjusted to pH 7 and derivatised in-cartridge with PFBBr and DBU, and then eluates from SPE were evaporated and sampled by HS-SPME [31]. The change of pH was necessary to eliminate interferences that were not retained by SPE at higher pH [31].

The above-mentioned PFBBr derivatisation approaches were followed by GC with chemical ionisation (CI) and MS detection in negative ion mode, instead of the more routinely available electron ionisation (EI) with MS detection in positive ion mode. As such, the PFBBr derivatisation methods were investigated for thiol analysis in wine while using GC with EI–MS, again in combination with HS–SPME of the derivatives [32,33]. Focusing on 3-SH, an extraction was proposed that involved LLE with pentane and back-extraction into cold aqueous NaOH, followed by direct PFBBr derivatisation (Table 1, Entry 11) [32]. This method showed that the analysis of a PFBBr thiol derivative could be achieved by EI–MS, although the sample volume (100 mL) was much larger than that required for NCI-MS detection, and only 3-SH was assayed (albeit at levels below its ODT) [32]. The method was improved by applying extractive arylation with PFBBr but still using GC–EI–MS (Table 1, Entry 12) [33]. The approach, which included 3-SHA and 4-MSP, along with 3-SH, employed PFBBr derivatisation of thiols in 40 mL of wine (pH adjusted to 12) with the simultaneous extraction of derivatives into pentane-diethyl ether. The extracts were dried, reconstituted, and subjected to HS-SPME [33]. The improved analytical performance, when compared to the previous method for 3-SH alone [32], was proposed to result from the removal of interferences, more optimal conditions for derivatisation or HS-SPME sampling, or having fewer steps that contribute to analyte losses during extraction [33]. In comparison to selective *p*-HMB extraction and the analysis of free thiols, the suggested PFBBr derivatisation-based methods for thiol extraction and analysis (either for CI–MS or EI–MS) have significantly lower sample volume and solvent consumption (especially with HS–SPME), less sample preparation steps, and no requirement for organomercurial compounds. However, the overall extraction processes are still lengthy and complicated (e.g., pH adjustment and multiple steps), and PFBBr is not entirely without safety concerns.

ETP is another reagent that has been investigated for the derivatisation of thiols (Table 1, Entries 12 and 13) in wine [19], beer [34], hops [34], and wort samples [34]. In the case of wine analysis, ETP rapidly reacts with thiols at basic pH (10 min), and, following an optimised SPE step, the ETP derivatives can be analysed by GC–MS (Table 1, Entry 12) [19]. The ETP-based method has further simplified the approach to thiol derivatisation when compared to *p*-HMB and PFBBr methods, but still requires pH adjustment of wine (not a trivial undertaking). The main shortcoming was the lack of sensitivity for 4-MSP in real wine samples as a result of poor derivatisation [19]. Nonetheless, ETP has also been evaluated in combination with SBSE for the analysis of 3-SH, 3-SHA, and 4-MSP in beer, hops, and wort samples (Table 1, Entry 13) [34]. The SBSE procedure is relatively simple to conduct and it requires less solvent, but the disadvantages included the need for pH adjustment of samples, that a single SBSE of derivatives required more than 3 h, that stir bars needed to be conditioned before use and reconditioned after each use, and the need for a thermal desorption unit (TDU) [34].

Finally, there is *o*-methylhydroxylamine for the derivatisation of a carbonyl group as a methoxime. Aside from its use for masking the keto functionality of 4-MSP to facilitate thiol derivatisation with PFBBr (Table 1, Entries 8 and 9), *o*-methylhydroxylamine derivatisation has been employed in an automated SIDA HS–SPME procedure for the analysis of 4-MSP at sub-ODT concentrations (Table 1, Entry 14) [35]. The procedure creates specific higher mass fragments that facilitate MS detection when

compared to the natural analyte (but using positive CI rather than the more routine EI), and it requires a small volume of sample (3 mL) and less sample preparation as compared to ETP [35]. However, despite being easier to undertake and very sensitive, the procedure is only applicable to 4-MSP, and it is complicated by the fact that two derivative isomers are formed from a single analyte, with the favoured (*E*)-isomer being selected for quantitation [35].

3.2.2. Derivatisations for LC Analysis of Thiols

The abovementioned specific extraction and derivatisation approaches that involve Hg^+, Ag^+, PFBBr, and ETP are for GC based systems. Emerging some time after many of the GC methods were developed, derivatisation-based procedures that were designed for LC analysis are perhaps one of the most important developments in thiol isolation. In comparison to their GC counterparts, the suggested derivatisation protocols for LC analysis of volatile thiols have tended to simplify the overall extraction protocol and offer excellent sensitivity in a variety of matrices. Derivatisation reagents applied for LC-based analysis of thiols (Table 1, Entries 15–19) include conventional reagents, like *o*-phthaldialdehyde (OPA) [36], 2-phenyl-1,2-benzisoselenazol-3(2H)-one (ebselen) [37–40], 4,4′-dithiodipyridine (DTDP) [7,41,42], and 1-(4-(1*H*-phenanthro[9,10-d]imidazol-2-yl)phenyl)-1*H*-pyrrole-5-dione (PIPD) [45], as well as stable isotope labelled reagent pairs d_0/d_7-ω-bromoacetonylquinolinium bromide (d_0/d_7-BQB) [43,49] and d_0/d_4-acridone-10-ethyl-*N*-maleimide (d_0/d_4-AENM) [44]. The reagents readily react with the sulfhydryl group, and derivatisation reactions are often performed in various formats, such as in conjunction with LLE, SPE, or GP–MSE prior to LC analysis, to assist with sample clean-up and enrichment.

OPA was considered for thiol derivatisation in white wine [36] due to the reactivity of the dialdehyde functionality with amino acids and other nucleophiles, including thiols [50], thus beginning the exploration of LC-based approaches for the analysis of volatile thiols in wine (Table 1, Entry 15). Derivatisation with OPA in the presence of ethanolamine under basic conditions is rapid (5 min at room temperature in borate buffer), but 4-MSP was unable to be derivatised and the pre-derivatisation sample preparation steps are rather complicated. Briefly, wine has to be treated with potassium metabisulfite and polyvinylpolypyrrolidone, followed by pH adjustment and reaction with borohydride, and then LLE with CH_2Cl_2 (possibility of forming an emulsion) and sample concentration steps prior to derivatisation [36]. Moreover, the OPA-thiol derivatives were unstable, even when stored at −80 °C, and their rapid and significant degradation would ultimately lead to a loss of sensitivity and inaccurate quantitation [36].

The Se-N-containing reagent 2-phenyl-1,2-benzisoselenazol-3(2H)-one (ebselen) selectively and efficiently reacts with thiols by the cleavage of Se-N bond and formation of an Se-S bond with the –SH group [51]. Ebselen has been used to derivatise a range of thiols (such as those in Figure 1b) in various matrices (lipid: olive oil [37]; hydroalcoholic: wine [38], beer [38]; aqueous: brewed coffee [39]; organic extract: roasted coffee [40]) (Table 1, Entry 16). The proposed ebselen derivatisation approaches are fast, single-step derivatisation/extraction (~ 1 min), with some slight variations in initial sample preparation, solvent choice, solvent volumes, and workup steps, depending on sample matrices. In general, a suitable solvent containing ebselen (or with ebselen introduced separately) is added to the solid or liquid sample. After vortexing for a short period of time, the organic phase is collected, concentrated, and the residue is re-dissolved for analysis (or analysed directly without concentration) [37–40]. The major advantages of these approaches when compared to aforementioned derivatisation procedures is they are much less complicated and they employ mild conditions, although they still require the handling of samples under N_2, and the instruments are all high resolution mass spectrometers as opposed to the more common triple quadrupole. As a slight aside, these derivatives are claimed to enhance ionisation and improve the signal response due to the ease of ionisation and the positive charge gained by the nitrogen. However, the nitrogen in ebselen derivatives is an amide (weak acid) and not

an amino nitrogen, so positive ionisation mode would rely on the protonation of the carboxamide oxygen [52].

Thiol derivatisation with DTDP has also been developed for wine analysis (Table 1, Entry 17) [7], due to its high derivatisation ability of sulfhydryl groups at acidic pH [53], whereby DTDP specifically and rapidly directly reacts with thiols in the natural wine pH range [7]. Avoiding pH adjustment throughout derivatisation and extraction is deemed to be an important point, and, when coupled with conventional SPE for clean-up and enrichment of derivatives, this procedure provides a relatively simple approach that affords the desired sensitivity for analysis of 3-SH, 3-SHA, 4-MSP, BT, and 2-FT [7]. The flexibility of the DTDP derivatisation and extraction method has been demonstrated in the chiral analysis of enantiomers of 3-SH and 3-SHA in wine [41], and in a refined form (no concentration after SPE) while using convergence chromatography for wine analysis [42]. The additional advantages of DTDP derivatisation include the formation of stable and easily ionisable derivatives (due to the pyridine moiety) that are ideal for electrospray ionisation (ESI), and, to a lesser extent, the inclusion of a chromophore, which may be useful for samples with high levels of thiols (3-SH in particular), although this was not tested. As a bonus, DTDP is a non-hazardous chemical and is safer to deal with than some other reagents, especially *p*-HMB or PFBBr.

More recently, 1-(4-(1*H*-phenanthro[9,10-d]imidazol-2-yl)phenyl)-1*H*-pyrrole-5-dione (PIPD) has been demonstrated as a new derivatisation reagent for volatile thiols (Table 1, Entry 20). The maleimide moiety in PIPD can rapidly react with thiols to form derivatives that are stable (4 °C for at least three days) and detectable by HPLC–fluorescence (with atmospheric pressure chemical ionisation (APCI)-MS used for identification) [45]. Thiol extraction was performed with GP–MSE (N_2 at 2.5 mL/min for 30 min at 190 °C) in a customised apparatus prior to derivatisation (10 min at 35 °C in phosphate buffered saline, pH 7.5) [54]. After extraction and derivatisation, the mixtures were diluted with methanol (MeOH), filtered, and directly injected [45]. This proposed extraction and derivatisation methodology is simple and fast, and the analytical method is precise and sensitive, but the approach requires a customised gas purge chamber and the sample is kept at a high temperature for 30 min, which seems unlikely to be applicable to liquid samples, such as wine or beer. In addition, PIPD has to be synthesised, as opposed to other commercially available reagents.

Whether for LC or GC, the derivatisation examples that have been mentioned so far require the use of reference standards and internal standards to establish the calibration curves for quantitative analysis. In many cases, the reference standards or internal standards (particularly deuterated internal standards) are not commercially available, or they are expensive to acquire or non-trivial to synthesise. These concerns can be somewhat simplified by the use of stable isotope labelled derivatisation-based methods [43,44,49], and, when considering that the derivatisation of volatile thiols appears to be essential for food and beverage analysis by LC, introducing stable isotope labelled derivatisation does not add any extra sample processing steps. Reagents for stable isotope labelled derivatisation can not only enhance the stability and detectability of thiols, just like the conventional reagents, but also provide advantages in facilitating untargeted identification and targeted quantitation based on the characteristic mass differences between the unlabelled/labelled derivative pair that are easily distinguishable by MS [55]. Stable isotope labelled derivatisation reagent pairs that have been used for thiol analysis in beverages include d_0/d_7-ω-bromoacetonylquinolinium bromide (d_0/d_7-BQB) for beer (Table 1, Entry 18) [43,49] and d_0/d_4-acridone-10-ethyl-*N*-maleimide (d_0/d_4-AENM) for wine (Table 1, Entry 19) [44]. Both of the reagents utilise a reactive group (bromide for BQB, maleimide in AENM), an ionisable group, and an isotopically labelled group in one of the pairs. BQB derivatisation consists of rather simple sample preparation steps, which only involve drying and derivatisation (60 °C, 60 min, pH = 3.5) [49]. AENM derivatisation is faster (40 °C, 10 min, pH = 7.4), but it requires a lengthy *p*-HMB-based SPE step before derivatisation [44]. AENM-thiol adducts were reported to be stable at room temperature for at least three days [44]. Despite the advantages of stable isotope labelled derivatisations, the reagent pairs have to be synthesised, which could be a potential downside of these approaches.

4. Analytical Instrumentation

Appropriate analytical instrumentation and techniques are required for either qualitative or quantitative analysis after thiol extraction and/or derivatisation. GC or LC coupled to various types of detectors, particular MS, are the leading separation and detection techniques used for volatile thiol analysis in wine, foods, and other beverages (Table 2). GC (single or multidimensional) has been coupled with olfactometry (O), flame ionisation detector (FID), flame photometric detector (FPD), pulsed flame photometric detector (PFPD), atomic emission detector (AED), electron-capture detector (ECD), sulfur chemiluminescence detector (SCD), and MS detector (single quadrupole, Q; triple quadrupole, QqQ; ion trap, IT; and, high resolution MS with time-of-flight (TOF, including quadrupole–TOF) or Orbitrap) in the majority of cases. Reversed-phase (RP)–LC conditions coupled with MS detectors (especially QqQ) are the most common configurations for LC-based instrumentation. When using MS detection, EI or CI modes are proposed for GC–MS analysis of thiols, and electrospray ionisation (ESI) in positive mode is frequently reported for the LC–MS methods.

4.1. Analysis by GC

GC separations in the gas phase are applicable for volatile analytes, such as thiols and some of their derivatised forms (e.g., PFBBr or ETP derivatives). For injection, a purge and trap injector (PTI) [21] and cool-on-column injection (20 °C [25], 35 °C [25], 40 °C [23]) have been reported for the GC separation of native forms of thiol analytes (e.g., Table 2, Entries 3 and 31). PTI has great extracting and concentrating ability for relatively large volumes of sample (8 mL) [21,56], and cool-on-column injection is preferred for labile analytes [56], hence they are both suitable for the analysis of trace to ultra-trace volatile thiols. The enhancement of thermal stability of analytes after derivatisation allows for the use of conventional injectors and injection modes for thiols, including splitless [18,19,32], large volume (20 µL) [29,30], splitless to split [33,35], TDU in splitless mode [26], and pulsed splitless [28] injection programs. After injection, most of the separations are performed in a one-dimensional GC system installed with fused silica capillary columns, either with non-polar (e.g., BPX-5 [22], DB-5ms [32]) or polar (e.g., HP-Innowax [19], DB-Wax [35]) stationary phases. Selecting the right column for thiol analysis still requires practical trial-and-error approaches, even though multiple options for GC capillary columns are available (Table 2). For example, large volume injection of PFBBr derivatives followed by separation on a VF-5ms column showed problematic chromatographic behaviour (dirty, distorted, broadened, and delayed peaks) and switching to a column with a more polar phase did not resolve this issue [29]. In another instance, peak interferences and tailing when separating PFBBr derivatives on a DB-5 column were overcome by using a DB-FFAP column [33]. In the case of large injection volumes (10 µL), a column with larger internal diameter (0.53 mm i.d.) was preferred [25]. Apart from one-dimensional GC, two-dimensional separations of volatile thiols have also been explored with heart cut GC or GC×GC systems (Table 2, Entry 24, 27, 31) [23,27,57]. However, it was worth noting that, even with the enhanced resolving power of GC×GC, conventional sample preparation procedures without specific chemical derivatisation failed to detect a targeted thiol (4-MSP) due to the high background noise [27].

The detectors of choice in GC applications are normally associated with the analytical aims of the methods; that is, whether for identification or quantitation purposes. O, FID, FPD, PFPD, SCD, and Q-TOF–MS appear to serve as detectors for identification purposes given the sensitivity and selectivity of detectors towards ultra-trace volatile thiols, whereas Q, QqQ, and ITMS are regularly used for quantitation (and can be coupled to GC–O as well). From some of the early work that was focused on screening/discovering volatile thiols in foods and beverages (e.g., Table 2, Entry 1) to as recent as 2017 (e.g., Table 2, Entry 30), GC–O has frequently been utilised to locate odour zones of interest and provide the odour quality of the analytes being isolated [12,21,23]. GC–O also serves as an important criterion for the identification of aroma compounds, and quite remarkably, the human olfactory organ has demonstrated greater sensitivity for certain thiols during GC–O analysis than PFPD or MS [12]. Such ultra-sensitivity towards volatile thiols has been related to specific thiol olfactory receptors (e.g., OR2T11, OR2W1, and OR2C1) in humans [58,59].

Table 2. Instrumental methods reported for potent volatile thiols in wine, foods, and other beverages.

Entry No. [1]	No. of Thiols	Year	Matrix	Analyte Form [2]	Analytical Instrumentation	Aim [3]	Major Separation Parameters [4]	ME [5]	LOD [6]	RSD [7] (%)	Recovery [8] (%)
1	1	1995 [21]	Wine	Free	GC–O, –FPD, –EI–MS	ID	• Multiple columns	–	–	–	–
2	5	1998 [22]	Wine	Free	GC–EI–MS	QT	• BP20 (50 m × 0.22 mm, 0.25 µm)	–	–	4–10	75–80
3	3	2003 [25]	Wine	Free	GC–NCI–ITMS/MS, –AED	QT	• DB WAX (30 m × 0.25 mm, 0.5 µm) for MS • DB WAX (30 m × 0.53, 0.5 µm) for AED	–	⇔	<12	–
4	12	2006 [12]	Beer	Free	GC–O, –PFPD, –FID, –EI–MS	ID	• CP-Sil 5 CB (50 m × 0.32 mm, 1.2 µm) or FFAP CB (25 m × 0.32 mm, 0.3 µm) for O, FID, PFPD • DB CP-Sil 5 CB-MS (50 m × 0.32 mm, 1.2 µm) for MS	–	–	–	–
5	2	2006 [18]	Wine	Deriv.	GC–ECD,–NCI–MS	QT	• VF-5ms (20 m × 0.15 mm, 0.15 µm)	Y	<	10–20	–
6	2	2007 [60]	Wine	Free	GC–EI–MS	QT	• INNOwax (30 m × 0.32 mm, 0.25 µm) connected to HP-1 (10 m × 0.32 mm, 0.25 µm)	N	>	–	≈100
7	4	2007 [29]	Wine	Deriv.	GC–CI–MS	QT	• VF-5ms (20 m × 0.15 mm, 0.15 µm)	Y/N	<	10–17	–
8	5	2008 [30]	Wine	Deriv.	GC–CI–MS	QT	• VF-5ms (20 m × 0.15 mm, 0.15 µm)	Y	<	1–20	47–123
9	2	2008 [61]	Wine	Free	GC–EI–MS	QT	• TR-5MS (30 m × 0.25 mm, 0.25 µm)	N	⇔	–	–
10	1	2008 [26]	Cheese	Free	GC–O, –PFPD, –EI–MS	ID, QT	• DB-XLB (30 m × 0.25 mm, 0. 5 µm) • HP-5ms (30 m × 0.25 mm, 0.25 µm)	–	–	–	>70
11	5	2009 [62]	Wine	Free	GC–EI–ITMS	QT	• DB-WAXetr (60 m × 0.25 mm, 0.25 µm)	–	⇔	6.5–12.3	28–123
12	3	2009 [31]	Wine	Deriv.	GC–CI–MS	QT	• Optima Wax (30 m × 0.25 mm, 0.25 µm)	N	<	<10	–
13	1	2011 [32]	Wine	Deriv.	GC–EI–MS	QT	• DB-5ms (60 m × 0.25 mm, 0.25 µm)	N	<	<2.5	–
14	3	2013 [19]	Wine	Deriv.	GC–EI–MS	QT	• HP-INNOwax (60 m × 0.25 mm, 0.25 µm)	N	⇔	1.9–17	94–112
15	7	2013 [37]	Olive oil	Deriv.	HPLC–ESI–Orbitrap MS	QT	• Luna C18 (150 mm × 2.1 mm, 5 µm) • A: 10 mM ammonium formate in water • B: 10 mM ammonium formate in MeOH	Y	<	≈13	79–20
16	1	2014 [43]	Beer	Deriv.	LC–ESI–MS/MS LC–Q–TOF	ID	• VP-ODS column (150 mm × 2.0 mm, 5 µm) • A: 0.1% formic acid in water • B: 0.1% formic acid in MeOH	–	–	–	–
17	1	2014 [35]	Wine	Deriv.	GC–EI–MS/MS	QT	• DB-WAX (60 m × 0.25 mm, 0.25 µm)	N	<	15	99–102
18	3	2015 [33]	Wine	Deriv.	GC–EI–MS	QT	• DB-FFAP (30 m × 0.25 mm, 0.25 µm)	N	⇔	5–11	90–109
19	5	2015 [63]	Wine	Free	GC–MS/MS(QqQ)	QT	• BP20 (2 m × 0.25 mm, 0.22 µm) connected to ZB-1ms (60 m × 0.25 mm, 1 µm)	N	<	5–18	86–110
20	3	2015 [34]	Beer, hops, wort	Deriv.	GC–EI–Q–TOF–MS/SCD GC–EI–MS/MS(QqQ)	ID, QT	• DB-WAX (30 m × 0.25 mm, 0.25 µm) • DB-WAX (15 m × 0.25 mm, 0.25 µm)	–	<	1.3–7.2	99–101
21	2	2015 [36]	Wine	Deriv.	UHPLC–ESI–MS/MS(QqQ)	QT	• Acquity UPLC BEH C18 (100 mm × 2.1 mm, 1.7 µm) • A: 10 mM ammonium acetate in water • B: MeOH:MeCN:isopropanol (49:49:2)	Y/N	<	0.6–11.9	98–128
22	5	2015 [7]	Wine	Deriv.	HPLC–ESI–MS/MS(QqQ)	QT	• Alltima C18 (250 mm × 2.1 mm, 5 µm) • A: 0.5% aqueous formic acid • B: 0.5% formic acid in acetonitrile	–	⇔	<8.5	94–103

Table 2. *Cont.*

Entry No. [1]	No. of Thiols	Year	Matrix	Analyte Form [2]	Analytical Instrumentation	Aim [3]	Major Separation Parameters [4]	ME [5]	LOD [6]	RSD [7] (%)	Recovery [8] (%)
23	1	2016 [64]	Wine	Free	GC–EI–MS/MS (QqQ)	QT	• ZB-1ms (60 m × 0.25 mm, 1 µm)	–	<	9	–
24	2	2017 [57]	Wine	Free	GC–EI–MS/MS (QqQ)	ID	• ZB-1ms (60 m × 0.25 mm, 1 µm)	–	–	–	–
25	6	2017 [28]	Beer, hops	Free	GC–EI–MS/MS(QqQ)	QT	• InertCap Pure-WAX (30 m × 0.25 mm, 0.25 µm)	–	<	2.8–8.4	74–113
26	6	2017 [44]	Wine	Deriv.	UHPLC–ESI–MS/MS(QqQ)	QT	• Eclipse Plus C18 column (50 mm × 2.1 mm, 1.8 µm) • A: 0.1% formic acid in 5% aqueous MeCN • B: 0.1% formic acid in MeCN	N	⇔	≤3.5	≥78
27	1	2017 [27]	Hops	Free	GC×GC–Q–TOF Heart cut 2D-GC–CI–ITMS	QT	• Q–TOF: 1st GC: DB-FFAP (30 m × 0.25 mm, 0.25 µm), 2nd GC: DB-5 (2 m × 0.15 mm, 0.30 µm) • ITMS: 1st GC: FFAP (30 m × 0.32 mm, 0.25 µm), cool-on-column injection; 2nd GC: DB-1701 (30 m × 0.25 mm, 0.25 µm)	N	<	<15	109 ± 6 104 ± 4
28	2	2018 [41]	Wine	Deriv.	HPLC–ESI–MS/MS(QqQ)	QT	• Polysaccharide Amylose-1 column (150 mm × 2.0 mm, 3 µm) • A: 5 mM aqueous ammonium bicarbonate • B: MeCN	Y/N	<	<8	90–110
29	8	2018 [45]	Coffee bean, cookie, fried nut, biscuit	Deriv.	HPLC–FLD-APCI-MS	QT	• Eclipse XDB-C18 column (150 mm × 4.6 mm, 5 µm) • A: 30% aq. MeCN • B: MeCN	N	>	4.98	86–97
30	4	2018 [42]	Wine	Deriv.	UPC^2–MS/MS(QqQ)	QT	• BEH 2-EP column (100 mm × 3 mm, 1.7 µm) • Solvent: CO_2 and MeOH	N	<	8–18	94–119
31	11	2019 [23]	Fruit, wine	Free	GC–O, –FID, –SCD, GC×GC–Q–TOF	ID, QT	• DB-FFAP (30 m × 0.32 mm, 0.25 µm) • 1st GC: DB-FFAP (30 m × 0.25 mm, 0.25 µm), 2nd GC: DB-17ms (2 m × 0.18 mm, 0.18 µm)	–	–	–	–

[1] Entry number in bold indicates the method is a stable isotope dilution assay (SIDA). [2] free: analytes in free thiol form; deriv.: analytes in thiol derivative form. [3] ID: identification, QT: quantitation. [4] GC column dimension expressed as (length × internal diameter, film thickness; LC column dimension expressed as (length × internal diameter, particle size); A: mobile phase A; B: mobile phase B; MeCN: acetonitrile; MeOH: methanol. [5] ME: matrix effect; Y: ME existed, N: ME not evident; Y/N: ME observed for some analytes; –: not evaluated. [6] LOD expressed in comparison to the odour detection thresholds (ODT) of the analytes; <: LOD < ODT; >: LOD > ODT; ⇔: methods involved multiple analytes where LOD > ODT for some analytes and LOD < ODT for others; –: not reported. [7] RSD: repeatability (%); –: not reported. [8] –: not reported.

FPD [21], PFPD, and SCD [23,34] are important sulfur-selective detectors [65] that are also useful when screening for volatile thiols. These detectors record signal responses of sulfur atoms in the compounds of interest, which provides preliminary chromatographic information. For instance, the retention indices that were calculated upon analysing linear thiol compounds with PFPD have been used for identification purposes (Table 2, Entry 10) [26]. Similar to FPD, SCD has also been applied for screening volatile thiols due to the demonstrated high selectivity and sensitivity (absolute limit of detection below 1 ng, Table 2, Entry 31) [23]. The chromatographic conditions (e.g., GC column and oven program) that are obtained from using detectors such as FPD can then be applied for GC–MS quantitation, as in the case of thiols in soy sauce [66]. Being less expensive to purchase and less complicated to operate and maintain are other practical reasons for using these detectors for routine thiol screening. For thiol quantitation, AED has been evaluated for three volatile thiols in wines (Table 2, Entry 3) and showed good detection performance for 4-MSP, but was not suitable for 3-SH and 3-SHA due to co-elution problems with the IS used [25]. Even so, the LOD of 5 ng/L with AED detection was a few times higher than the ODT of 4-MSP (0.8 ng/L) [25].

MS detectors are still required because of their indispensable ability of obtaining mass spectra for the identification of analytes of interest and the superior quantitation capacity, despite the highly specific coverage of FPD, PFPD, and SCD towards sulfur-containing volatiles. Other than retention index, MS spectra of peaks of interest can be compared to those in commercial databases (e.g., NIST [67] or Wiley [68]), or an in-house thiol database [23]. Accordingly, identity confirmation of new thiols then necessitates the synthesis of reference standards if they are not readily available from commercial suppliers [23,64,69]. The majority of GC-based quantitative analyses are conducted with MS detectors, with electron ionisation (EI) (Table 2, multiple entries) or less frequently used chemical ionisation (CI) (Entries 3, 5, 7, 8, 12) [18,25,29–31] being applied for volatile thiol detection. Selected ion monitoring (SIM) has been frequently used over full scan mode, with one quantifier ion and desirably at least two other qualifier ions when single stage MS is applied for quantitative analysis. MS/MS is used in multiple reaction monitoring (MRM), selected reaction monitoring (SRM), or consecutive reaction monitoring (CRM) mode, depending on the detector (QqQ and ITMS), which provides better selectivity and sensitivity than single stage MS. Taking 3-SH, which is probably the most evaluated thiol in wine and beer, as an example, many methods have used MS for its detection. When detected in the native form, SIM ions at *m*/*z* 134, 100, 82, and 67 [19,60] were chosen as the qualifiers and quantifier for single stage MS, whereas transitions *m*/*z* 134→82 and 100→82 were the pairs used for MRM with MS/MS [28]. If 3-SH has been derivatised, ions that were selected in EI–MS have higher *m*/*z*, for instance, 314, 181, and 133 for the PFBBr derivative [32] and 232, 187, and 132 for the ETP derivative [19]. The reported LOD values for 3-SH when using single stage MS were 30 ng/L [32], 69 ng/L [60], 7 ng/L [29], and 2 ng/L [30], which were higher than those values that were obtained with MS/MS (1.9 ng/L [28] and 0.7 ng/L [25]).

4.2. Analysis by LC

In recent years, LC-based analytical methods have emerged as promising and novel alternatives for volatile thiol analysis (both screening and quantitation) in foods and beverages (Table 2, Entries 15, 16, 21, 22, 26, 28, 29). Although LC has been used to assess non-volatile thiols, such as cysteine and glutathione, in biological samples for some time [70–72], the LC analysis of volatile thiols in foods and beverages has arisen more recently. The high volatility and low abundance of the analytes meant that they were more compatible with GC separations rather than LC with liquid-phase MS detection. Even when bypassing the chromatographic system, underivatised 3-SH was undetectable by direct liquid infusion MS in positive or negative ion mode [43], so to facilitate the potential application of LC to the analysis of volatile thiols, the analytes first have to be converted into non-volatile derivatives with suitable reagents. Indeed, with promising derivatisation reagents having been specifically suggested (Section 3.2.2 and Table 1), complementary LC methods have necessarily been developed in

tandem (Table 2, Entries 16, 21, 22, 26, 28), some of which even rival the analytical performance of the GC methods.

RP–LC separations with C18 stationary phases are commonly used for derivatised volatile thiols. The injection volumes and flow rates (0.20–0.40 mL/min) varied slightly, depending on the column and instrument used (Table 2). For example, slightly higher flow rates (0.35 mL/min and 0.40 mL/min) were utilised in ultra-performance (UP) LC with a column containing 1.7 µm particles [36]. Mobile phases are the same as commonly used for RP–LC, for instance, aqueous MeOH [37,43] and aqueous acetonitrile (MeCN) [7,41,44]. Proposed RP–LC methods have been able to resolve the analytes in a relatively shorter period of time (e.g., 17 min with UPLC [36]) compared to GC methods (e.g., 55 min [33]).

MS is still the preferred detection technique for LC methods, even when derivatisation introduces a chromophore into volatile thiols (which may facilitate UV [41] or fluorescence detection [36,45]) (Table 2). In fact, detection is typically undertaken with MS/MS, and instruments, including QqQ, Q-TOF, and Orbitrap operated in ESI mode have been described for identifying or quantitating volatile thiols, with superb sensitivity and selectivity. More specifically, with a QqQ, MRM [7,36,41] is the main mode that is utilised for quantitation and double precursor ion scan (DPIS) mode has been applied more for qualitative purposes [43,49]. The selection of mass transition pairs for MRM typically includes one quantifier pair and at least one qualifier pair as the minimum requirement, with the transitions being chosen from direct infusion product ion MS experiments of the reference standard derivatives [7]. MRM of unique mass transition pairs provides a cleaner chromatographic background, which directly results in greater sensitivity [44]. QqQ with MRM mode has been employed for thiol derivatives that were obtained either with conventional derivatisation reagents [7,33,36,41] or stable isotope derivatisation reagent pairs [44].

DPIS combined with stable isotope labelled derivatisation has been investigated for thiol profiling in beers (Table 2, Entry 16) [43,49]. Admittedly, the thiols that were tentatively identified in beer with this method were biological thiols, but this could be of potential use for volatile thiols analysis. Light- and heavy-labelled thiol derivatives have characteristic ions with a fixed mass shift that can be distinguished by DPIS with QqQ by employing stable isotope labelled derivatisation, and identities can then elucidated by product ion scan and Q–TOF [43]. Only peak pairs from extracted DPIS chromatograms with the same retention time and peak intensity are considered and relative quantitation can be readily achieved by varying the ratios of light- and heavy-labelled samples being mixed [43]. This overall approach could be an attractive option for volatile thiol discovery in foods and beverages.

The application of Orbitrap MS for volatile thiol analysis has been reported for olive oil [37], wine [38], beer [38], and roasted coffee [40]. While using Se-containing ebselen derivatisation, thiol derivatives inherit the selenium isotopic pattern (^{80}Se, ^{78}Se) and the corresponding accurate masses that were recorded by using Orbitrap MS (mass error tolerance <2 ppm) have been used for tentative identification and quantitation (when thiol reference standards were used) [37]. An Orbitrap MS generated chromatograms with almost no background noise due to its superior sensitivity and selectivity, and the resulting limit of quantitation values were extremely low for 3-SH, at 0.1 ng/kg in olive oil (Table 2, Entry 15) [37], and 0.01 ng/L in wine [38].

4.3. LC vs. GC

Overall, the LC methods have been demonstrated to be more sensitive, selective, and faster than GC approaches for quantitative volatile thiol analysis in foods and beverages. Perhaps more importantly, another significant advantage of LC-based methods is that the sample preparation steps are less complicated, markedly so in a number of cases. Furthermore, LC with MS/MS in DPIS mode has potential for untargeted thiol profiling and screening (Table 2, Entry 16). However, as discussed in the next section, the LC–MS approaches have to be treated carefully (particularly for quantitation) to solve analytical challenges from matrix effects [73].

GC approaches generally involve more complicated sample preparation, but are still attractive when compared to LC–MS methods, particularly with respect to the aroma qualities that are obtained by GC–O, which can provide valuable information for thiol identification (or verification) [23] and related sensory studies [74]. In addition, multidimensional GC offers great separation power [75], and with fast GC in the second dimension improving the detection limits and reducing the matrix background, GC×GC coupled with different types of detectors could serve as a powerful tool for discovering new thiols. For instance, GC×GC–TOF–MS has been recently used for thiol screening in wine and fruit, where a total of 11 volatile thiols were identified (Table 2, Entry 31) [23].

GC and LC based methods for thiol analysis are both very versatile and they have great potential in identification and quantitation. The choice of method is often based on instrument availability in the given laboratory, along with capital, operating, and maintenance costs, and most importantly, the analytical methods that are required to address the aims of the research.

4.4. Other Instruments

Beyond GC and LC, an ultraperformance convergence chromatography–tandem mass spectrometry (UPC^2–MS/MS) method has been developed to quantitate volatile thiols in wine [42] after DTDP derivatisation [7]. This proposed method uses supercritical CO_2 as the primary mobile phase (along with MeOH) at high flow rate (1.5 mL/min), with this convergence chromatography approach providing great efficiency (7 min run time per sample) and being coupled with QqQ (MRM mode) for sensitive detection (with all LODs below the thiol ODT values). Such instrumentation is not common in many laboratories, which may limit application of the method, although this novel approach offers potential high throughput thiol analysis without compromising sensitivity [42].

4.5. Matrix Effects and Quantitative Analysis

With the advances in chromatography and MS capabilities, instrumentation with higher separation ability and detection sensitivity has become more available, which leads to a greater number of analytical methods being reported for volatile thiols in more foods and beverages. However, matrix effects in trace analysis have been noted in modern analytical methods that were developed for agricultural samples [76]. In addition, the concentrations of these unstable compounds are at trace to ultra-trace levels and in complex matrices, so their quantitative analysis has to be treated with great care to avoid inaccurate or inconclusive outcomes regarding these potent odour-active molecules.

Matrix effects should be critically evaluated when developing quantitative methods to achieve accurate and reliable results. It is well known that matrix interferences have significant impacts on the extraction, separation, detection, and consequently the quantitation of analytes. This is of particular importance, given both the reactivity and trace concentrations of potent volatile thiols. Matrix effects have been clearly noticed and explored during method development for volatile thiol analysis [18,41]. One way to distinguish the extent of matrix effects is to compare the slopes of calibration curves that have been obtained from different matrices [18,31]. While using this approach, a matrix effect was evidently observed in model wine vs. real wines [18,41] and oxidised vs. non-oxidised wines [30], with various impacts during the extraction, separation, or detection steps potentially leading to differences in the results. For instance, undiluted solvent-assisted flavour evaporation (SAFE) distillate [23], competitive absorption on an HS–SPME fibre [25], or ESI–MS signal enhancement/suppression [77] could cause large matrix effects.

Even when using matrix-matched calibration approaches, the choice of IS can be extremely important in minimising or compensating for matrix effects. Many compounds with similar properties to volatile thiols that showed a negligible matrix effect have been suggested as internal standards, such as 4-methoxy-2-methyl-2-mercaptobutane [22] and 6-sulfanylhexan-1-ol (6-SH) [60]. Even so, stable isotope labelled IS are the best option in a stable isotope dilution assay (SIDA), which is arguably the most accurate analytical approach, and, in most cases, can efficiently eliminate a matrix effect by compensating extraction, separation, and detection variabilities due to the almost identical

properties between analytes and their stable isotope, labelled analogues [78]. SIDA has been widely employed for LC–MS [7,36,41] and GC–MS [19,25,27,30–33] methods for volatile thiols analysis, and both ^{2}H [7,19,25,30,32,33,36,41] and ^{13}C labelled [27] internal standards have been used in various cases (Table 2, Entries with bolded numbers). Typically, the ideal degree of atom labelling should be greater than two [76], but d_2-3-SHA [36] and d_2-3-SH [30,36] have been reported in a few cases as stable isotope labelled IS. Additionally, the elution of stable isotope labelled IS generally occurs slightly earlier than analytes depending on the extent of isotope incorporation, and such examples can be seen for both GC [31,32] or LC [7,41]. However, the co-elution of analytes and stable isotope labelled IS would be desirable to completely compensate for matrix effects [78].

Indeed, SIDA does not guarantee the complete elimination of matrix effects for thiol analysis in wine. It has been shown that deuterated IS spiked into an oxidised wine somehow underwent a faster oxidation than native analyte, which led to a persistent matrix effect [30]. On the other hand, phenols or pigments in red wines have a potentially significant impact on LC–MS/MS signal responses, as evident when comparing calibration curve slopes from different wine and model wine matrices [41]. However, the main limitation with SIDA for thiol analysis is that stable isotope labelled internal standards are often not commercially available or they are expensive to obtain [79]. The same issue applies to stable isotope labelled derivatisation, but it does offer a straightforward and accurate method for quantitative analysis [44] once the reagents have been prepared. Finally, even though stable isotope labelled IS cannot always correct for matrix effects occurring during detection, they are still ideal for overcoming variabilities in extraction, reaction, or adsorptive losses, for instance, which is essential for accurate quantitative analysis.

5. Conclusions and Outlook

This review presents an up-to-date overview of the analysis of potent volatile thiols in foods and beverages, with a focus on wine analysis, because that is where many of the methodological advances have arisen. It covers topics from traditional selective extraction, chemical derivatisation (for LC or GC), chromatographic and MS instrumentation, matrix effects, and quantitation considerations. The identification of new volatile thiols and quantitation of known thiols have been made possible over the past three decades thanks to the development of specific thiol extraction methods in combination with sensitive analytical instrumentation. The major observations regarding the current state of potent volatile thiol analysis are: (1) extractions consisting of some form of thiol derivatisation are a popular choices due to their efficiency and simplicity; (2) GC coupled to different detectors (e.g., O, sulfur selective detectors, and MS) are still of considerable utility for discovering new thiol odorants, although newer LC–MS/MS approaches with thiol-specific derivatisation and precursor ion scan experiments also look promising; (3) recently developed quantitative LC–MS methods usually outperform existing GC–MS counterparts when considering the whole protocol, from isolation to analysis; and, (4) SIDA approaches are frequently applied for reliable quantitation.

Undoubtedly, the analysis of potent volatile thiols has been greatly advanced; however, there is still room to further improve the analytical performances to develop faster, more cost-effective, and greener methods that can provide more comprehensive information. In terms of specific extraction, currently available techniques could be coupled with the popular MS detection for the sensitive analysis of volatile thiols. For instance, inspiration could be drawn from the analysis of low molecular weight thiols in water as their *p*-HMB–thiol complexes by LC–ESI–MS/MS after online SPE preconcentration [16]. This offers a stable, sensitive, and selective means for thiol analysis, although the use of mercury features again, and its application to potent volatile thiols in foods and beverages would still need to be investigated. New approaches that apply novel extraction materials should be continuously designed for low cost and effective isolation of volatile thiols, apart from maximising the potential of currently available extraction methods. For example, the potential of novel molecularly imprinted polymer SPME fibres for volatile analysis keeps growing [80], and it seems plausible that SPME fibre coatings could also be customised for volatile thiol extraction. The same can be suggested for SPE

sorbent materials, given the recent example of an Ag^+ based SPE cartridge that has been proposed for volatile thiol analysis [28].

Regarding the future of chromatographic separation in the analysis of volatile thiols, emerging trends include the testing of novel stationary phases and new separation techniques. With respect to the stationary phases, new generation superficially porous silica LC columns have been made commercially available and reported for the separation of a variety analytes [81]. These columns are compatible with conventional HPLC instrumentation (including MS), and the chemistries of the stationary phases are similar to conventional C18 columns, but they offer faster and more efficient separation [81]. Other than new generation LC columns, there are also other stationary phases of potential interest. As an example, a polysaccharide-based chiral LC column has not only demonstrated good analytical performance for separation of thiol enantiomers, but it also revealed the possibility to simultaneously analyse other important achiral volatile thiols [41]. Alternative separation techniques should be also considered, besides improving separation through new columns. The excellent separation efficiency achieved for volatile thiol derivatives by ultraperformance convergence chromatography [42] offers a glimpse of what the future may hold in terms of speed and sensitivity.

The trend for detection is that QqQ and high resolution (Q–TOF or Orbitrap) MS will be more prevalent for both the identification and quantitation of potent volatile thiols. The use of QqQ for LC–MS/MS analyses of thiols has grown strongly in recent years and offers a number of benefits in comparison to GC–MS methods. Future advances should take advantage of the unique fragmentation patterns (with diagnostic fragmented ions) and precursor ion scan mode in LC–MS/MS for the preliminary screening of unknown volatile thiols. Q–TOF and Orbitrap MS detection will also be ideal for such purposes (based on unique isotope pattern of diagnostic ion) due to their unparalleled resolution power and the ability to determine molecular formulas. A few recent reports [37,38,43,49] have explored this non-targeted approach, but ongoing research is required to better answer the complex sensorial, (bio)chemical, and microbiological questions that surround potent thiol odorants in foods and beverages.

Author Contributions: Conceptualisation: L.C., D.L.C., and D.W.J.; writing—original draft preparation, L.C.; writing—reviewing and editing, L.C., D.L.C., and D.W.J.

Funding: L.C. was a recipient of the joint scholarship of UA and China Scholarship Council (CSC201506350012), and was also supported by a Wine Australia supplementary scholarship (AGW Ph1512). The Australian Research Council Training Centre for Innovative Wine Production (www.ARCwinecentre.org.au; project number IC170100008) is funded by the Australian Government with additional support from Wine Australia and industry partners. The University of Adelaide is the member of the Wine Innovation Cluster.

Conflicts of Interest: The authors declare no conflict of interest.

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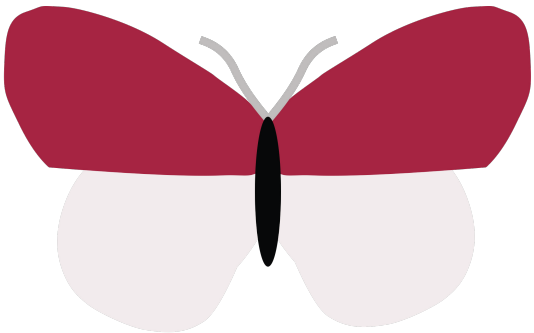
Chapter 3

Chiral analysis of 3-sulfanylhexan-1-ol and 3-sulfanylhexyl acetate in wine by high-performance liquid chromatography–tandem mass spectrometry.

Analytica Chimica Acta 2018, 998, 83–92. DOI.

Chen L., Capone D. L., Jeffery D. W.



Title: **Chirality**.

Illustration of a butterfly of Adelaide origin.

Statement of Authorship

Title of Paper	Chiral analysis of 3-sulfanylhexan-1-ol and 3-sulfanylhexyl acetate in wine by high-performance liquid chromatography–tandem mass spectrometry
Publication Status	☑ Published ☐ Accepted for Publication ☐ Submitted for Publication ☐ Unpublished and Unsubmitted w ork w ritten in manuscript style
Publication Details	Chen, L.; Capone, D.L.; Jeffery, D.W. (**2018**) Chiral analysis of 3-sulfanylhexan-1-ol and 3-sulfanylhexyl acetate in wine by high-performance liquid chromatography–tandem mass spectrometry. *Analytica Chimica Acta*, **998**, 83–92.

Principal Author

Name of Principal Author (Candidate)	Liang Chen
Contribution to the Paper	Designed and performed chemical synthesis. Analysed HPLC-MS, HPLC-MS/MS, and NMR data for characterisation of synthesised compounds. Optimised the chromatographic conditions for the analytical method. Prepared the samples by solid-phase extraction and analysed samples by HPLC-MS/MS. Processed HPLC-MS/MS chromatograms, interpreted data, and carried out statistical analyses. Produced a complete first draft and revised the manuscript.
Overall percentage (%)	70%
Certification:	This paper reports on original research I conducted during the period of my Higher Degree by Research candidature and is not subject to any obligations or contractual agreements with a third party that would constrain its inclusion in this thesis. I am the primary author of this paper.
Signature	Date 31-07-2018

Co-Author Contributions

By signing the Statement of Authorship, each author certifies that:

i. the candidate's stated contribution to the publication is accurate (as detailed above);

ii. permission is granted for the candidate in include the publication in the thesis; and

iii. the sum of all co-author contributions is equal to 100% less the candidate's stated contribution.

Name of Co-Author	Dimitra L. Capone
Contribution to the Paper	Supervised the study, and led the analytical experimental design and validation. Collected, processed, and interpreted data. Wrote, edited, and revised the paper.
Signature	Date 31/07/2018

Name of Co-Author	David W. Jeffery
Contribution to the Paper	Conceived the original concept of the study. Supervised and directed the project. Interpreted the data. Wrote, edited, revised, and submitted the paper.
Signature	Date 31/07/2018

Please cut and paste additional co-author panels here as required.

Analytica Chimica Acta 998 (2018) 83–92

Contents lists available at ScienceDirect

Analytica Chimica Acta

journal homepage: www.elsevier.com/locate/aca

Chiral analysis of 3-sulfanylhexan-1-ol and 3-sulfanylhexyl acetate in wine by high-performance liquid chromatography–tandem mass spectrometry

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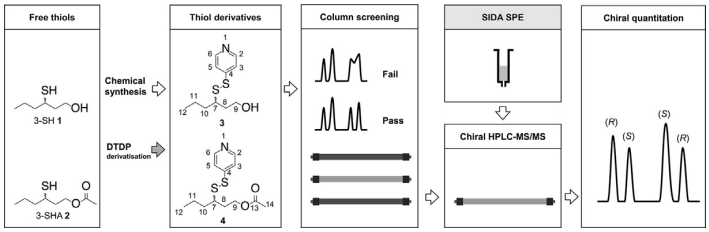
HIGHLIGHTS

- A chiral HPLC-MS/MS method was developed for analysis of thiol enantiomers after derivatisation and extraction from wine.
- The validated method yielded excellent performance characteristics for quantitation of the enantiomers of 3-SH and 3-SHA.
- The potential exists for simultaneous determination of several important achiral thiols.
- Enantiomer profiles of 3-SH and 3-SHA were determined for a selection of commercial white, rosé, and red wines.

GRAPHICAL ABSTRACT



ARTICLE INFO

Article history:
Received 17 August 2017
Received in revised form
17 October 2017
Accepted 21 October 2017
Available online 27 October 2017

Keywords:
Aroma
Varietal thiols
Enantiomers
Chirality
Derivatisation
Stable isotope dilution analysis

ABSTRACT

A number of polyfunctional thiols are well-recognised as some of the most potent aroma compounds in nature and have been identified in various foods, and beverages such as wine. These potent character impact compounds are present at nanogram-per-litre levels and are particularly challenging to measure. Where present, enantiomeric forms having different odour qualities further complicate the analysis. In this work, a chiral high-performance liquid chromatography–tandem mass spectrometry (HPLC-MS/MS) method using stable isotope dilution analysis (SIDA) has been developed and validated for the individual enantiomers of 3-sulfanylhexan-1-ol (3-SH, **1**) and its *O*-acetate, 3-sulfanylhexyl acetate (3-SHA, **2**), in wine after extraction as their 4-thiopyridine derivatives. Authentic derivatised thiols were first synthesised and used for chiral column screening and method development with polysaccharide-based HPLC phases. Validation of the method using a Lux Amylose-1 column for quantification of the enantiomers of **1** and **2** gave linear calibrations, and excellent figures for accuracy (recovery: 90%–111%, Z-score: −1.8–1.9), precision (RSD_r<8%), and limits of detection (0.7 ng L^{-1} or less) in model media and different wine matrices. The newly developed method was applied to determine the enantiomer profiles of **1** and **2** in range of commercial wines.

Abbreviations: 3-SH, 3-sulfanylhexan-1-ol; 3-SHA, 3-sulfanylhexyl acetate; 4-MSP, 4-methyl-4-sulfanylpentan-2-one; BMT, benzenemethanethiol; CSP, chiral stationary phase; DTDP, 4,4′-dithiodipyridine; FT, 2-furfurylthiol; ME, matrix effects; SIDA, stable isotope dilution analysis.

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https://doi.org/10.1016/j.aca.2017.10.031
0003-2670/

1. Introduction

Aroma and flavour analysis is a complex and important scientific field that contributes to the understanding of volatile compounds that are crucial to sensory properties of (and preference for) foods and beverages. Of particular interest are certain key odorants, designated character impact compounds [1], which can be associated with the characteristic aromas of a product such as coffee, pepper, or wine. Indeed, passionfruit and citrus aromas emanating from Sauvignon blanc wines are primarily due to polyfunctional thiols, especially 3-sulfanylhexan-1-ol (3-SH, **1**) and related 3-sulfanylhexyl acetate (3-SHA, **2**), which are potent volatiles found at nanogram-per-litre concentrations [2,3]. Notably, these varietal thiols, as they are also known, are present as pairs of enantiomers due to the chiral centre at position C-3. The racemic mixtures of **1** and **2** have reported odour detection thresholds in hydroalcoholic solution of 60 and 4.2 ng/L, respectively [3], but the enantiomers exhibit different detection thresholds and aroma qualities (Table 1) [4], such that the sensory profiles of wine can be affected by different enantiomer ratios [5]. However, quantitative data for the enantiomers of **1** or **2** in wines are scarce and understanding of their sensory effects in different wines in relation to their concentrations, and of the factors affecting their distribution profiles, is currently very limited.

Perhaps the main reason that enantiomers of **1** and **2** have been ignored by wine chemists is due to the analytical challenges associated with the ultra-trace occurrence of these reactive thiols [7]. This is coupled with the demanding task of synthesising and purifying authentic standards and labelled analogues. To our knowledge, work addressing these enantiomers in wine is apparently limited to a 2006 report of Tominaga et al., in which enantiomers of **2** were not fully resolved and no quantitative data were given for either set of enantiomers [4], and to a Master thesis, where only **1** was analysed [6]. In those studies the thiols were isolated using *p*-hydroxymercuribenzoate (*p*-HMB) [4] or derivatised with 2,3,4,5,6-pentafluorobenzyl bromide (PFBBr) [6] and subjected to GC-MS analysis using cyclodextrin-based capillary columns. The only reported concentrations for enantiomers of **1** ranged from 368 to 1129 ng L^{-1} for (*S*)-**1** and 275–1031 ng L^{-1} for (*R*)-**1** in a small survey of predominantly commercial Australian Sauvignon blanc and Chardonnay wines (Table 1), with ratios of approximately 50:50 but slightly favouring the (*S*)-enantiomer in most cases (although the two botrytised wines included in Table 1 were reported to be closer to 60:40 for (*S*):(*R*)) [6]. A similar uniform ratio for **1** (with slight preponderance for (*S*)-**1**) and somewhat different ratio for **1** in botrytised wines (70:30 for (*S*):(*R*)) was observed in the 2006 study involving some French white wines, in which **2** was also found with (*S*):(*R*) ratios of around 70:30 [4]. Note that resolution of enantiomers of **1** and **2** has been reported for yellow passionfruit using multi-dimensional GC with flame photometric detection [8,9], but as with many methods for analysis of polyfunctional thiols, the sample preparation is tedious.

We recently reported a stable isotope dilution analysis (SIDA) method for determination of polyfunctional thiols in wine that greatly simplified the sample preparation phase. In that work, thiols were derivatised in situ with 4,4′-dithiodipyridine (DTDP) followed by solid-phase extraction (SPE) and HPLC-MS/MS analysis of the reconstituted extracts. We now report an important expansion of this method to encompass quantitation of the enantiomers of **1** and **2** using a chiral HPLC column. This necessitated synthesis of authentic thiol–4-thiopyridine disulfides of **1** and **2** (i.e., **3** and **4**, see Fig. 1) for chiral column screening and further method development. The method was fully validated in different wine matrices and was applied to a range of commercial wines to investigate the enantiomer profiles of **1** and **2**.

2. Material and methods

2.1. Chemicals

The following chemicals and reagents were purchased from commercial suppliers: 1H-benzotriazole (BtH, 99%), 4-thiopyridine, 4,4′-DTDP, acetaldehyde, formic acid, ammonium bicarbonate, ethylenediaminetetraacetic acid (EDTA), and ethylenediaminetetraacetic acid disodium salt (EDTA 2Na) (Sigma-Aldrich, Castle Hill, NSW, Australia); acetic acid (Chem-Supply, Gillman, SA, Australia); HPLC-grade acetonitrile (MeCN), ethanol (EtOH), and methanol (MeOH) (Merck, VWR, Tingalpa, QLD, Australia); C18 Bont Elut SPE cartridges (500 mg, 6 mL, Agilent, Mulgrave, VIC, Australia). Water was obtained from a Milli-Q system (Millipore, North Ryde, NSW, Australia).

The following compounds were previously prepared in-house or synthesised according to published procedures: 1-chlorobenzotriazole (BtCl) [10]; disulfide standards **3** and **4** [11]; thiol standards **1** and **2** [12]; deuterium-labelled internal standards d_{10}-**1** [13] and d_5-**2** [12]; pure enantiomers of (*R*)- and (*S*)-**1** [4], and (*R*)- and (*S*)-**2** [4].

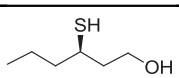
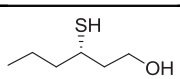
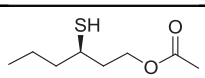
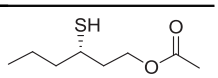
2.2. NMR spectroscopy

Proton (^{1}H) and carbon (^{13}C) nuclear magnetic resonance (NMR) spectra were recorded with a Varian spectrometer operating at 500 MHz for proton and 125 MHz for carbon nuclei, respectively. Chemical shifts were recorded as δ values in parts per million (ppm). Spectra were acquired in $CDCl_3$ at 26 °C, and the resonances were assigned by routine 2D correlation experiments.

2.3. High-resolution mass spectrometry (HRMS)

Spectra were obtained on an Agilent G6530B Q-TOF mass spectrometer with electrospray ionisation (ESI) in positive mode using solutions prepared in water at approximately 10 mg L^{-1}.

Table 1
Structures, sensory thresholds, sensory descriptors, and concentrations of enantiomers of 3-SH **1** and 3-SHA **2** previously reported in wine.

	(*R*)-3-SH	(*S*)-3-SH	(*R*)-3-SHA	(*S*)-3-SHA
Structure				
Threshold[a] (ng L^{-1}) [4]	50	60	9	2.5
Sensory description [4]	grapefruits, citrus peel	passionfruit	passionfruit	boxwood
Concentration[b] (ng L^{-1}) [6]	275–1031 (219, 2998)[c]	368–1129 (356, 4396)[c]	not reported	not reported

[a] In model wine media.
[b] Commercial Sauvignon blanc ($n = 12$) and Chardonnay ($n = 1$).
[c] Values in parentheses are for botrytised Semillon ($n = 1$) and botrytised Sauvignon blanc ($n = 1$), respectively.

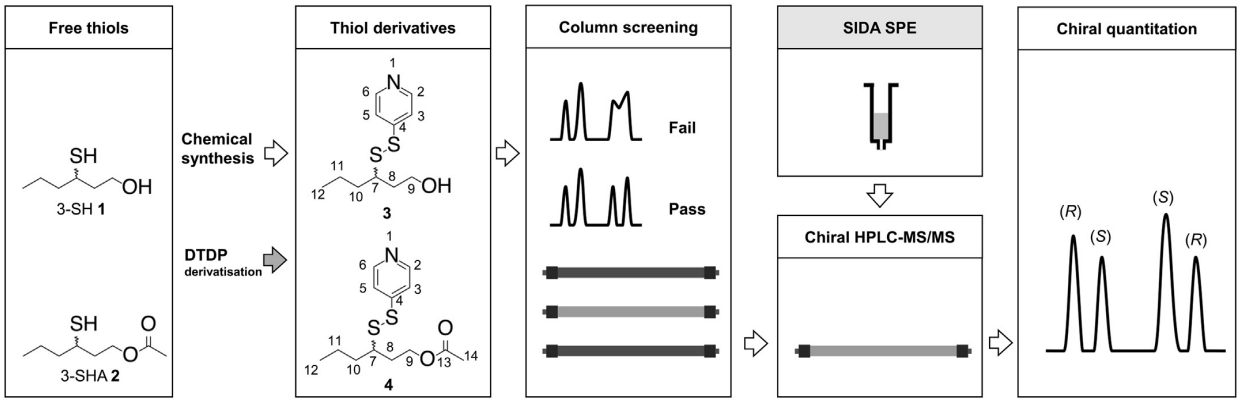


Fig. 1. Approach to resolving and determining enantiomers of 3-SH **1** and 3-SHA **2** in wine using chemical synthesis of thiol–DTDP derivatives, chiral column screening, derivatisation in wine and SPE clean-up, and precise quantitation by SIDA with chiral HPLC-MS/MS. Steps marked with grey shading were based on the report of Capone et al. [14] for racemic thiol analysis. Atom numbering of **3** and **4** relates to the numbering used for NMR structural assignments (see Figs. S2–S5 of the Supplementary material for NMR spectra).

2.4. Synthesis of standards 3 and 4 for chiral screening

2.4.1. 3-(Pyridin-4-yldisulfanyl)hexan-1-ol (*3*)

4-Thiopyridine (104 mg, 0.93 mmol) was added to dry dichloromethane (1 mL) containing BtCl (220 mg, 1.43 mmol) and BtH (109 mg, 0.91 mmol) at −78 °C and under N_2. The solution was stirred at −78 °C for 2 h and then 3-SH **1** (185 μL, 1.35 mmol) was added at −20 °C and stirring continued at this temperature for 0.5 h. The reaction was quenched with aqueous solutions of $Na_2S_2O_3$ (3.4 mL) and saturated $NaHCO_3$ (6.8 mL) with rapid stirring at 0 °C for 20 min. The reaction mixture was extracted with dichloromethane (3 × 30 mL) and the combined organic extracts were dried over $MgSO_4$ and filtered. The residual solvent was removed in vacuo to afford a white solid (468 mg), which was purified by flash chromatography on silica (10% MeCN/CH_2Cl_2, R_f = 0.50) to give compound **3** as a pale yellow oil (43 mg, 0.18 mmol, yield 19%) with purity > 95% (by ^{1}H NMR).

^{1}H NMR (500 MHz, $CDCl_3$, δ) (Atom numbering as in Fig. 1): 8.45 (2H, d, J = 5.0 Hz, $H_{2,6}$), 7.46 (2H, d, J = 5.0 Hz, $H_{3,5}$), 3.84–3.73 (2H, m, H_9), 2.99 (1H, quint, J = 6.2 Hz, H_7), 1.86 (2H, q, J = 6.7 Hz, H_8), 1.72 (1H, s, OH), 1.62–1.57 (2H, m, H_{10}), 1.54–1.41 (2H, m, H_{11}), 0.87 (3H, t, J = 7.5 Hz, H_{12}); ^{13}C NMR (125 MHz, $CDCl_3$, δ): 149.68 (C_4), 149.26 ($C_{2,6}$), 120.25 ($C_{3,5}$), 59.89 (C_9), 49.21 (C_7), 36.61 (C_8), 36.16 (C_{10}), 19.92 (C_{11}), 13.70 (C_{12}). ESI-HRMS (m/z): $[M+H]^+$ calcd. for $C_{11}H_{17}NOS_2^+$, 244.08299; found, 244.08277; HPLC-ESI-MS/MS of m/z 244 (m/z, %): 144 (100), 111 (1).

2.4.2. 3-(Pyridin-4-yldisulfanyl)hexyl acetate (*4*)

The same synthetic procedure described above was employed, using 4-thiopyridine (108 mg, 0.97 mmol), BtCl (228 mg, 1.49 mmol), BtH (115 mg, 0.97 mmol) and 3-SHA **2** (243 μL, 1.35 mmol), affording compound **4** as a colourless oil (57 mg, 0.2 mmol, 21%) with >97% purity (by ^{1}H NMR) after purification (40% MeCN/CH_2Cl_2, R_f = 0.45).

^{1}H NMR (500 MHz, $CDCl_3$, δ) (Atom numbering as in Fig. 1): 8.47 (2H, d, J = 5 Hz, $H_{2,6}$), 7.46 (2H, d, J = 5 Hz, $H_{3,5}$), 4.23–4.15 (2H, m, H_9), 2.87 (1H, quint, J = 6.2 Hz, H_7), 2.02 (3H, s, H_{14}), 1.93 (2H, q, J = 6.7 Hz, H_8), 1.63–1.57 (2H, m, H_{10}), 1.51–1.39 (2H, m, H_{11}), 0.87 (3H, t, J = 7.5 Hz, H_{12}). ^{13}C NMR (125 MHz, $CDCl_3$, δ): 171.31 (C_{13}), 149.91 ($C_{2,4,6}$), 120.74 ($C_{3,5}$), 62.12 (C_9), 49.64 (C_7), 36.42 (C_{10}), 33.15 (C_8), 21.23 (C_{14}), 20.41 (C_{11}), 14.16 (C_{12}). ESI-HRMS (m/z): $[M+H]^+$ calcd. for $C_{13}H_{19}NO_2S_2^+$, 286.09354; found, 286.09379; HPLC-ESI-MS/MS of m/z 286 (m/z, %): 226 (49), 144 (100), 143 (63), 115 (27), 111 (3).

2.5. Stock and working solutions

Individual stock solutions of racemic **1** and **2** were prepared volumetrically with pure standards in ethanol. Appropriate aliquots of the stock solutions were combined and diluted volumetrically with ethanol to yield ten different working solutions. Internal standard solution (combined deuterated IS) and pure single enantiomer solutions were prepared volumetrically in ethanol. Solutions containing free thiols had EDTA (3 g L^{-1}) added, were flushed with N_2, and stored in glassware at −20 °C. Synthetically-prepared **3** and **4** were dissolved in 10% aqueous ethanol solution and stored at 4 °C.

2.6. Wine samples

Commercial wines obtained from retail outlets were used for method validation (a Sauvignon blanc (SAB), a rosé, and a red blend) and a survey (Table S1 of the Supplementary material). Model wine (MW) solution used for validation consisted of 10% (v/v) aqueous ethanol saturated with potassium hydrogen tartrate and pH adjusted to 3.4 with tartaric acid solution.

2.7. Sample preparation

Thiols were derivatised and extracted as detailed previously [14]. A fresh set of matrix-matched calibration samples and quality control samples (QCs) were prepared with each batch of samples to be analysed. Reconstituted extracts were run immediately or stored at −20 °C before HPLC-MS/MS analysis.

2.8. Analytical method development

2.8.1. Chiral column screening

Lux Amylose-1, Amylose-2 and Cellulose-1 were screened for their chiral resolution ability using synthetic **3** and **4**. Chiral screening was performed by PhenoLogix [15] using columns that were 100–250 mm in length with 4.6 mm i.d. and 5 μm particle size (Phenomenex, Torrance, CA, USA). Mobile phase compositions used for column screening are summarised in Table S2 of the Supplementary material. Solutions of **3** and **4** were prepared in the mobile phases being tested at concentrations of 1 mg mL^{-1}, and detected at 254 nm.

2.8.2. Optimisation of enantiomer separation

Lux Amylose-1 and Amylose-2 columns (150 × 2.0 mm, 3 μm particle size, Phenomenex, CA, USA) protected by guard cartridges (4 × 2.0 mm) of the same material were selected for method development and optimisation. A ThermoFinnigan instrument consisting of a Surveyor HPLC and a LCQ Deca XP Plus mass spectrometer fitted with an electrospray ionisation source (ESI) was used. HPLC parameters trialled included: eluents consisting of different percentages of MeCN or MeOH and water, and organic additive (either formic acid, ammonium formate, or ammonium bicarbonate); flow rate at either 0.150, 0.180, 0.200 or 0.300 mL min^{-1}; column temperature at either 15, 20 or 25 °C; injection volume of 10 or 20 μL; various isocratic or gradient methods. MS data were recorded in full scan mode at *m/z* 50–1000 amu. Instrument control and data acquisition were managed using Xcalibur software (version 1.3).

2.9. HPLC-MS/MS method validation

Method validation and quantitative analyses were performed with an Amylose-1 column (150 × 2.0 mm, 3 μm particle size) protected by a guard cartridge (4 × 2.0 mm) and connected to either an Agilent 1200 HPLC (including a 1290 binary pump) equipped with an Applied Biosystems 4000 QTRAP hybrid tandem mass spectrometer or an Sciex ExionLC AD connected to a Sciex QTRAP 6500^{+} tandem mass spectrometer, both with a TurboV source (with IonDrive for the 6500) fitted with TurboIonSpray probe. Positive ESI with a source voltage of 5500 V was used for all analyses with an injection volume of 10 μL and column temperature set at 25 °C. Eluents were prepared freshly before use and consisted of 5 mM aqueous ammonium bicarbonate (A, pH ≈ 8.7) and acetonitrile (B), with a flow rate of 0.300 mL min^{-1}. The program for solvent B was 0 min, 65%; 10 min, 65%; 10.5 min, 90%; 25 min, 90%; 25.1 min, 65%; 30 min, 65%. MS data were recorded in multiple reaction monitoring (MRM) mode with the same MRM transition pairs for analytes and deuterium internal standards as previously reported [14], and optimised source parameters (gases, temperature) for each instrument. Analyst software (versions 1.6.2 and 1.6.3) was used for instrument control and data acquisition. The optimised method was validated according to standard procedures [16]. Other polyfunctional thiols could also potentially be evaluated but were not the focus of this work (Fig. S1 of the Supplementary material).

2.9.1. Elution order assignment

Elution orders of the enantiomers of **1** and **2** were initially tested using pure enantiomers spiked in model wine and derivatised with DTDP. Peak identity was also confirmed in wine by fortifying pure enantiomers in a charcoal-stripped Sauvignon blanc wine (100 g L^{-1} charcoal with stirring for 2 h followed by filtration through a 2.5 μm filter paper) followed by derivatisation, SPE and HPLC-MS/MS analysis. (*R*)-**1** (0, 500, 1500 ng L^{-1}) and (*R*)-**2** (0, 100, 300 ng L^{-1}) were spiked into the charcoal-stripped wine that was previously spiked with racemic **1** (1000 ng L^{-1}) and **2** (200 ng L^{-1}). This led to wine samples containing consistent concentrations of (*S*)-enantiomers (500 ng L^{-1} for 3-SH **1** and 100 ng L^{-1} for 3-SHA **2**) and increasing concentrations of (*R*)-enantiomers (500, 1000, and 2000 ng L^{-1} for 3-SH **1**; 100, 200, and 400 ng L^{-1} for 3-SHA **2**). The samples were prepared and analysed according to the optimised method.

2.9.2. Linearity and matrix effects

Racemic calibration solutions were prepared in duplicate at ten concentrations (0, 156.3, 312.5, 625, 937.5, 1250, 1562.5, 1875, 2187.5 and 2500 ng L^{-1} for each enantiomer of **1**; 0, 31.3, 62.5, 125, 187.5, 250, 312.5, 375, 437.5 and 500 ng L^{-1} for each enantiomer of **2**) using MW, and a Sauvignon blanc, a rosé, and a red wine. Linearity was tested across the given concentration range by regression analysis and expressed as coefficients of determination (R^2). Regression models were evaluated by a D'Agostino-Pearson omnibus K^2 normality test of residuals [17]. For matrix effects, slopes of curves in different matrices were compared using the following equation [18]:

$$Matrix\,effect\,(\%) = \left(1 - \frac{Slope\,of\,calibration\,curve\,in\,real\,wine}{Slope\,of\,calibration\,curve\,in\,model\,wine}\right) \times 100$$

2.9.3. Accuracy and precision

Accuracy was evaluated through recovery (%) and Z-score of six replicate samples spiked with analytes at low and high levels in each matrix. Precision was expressed using within-laboratory (indicated by subscript r) relative standard deviation (RSD_r) and Horwitz ratio ($HorRat_r$) [19]. Six replicates spiked at two concentrations (312.5 and 1562.5 ng L^{-1} for enantiomers of **1**; 62.5 and 312.5 ng L^{-1} for enantiomers of **2**) within the calibration range were prepared and analysed in one batch along with calibration samples in each matrix.

2.9.4. Limits of detection and quantitation

The limit of detection (LOD) and limit of quantitation (LOQ) were estimated based on 3 and 10 times the error of *y*-intercept divided by the slope obtained from full calibration curves [20].

2.10. Statistical analysis

Mean values, standard deviations (SD), RSD_r, $HorRat_r$, linear regressions, and Pearson correlations were calculated in Microsoft Excel (Professional Plus version, 2013). Normality testing ($\alpha = 0.05$) for evaluation of regression residuals was performed using GraphPad Prism 7 (GraphPad Software, Inc.).

3. Results and discussion

*3.1. Method development for chiral analysis of **1** and **2***

3.1.1. Experiment design and column screening

A recently developed HPLC-MS/MS method for determination of polyfunctional thiols in wine using in situ derivatisation with DTDP [14] was also suited to chiral analysis of **1** and **2** as their mixed disulfide derivatives **3** and **4**. This necessitated switching from an achiral C18 phase to a chiral stationary phase (CSP) but to our knowledge there was no relevant literature precedent for the separation of the enantiomers similar to **3** and **4**. The expense of chiral HPLC columns discounted a trial and error approach, so chiral screening by PhenoLogix [15] was used to determine the most appropriate CSP. First, authentic standards **3** and **4** were synthesised using a one-pot procedure for asymmetric disulfide formation mediated by BtCl [11]. New compounds **3** and **4** were fully characterised and used for screening of commercially-available polysaccharide chiral HPLC columns (Fig. 1).

Initial screening was conducted with three Lux polysaccharide-based columns (4.6 mm i.d.) under various MS compatible mobile phase conditions (Table S2 of the Supplementary material). Amylose-1 and Amylose-2 columns were the best options based on the screening, having successfully resolved the enantiomers of **3** and **4**, with Amylose-2 affording a run time of about 15 min and

Amylose-1 greater than 30 min (Table S2 of the Supplementary material). We chose a column diameter (2.0 mm) that used less solvent and was more compatible with HPLC-MS and began with the chromatographic conditions used for column screening as a starting point for HPLC-MS/MS method optimisation.

3.1.2. HPLC-MS/MS method optimisation

When transferring the chromatographic conditions used for column screening to HPLC-MS/MS for optimisation, the previously observed separation of enantiomers using a 4.6 mm i.d. Amylose-2 column could not be achieved with the 2.0 mm counterpart. Enantiomers of **3** remained well resolved but the baseline resolution for **4** could not be replicated ($\alpha = 1.04$), even though a number of HPLC parameters (see section 2.8.2) including mobile phase composition, organic additive, flow rate, column temperature, and gradient program were trialled (data not shown).

The chiral resolution of **3** and **4** on a 2.0 mm i.d. Amylose-1 column was better than that obtained on Amylose-2. Isocratic elution with 5 mM ammonium bicarbonate water:acetonitrile (40:60, *v*/*v*) achieved full enantioseparation of both thiol derivatives, but the initial HPLC-MS/MS run lasted almost 50 min (data not shown). Curiously, three out of the four peaks arising from the two pairs of enantiomers eluted within the first 10 min, whereas extreme retention was noted for one enantiomer of 3-SH derivative **3**, necessitating the long run time. Therefore, the isocratic method was changed to incorporate an isocratic stage and essentially a step gradient (see section 2.9), with a higher flow rate to decrease the run time to 30 min.

The separation results achieved with the two chiral columns demonstrated the profound effect of CSPs on chiral resolutions of given compounds. The retention factors and selectivity values of **3** and **4** are given in Table 2. Under optimised chromatographic conditions, the enantiomers of **3** and **4** were separated on an Amylose-1 column with respective selectivity values $\alpha = 5.69$ and 1.73, which were higher than those on Amylose-2 ($\alpha = 3.67$ and 1.04 for the enantiomers of **3** and **4**, respectively). The CSPs of Amylose-1 and Amylose-2 are amylose 3,5-dimethylphenylcarbamates (ADMPC) and amylose 3-chloro-5-methylphenylcarbamates (ACMPC), respectively, which differ at the 3-position of the phenyl moiety (electron-donating methyl group on Amylose-1 vs. electron-withdrawing chloro group on Amylose-2). These substituents, along with the carbamate and aromatic functionalities, are key factors affecting chiral recognition (via hydrogen bonding, dipole-dipole and π-π interactions); intramolecular hydrogen bonding of adjacent carbamates is also important as it gives these CSPs higher-order structure [21]. A previous study indicated that higher hydrogen-bonding ability would be expected from polysaccharide CSPs substituted with a halogen rather than an alkyl group, which explained the better separation of chiral sulfoxides on halogen-containing CSPs [22]. In our case, we observed opposite results on the two CSPs, which may suggest that the chiral recognition between **3**, **4** and the CSPs involved interactions other than hydrogen-bonding alone.

Table 2
Retention factors (k_1 and k_2) and selectivity values (α) for 3-SH and 3-SHA derivatives on Amylose-1 and Amylose-2 columns under respective optimised HPLC-MS chromatographic conditions.[a]

Analytes	Amylose-1 (ADMPC)			Amylose-2 (ACMPC)		
	k_1	k_2	α	k_1	k_2	α
3-SH derivative **3**	1.89	10.76	5.69	1.56	5.73	3.67
3-SHA derivative **4**	1.31	2.27	1.73	6.38	6.61	1.04

[a] Detailed in section 2.9 for Amylose-1. Amylose-2 used isocratic 5 mM aqueous ammonium bicarbonate:MeCN (60:40) and a flow rate of 0.200 mL min^{-1}.

Other factors such as chromatographic conditions and chemistry of chiral analytes may also account for separation differences. The chiral derivatives **3** and **4** are structurally similar, only differing at the C-1 position, where **3** (and 3-SH **1**) possesses a hydroxyl group whereas **4** (and 3-SHA **2**) has an acetoxy group. On ADMPC (Amylose-1), stronger retention was observed for enantiomers of **3** ($k_1 = 1.89$, $k_2 = 10.76$) than **4** ($k_1 = 1.31$, $k_2 = 2.27$), which indicated that **3** could interact strongly through the hydroxyl group with ADMPC compared to the acetoxy group. However, the opposite trend was seen on ACMPC where **4** ($k_1 = 6.38$, $k_2 = 6.61$) showed greater retention than **3** ($k_1 = 1.56$, $k_2 = 5.73$, Table 2). These results mirrored the previous observation of better chiral resolution of hydroxyl compounds (cannabinoids) on ADMPC compared with their acetylated analogues [23]. The theory that blocking the hydroxyl group by acetylation would result in lower separation was previously confirmed by conformational analysis [23], although the effect may be solvent-dependent [24].

Apart from the abovementioned observations, the obvious enantiomeric bias of ADMPC for **3**, with a selectivity factor $\alpha = 5.69$, was noteworthy. Extreme HPLC enantioseparation cases on ADMPC have previously been reported for other compounds [25], but the exact reasons for such retention of one enantiomer of **3** were not investigated further in the present study.

3.1.3. Elution order assignment

The order of elution for enantiomers of **3** and **4** was determined using enantiopure synthetic (*R*)- and (*S*)-forms of **1** and **2**. Model wine spiked with individual pure enantiomers yielded a single peak per enantiomer that had the same retention time and mass spectrum as one of the peaks arising from the respective synthetic racemate of **1** and **2** (data not shown). This was further confirmed by fortifying a charcoal-stripped Sauvignon blanc wine with pure enantiomers; this involved spiking with increasing amounts of (*R*)-enantiomers while the amounts of (*S*)-enantiomers, originating from a spike of racemic **1** and **2**, remained constant. Deuterated internal standards were also added, and area ratios of analyte/IS were calculated. Samples spiked with (*R*)-enantiomers of **1** and **2** yielded corresponding increases in area ratios, confirming the peak identities (Fig. 2).

The retention times of enantiomers of **1** and **2** as their derivatives were: (*R*)-**4**, 4.15 min; (*S*)-**3**, 5.21 min; (*S*)-**4**, 5.80 min; (*R*)-**3**, 21.18 min. An example MRM chromatogram is shown in Fig. 3. Notably, **1** enantiomers were remarkably resolved on Amylose-1, with extreme retention of (*R*)-**1** (and a broader peak, Fig. 3), whose retention time could not be decreased any further.

3.1.4. HPLC-MS/MS method validation

Validation of this SIDA method [16] encompassed the evaluation of linearity, matrix effects (ME), accuracy, precision, and sensitivity (LOD and LOQ) with the primary focus on the enantiomers of **1** and **2** (Table 3). Nonetheless, achiral polyfunctional thiols including 4-methyl-4-sulfanylpentan-2-one (4-MSP), 2-furfurylthiol (FT), and benzenemethanethiol (BMT) could potentially be evaluated on this column (Fig. S1 of the Supplementary material) but this was not pursued further for this study.

3.1.4.1. Linearity and matrix effects. Linearity and matrix effects were evaluated from calibration curves prepared with racemic **1** and **2** over the spiking range 0–2500 ng L^{-1} per individual 3-SH **1** enantiomer and 0–500 ng L^{-1} per individual 3-SHA **2** enantiomer in model wine and in a white, a rosé and a red wine. Calibrations encompassed a realistic range of these thiols in wine [14]. The curves in all matrices were fitted with simple linear regression, which afforded R^2 values greater than 0.98 and mostly above 0.99 for each enantiomer (Table 3). Normality testing of residuals gave

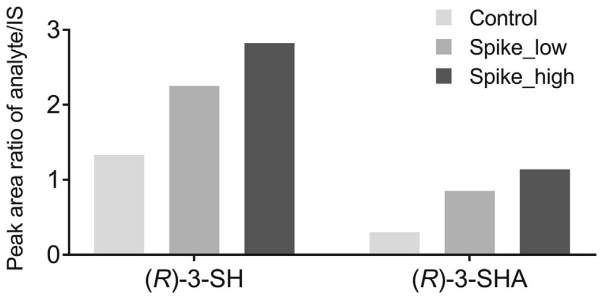


Fig. 2. Bar chart showing the increased analyte/IS ratios after spiking (*R*)-3-SH and (*R*)-3-SHA. All samples contained racemic **1** at 1000 ng L^{-1} and racemic **2** at 200 ng L^{-1}; (*R*)-**1** and (*R*)-**2** were spiked at 500 and 100 ng L^{-1}, respectively, for the low-level spiked samples and at 1500 and 300 ng L^{-1}, respectively, for high-level spiked samples.

insignificant *p*-values (0.1275–0.9708), further showing that a linear model was appropriate.

The effect of different matrices on this method was evaluated by comparison of slopes obtained from model wine and those from the three wine matrices. Deuterium labelled internal standards and SPE clean-up were employed to minimise interferences. The calculated ME are included in Table 3. Compared with model wine, Sauvignon blanc wine showed the least effect of the matrix for all enantiomers with ME values below 10%, which were within soft matrix effect range (<20%) considered to be a mild signal suppression/enhancement effect [18]. However, strong matrix effects (ME>50%) were observed for (*S*)-**1** in the red wine and (*R*)-**1** in the red and rosé wines. In contrast, comparing the slopes of the red and rosé wines against each other revealed only 10% variation, indicating similar impacts of the matrix for red and rosé wines (and implying a role of phenolic compounds and pigments present in such wines). Based on these evaluations, we recommend preparing different matrix-matched calibration standards for specific sample sets, i.e., calibration curve in model or white wine for white wine samples, calibration curve in red (or rosé) wine for red and rosé wine samples.

3.1.4.2. Accuracy and precision. Six replicates of standards of **1** and **2** were spiked into model and wine matrices at low and high concentrations and analysed to determine the accuracy and precision of the overall analytical procedure (Table 3). In terms of accuracy, mean recoveries for all analytes in the four matrices ranged from 90% to 111% and Z-scores were within the range of −1.8 to 1.9. Precision (repeatability) of the determinations expressed as RSD_r was between 1% and 8% depending on spiked analyte/matrix combination. The Horwitz ratio for a single laboratory ($HorRat_r$) [19] was also determined as a further description of method precision. $HorRat_r$ values gained here were consistently less than 0.1 and considerably below the lower limit (0.5) of the generally accepted range [19]. However, the analytes being considered were at ng L^{-1} concentrations, which leads to large predicted RSD values and consequently low HorRat values when the results are very precise (as is the case with our SIDA approach). Further supporting the low $HorRat_r$ values, better precision would be expected from intralaboratory compared to interlaboatory validation due to the lack of biases from different instruments, laboratory operations, etc. [19].

3.1.4.3. Limits of detection and quantitation. The obtained LOD values of the enantiomers of 3-SH **1** and 3-SHA **2** in the four matrices (≤0.7 ng L^{-1} for **1** enantiomers and 0.1 ng L^{-1} for **2**) were all well below their respective sensory thresholds (see Table 1). Similarly low LODs were reported for achiral methods of analysis for **1** and **2** such as the previously reported DTDP derivatisation method [14] that the current one is based upon, and a SIDA method for maleimide derivatives using HPLC-MS/MS [26].

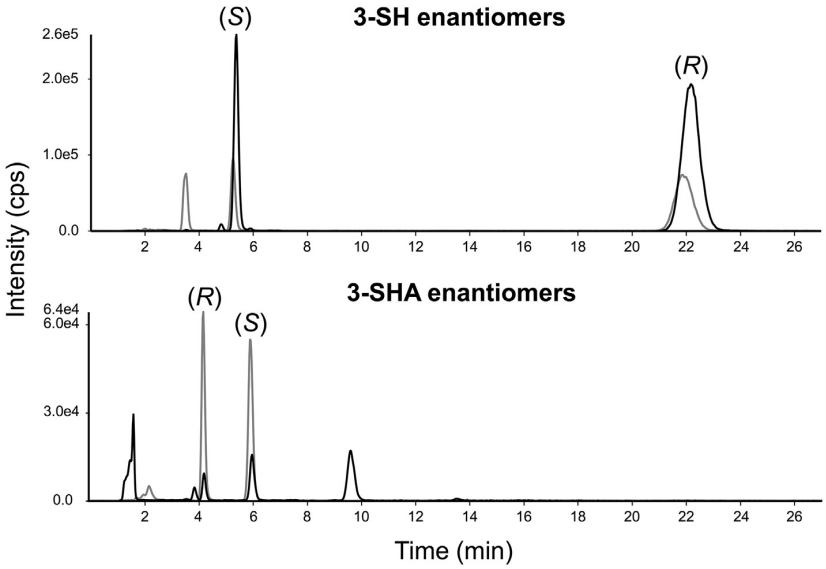


Fig. 3. MRM chromatograms of enantiomers of 3-SH **1** and 3-SHA **2** isolated from a Sauvignon blanc wine (as their derivatives) using the optimised chiral HPLC-MS/MS method (Section 2.9) with an Amylose-1 column. Grey line: MRM chromatograms of internal standards (d_{10}-**1**, *m*/*z* 254.5 → 144.9; d_5-**2**, 291.3 → 144.1), black line: MRM chromatograms of natural **1** and **2** in the sample (**1**, *m*/*z* 244.5 → 144.1; **2**, 286.4 → 144.2).

Table 3
Validation data in four matrices for the SIDA HPLC-MS/MS method developed for quantitation of enantiomers of 3-SH **1** and 3-SHA **2**.

Analyte	Matrix[a]	Linearity	ME	Accuracy				Precision				LOD[d]	LOQ[d]
		R^2	%	Recovery (%)		Z-score		RSD_r (%)		$HorRat_r$			
				L[b]	H[c]	L	H	L	H	L	H		
(*S*)-3-SH	MW	0.9832		93–102	93–104	−0.6–1.9	−0.9–1.4	3.5	4.8	0.07	0.14	0.6	1.8
	SAB	0.9906	10	92–111	91–108	−1.1–1.6	−1.0–1.5	7.0	7.3	0.13	0.17	0.4	1.2
	Rosé	0.9816	−20	90–104	95–104	−1.8–0.2	−1.2–1.4	6.6	3.2	0.12	0.08	0.6	1.8
	Red	0.9965	61	91–109	97–107	−1.1–1.3	−1.0–1.7	7.9	3.6	0.15	0.09	0.3	0.9
(*R*)-3-SH	MW	0.9901		95–100	92–107	−1.2–1.5	−1.1–1.7	2.1	5.6	0.04	0.13	0.4	1.2
	SAB	0.9965	−2	90–99	91–101	−0.7–1.5	−1.6–1.2	3.1	3.7	0.06	0.09	0.3	0.9
	Rosé	0.9755	61	91–103	96–101	−1.3–1.4	−1.6–1.1	4.4	1.7	0.08	0.04	0.7	2.1
	Red	0.9966	56	91–107	96–99	−1.3–1.3	−1.4–1.0	6.0	1.4	0.11	0.03	0.3	0.9
(*S*)-3-SHA	MW	0.9842		91–99	92–95	−1.4–1.1	−1.4–1.4	3.2	1.2	0.05	0.02	0.1	0.3
	SAB	0.9931	1	91–96	99–109	−1.2–1.7	−1.1–1.3	1.6	4.2	0.01	0.08	0.1	0.3
	Rosé	0.9941	21	92–100	98–101	−0.8–1.7	−1.1–1.3	3.4	2.0	0.05	0.04	0.1	0.3
	Red	0.9957	17	92–94	96–104	−1.0–1.6	−1.4–1.3	1.1	2.9	0.06	0.06	0.1	0.3
(*R*)-3-SHA	MW	0.9915		93–102	90–97	−1.1–1.6	−1.1–0.7	4.0	2.8	0.06	0.05	0.1	0.3
	SAB	0.9919	−1	93–103	94–109	−1.0–1.6	−1.2–1.7	4.1	5.0	0.06	0.09	0.1	0.3
	Rosé	0.9944	22	96–108	98–103	−0.8–1.6	−1.1–1.6	5.0	1.8	0.07	0.03	0.1	0.3
	Red	0.9958	13	90–100	96–101	−1.2–1.4	−0.9–1.6	3.9	3.3	0.02	0.05	0.1	0.3

[a] MW, model wine; SAB, Sauvignon blanc wine. Concentrations of endogenous analytes occurring in the wines used for validation are shown in Table S3 of Supplementary material.
[b] L, low-level standard spiked sample.
[c] H, high-level standard spiked sample.
[d] ng L^{-1}.

3.2. Distribution of enantiomers of 3-SH and 3-SHA in commercial wines

The validated method was applied to evaluate the distributions of enantiomers of 3-SH **1** and 3-SHA **2** in a small number of commercial wines (Table S1 of the Supplementary material). Out of 23 wines, enantiomers of **1** presented above their sensory thresholds in each (Fig. 4a): in dry wines, (*S*)-**1** ranged from 72 to 1663 ng L^{-1} and (*R*)-**1** ranged from 69 to 1320 ng L^{-1}, whereas considerably higher amounts[1] of both enantiomers of **1** were detected in one botrytised wine (4865 and 1755 ng L^{-1} for (*S*)- and (*R*)-forms, respectively). These results (in terms of summed enantiomers) mirrored reported concentration ranges of **1** in wines [14,27–29]. Sauvignon blanc generally exhibited higher concentrations of enantiomers of **1** compared to the other varieties, followed by Chardonnay and rosé wines. This agreed with the common observation that Sauvignon blanc is characterised by high varietal thiol concentrations [14] but **1** has also been found to be abundant in rosé [27,28] and Chardonnay [14] wines. Sauvignon blanc from Marlborough in New Zealand (i.e., SAB 1–3) contained higher amounts of **1** with up to 2983 ng L^{-1} combined total, which accords with previous data showing Marlborough Sauvignon blanc wines are normally higher in these thiols [2,29]. The red wines contained relatively much lower concentrations of the enantiomers of **1** compared with the white and rosé styles assessed.

With respect to **2**, 16 of 23 wines contained enantiomers above the LOQ, with (*S*)-**2** and (*R*)-**2** ranging between 1.1–130 and 1.2–58 ng L^{-1}, respectively (Fig. 4b). Among wines with detectable levels of **2**, 12 of them contained concentrations of (*S*)-**2** above its sensory threshold (2.5 ng L^{-1}, Table 1), whereas for (*R*)-**2**, only 4 wines had concentrations higher than threshold (9 ng L^{-1}). The odour activity values (OAV) of enantiomers of **2** implied a higher sensory impact from (*S*)-**2** (OAV up to 52) than (*R*)-**2** (OAV up to 6) in these wines. In general, Sauvignon blanc wines were frequently seen with high levels of **2**, with the highest concentration observed in a Sauvignon blanc wine of New Zealand origin (SAB 3). The total concentrations of **2** in Sauvignon blanc wines were consistent with previous data on wines produced in Australia [14] and New Zealand [2]. Rosé wines showed relatively higher concentrations of the enantiomers of **2** among the non-Sauvignon blanc varieties, in accord with that reported for racemic **2** [14]. In particular, a young Shiraz rosé wine (R3) from vintage 2017 had higher levels of **2** (especially of (*S*)-**2**) than older vintage wines (Table S1 of the Supplementary material), which can be attributed to a relative lack of acetate hydrolysis of **2** (i.e., conversion of **2** back to **1**) compared to aged wines [30]. Enantiomers of **2** in the botrytised wine were present in smaller amounts, in contrast to the results for **1**. In red wines, the concentrations of enantiomers of **2** were under the LOQ.

Possible relationships within and across (*S*)- and (*R*)- forms of **1** and **2** were checked by Pearson correlation analysis. Very strong correlations were seen for the enantiomer pairs of **1** and **2** ($r > 0.99$ for both; note, the botrytised sample was excluded from each correlation analysis). Additionally, reasonable correlations existed between the respective analyte enantiomers; that is, comparing (*S*)-**1** and (*S*)-**2** ($r = 0.55$), and (*R*)-**1** and (*R*)-**2** ($r = 0.54$). Despite the general chemical and biological complexity of wines, good correlations presented here were not entirely surprising given that compound **2** is derived from **1** during fermentation [7].

The relative amounts (%) of the enantiomers of **1** and **2** are presented in Fig. 5 (excluding CH2 and WB1 for 3-SHA **2**, where only one enantiomer was detected). Across all dry wines, even though enantiomers for **1** were roughly equally distributed (Fig. 5a), (*S*)-**1** appeared to be slightly more abundant than (*R*)-**1** in most wines; the percentage of (*S*)-**1** ranged between 44% and 57%, with a mean value of 52%. In the botrytised wine, (*S*)-**1** accounted for 74% of total 3-SH. In the case of **2**, the proportion of (*S*)-**2** dominated over (*R*)-**2** in most wines (Fig. 5b), ranging from 48% to 71% with a mean value of 60%. The botrytised wine and SAB8 had the highest proportion of (*S*)-**2**, at 71% in each.

The present results for 3-SH **1** were in accordance with the previous observations that the enantiomers were more or less uniformly distributed in dry Sauvignon blanc and Semillon wines, but often with a slight excess of (*S*)-**1** (at a consistent average value of around 53%) [4,6]. Furthermore, the higher proportion of (*S*)-**1** in botrytised wines was also observed in those two previous studies

[1] Extrapolated, (*S*)-3-SH outside of the calibrated range of the method.

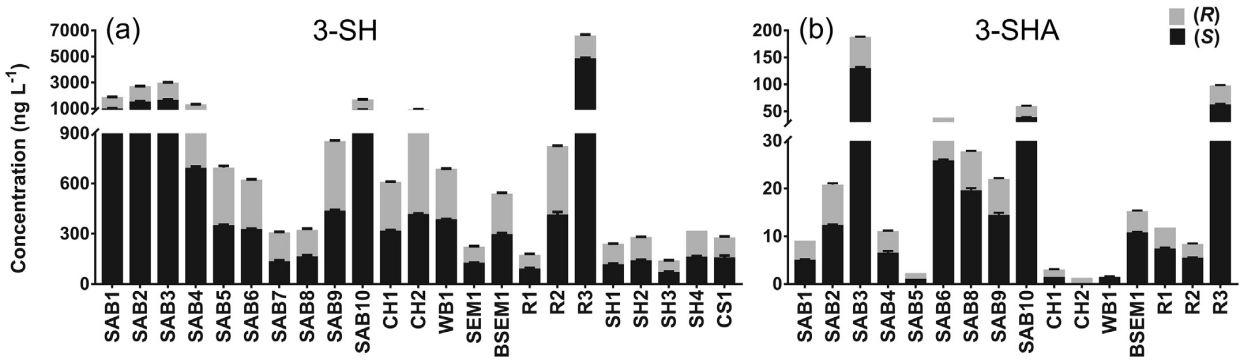


Fig. 4. Concentrations of enantiomers of (**a**) 3-SH **1** and (**b**) 3-SHA **2** in a selection of commercial wine samples. Error bars indicate SD between duplicate analyses. (*S*): enantiomers in (*S*)-form; (*R*): enantiomers in (*R*)-form; SAB, Sauvignon blanc; CH, Chardonnay; WB, white blend; SEM, Semillon; BSEM, botrytised Semillon; R, rosé; CS, Cabernet Sauvignon. For sample details refer to Table S1 of Supplementary material.

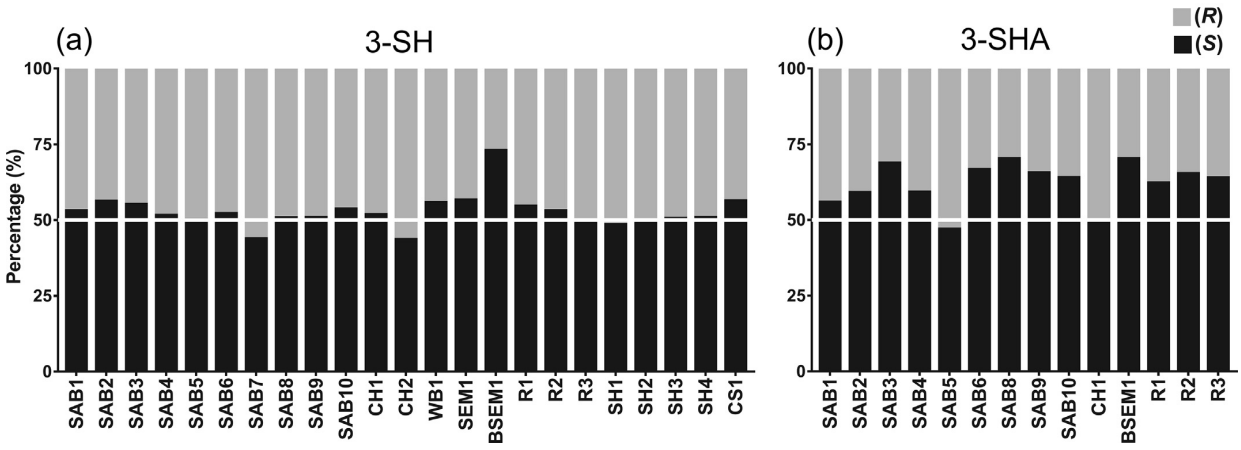


Fig. 5. Relative distribution of enantiomers of (**a**) 3-SH **1** and (**b**) 3-SHA **2** determined in commercial wines. (*S*): enantiomers in (*S*)-form; (*R*): enantiomers in (*R*)-form; SAB, Sauvignon blanc; CH, Chardonnay; WB, white blend; SEM, Semillon; BSEM, botrytised Semillon; R, rosé; CS, Cabernet Sauvignon. For sample details refer to Table S1 of Supplementary material.

and appears to be more broadly applicable. For 3-SHA **2**, the results of Tominaga et al. showed that the (*S*):(*R*) ratio was always around 70:30 whenever **2** was detected in dry Sauvignon blanc and Semillon wines (40% of wines assessed) [4]. The skewed distribution of **2** [4] was more or less mirrored in our data set, where the majority of the wines contained (*S*)-**2** ranging from 60 to 70%, but certain wines had slightly less (*S*)-**2** or almost equal amounts of (*S*)- and (*R*)-**2** (i.e., a racemic mixture, as in SAB5 and CH1). However, in the wines where **2** was virtually racemic, the levels were very low and just above the LOQ, so any baseline noise could have influenced the quantitation results.

Given the relatively small number of wines that have undergone chiral analysis of **1** and **2** in the current and previous work, further studies are required to verify the enantiomer distribution phenomenon. The simple chiral method presented here will facilitate additional studies, such as linking the stereochemistry of precursor diastereomers in grapes to the free thiol enantiomers in wine, to better elucidate (bio)chemical pathways.

3.3. Implications for relating enantiomers of 3-SH and 3-SHA to reaction stereospecificity

With respect to the production of 3-SH **1** and 3-SHA **2** in wine, on the basis of current knowledge these thiols are originally absent in grapes[2] [31], but **1** can be generated from precursors in grape juice by *Saccharomyces cerevisiae* metabolism during alcoholic fermentation, and **2** is then derived from **1** [7]. Two major types of precursors of **1** found in grapes are conjugates of cysteine (i.e., Cys-**1**) and glutathione (i.e., GSH-**1**), which have been determined at μg L^{-1} levels [32–34]. In fact there exist two diastereomeric forms of Cys-**1** and GSH-**1**, which then link to the respective enantiomers of **1** (and thus **2**) in wine [35]. In grape juice, (*S*)-GSH-**1** and (*S*)-Cys-**1** (referring to the stereocentre at C-3 of the hexan-1-ol chain) have

[2] Approximately 100 ng L^{-1} of **1** has been shown for Sauvignon blanc grapes during ripening.

routinely been found in greater amounts than their (*R*)-counterparts [31–33]. The dominance of (*S*)-GSH-**1** (and thus (*S*)-Cys-**1** due to its role in glutathione degradation pathways) indicated reaction stereospecificity in the conjugation between 2-hexenal and GSH, likely as a result of an enzymatic process [32]. Stereoselectivity studies on thiol formation during fermentation revealed that the alkyl chain stereocentre configuration is retained during conversion of (*R*)-GSH-**1** precursor to (*R*)-**1** (via (*R*)-Cys-**1**) [34], of (*R*)-Cys-**1** to (*R*)-**1** [13], and of (*S*)-Cys-**1** to (*S*)-**1** [13]. Furthermore, upon fermentation of a diastereomeric mixture of (*R*)- and (*S*)-Cys-**1**, (*S*)-**1** was produced in amounts that were almost three times higher than (*R*)-**1** in the final ferments [34], although almost equal amounts of (*R*)- and (*S*)-**1** were reported elsewhere [13]. The differences between production of (*R*)- and (*S*)-**1** could result from different expression levels of tryptophanase enzymes [35] in yeast, and precursor diastereomer ratios are likely to play a role (if they are indeed important precursors to thiols in wine).

A reasonably high proportion of (*S*)-**1** (over 60%) was observed by Tominaga et al. at the beginning of fermentation but by the end, both (*S*)- and (*R*)-**1** were present in similar amounts (albeit slightly in favour of (*S*)-**1** as noted above) [4]. The initial difference could potentially relate to the greater abundance of (*S*)-Cys-**1** and (*S*)-GSH-**1** in grapes and juices together with the favoured selectivity of β-lyase towards producing (*S*)-**1** at the beginning of fermentation. The reason for the subsequent equalisation of (*S*)- and (*R*)-**1** during fermentation is presently unknown, but could potentially arise due to differences in enzyme expression or activity as fermentation progresses [4]. In contrast, **2** was observed to be produced consistently in favour of its (*S*)-enantiomer throughout fermentation, leading to the predominance of (*S*)-**2** in the end [4]. The disproportionate production of (*S*)-**2** from the beginning of fermentation could be related to the higher occurrence of its precursor (*S*)-**1** at the same time; hypothetically, the enzymatic reaction yielding (*S*)-**2** was triggered at an early stage of fermentation such that the proportion of (*S*)-**2** did not change thereafter. Substrate selectivity as a function of the enzymes involved in *O*-acylation of **1** has previously been proposed but three different yeast strains had no influence on enantiomer distributions of **1** and **2** during fermentation [4]. Interestingly, the greater abundance of (*S*)-**1** found in botrytised wine (above 70%), including throughout fermentation [4], in conjunction with a relatively stable proportion of (*S*)-**2** (around 70%) suggests that botrytis infection and elevated (*S*)-**1** proportions have little effect on 3-SHA **2** enantiomer distribution. Whatever the reasons, it should be noted that monitoring evolution of enantiomers during fermentation has only been tested under limited circumstances [4] and much more could be done to understand the factors involved.

4. Conclusions

This study presents a new SIDA chiral HPLC-MS/MS method for quantitation of enantiomers of polyfunctional thiols 3-SH **1** and 3-SHA **2** in wine. Method development consisted of the synthesis of authentic DTDP derivatives, chiral column screening, and chromatographic optimisation. Enantiomers of **1** and **2** in wine were separated using a polysaccharide based chiral column and the method was fully validated in model and real wine matrices, revealing excellent performance with respect to linearity, accuracy, precision, and sensitivity. The method was successfully applied to a selection of commercial white, red, and rosé wines to examine the enantiomer profiles, revealing that enantiomers of 3-SH **1** were almost equally distributed in dry wines regardless of variety, whereas a botrytised wine showed elevated (*S*)-**1**. Enantiomers of **2** in dry and botrytised wines were nearly always present in favour of (*S*)-**2** over (*R*)-**2**, with a ratio of up to 70:30. This method enables further investigation of additional aspects associated with the enantiomer profiles of **1** and **2** in wine, including studies on their relationship to the diastereomeric precursors, changes in ratios during fermentation, and their sensory impact on wines. Finally, it is envisaged that the method could be extended to the evaluation of the enantiomers of **1** and **2** in other food and beverage products.

Acknowledgments

We thank Christine Böttcher (CSIRO) for undertaking HRMS analysis, Rochelle Schlank (UA) for assistance with NMR, and Yoji Hayasaka (AWRI) for expert advice with HPLC-MS/MS instrumentation. L.C. was a recipient of the joint scholarship of UA and China Scholarship Council, and was also supported by an Australian Grape and Wine Authority supplementary Scholarship (AGWA 1512). UA and AWRI are members of the Wine Innovation Cluster at the Waite Precinct in Adelaide. AWRI is supported by Australia's grape-growers and winemakers through their investment body, Wine Australia, with matching funds from the Australian government.

Appendix A. Supplementary data

Supplementary data related to this article can be found at https://doi.org/10.1016/j.aca.2017.10.031.

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SUPPLEMENTARY MATERIAL FOR

Chiral analysis of 3-sulfanylhexan-1-ol and 3-sulfanylhexyl acetate in wine by high-performance liquid chromatography–tandem mass spectrometry

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Table S1.

Commercial wines assessed for the distribution of enantiomers of 3-SH and 3-SHA in this study (from Australia unless indicated with NZ for New Zealand).

Variety	Code	Vintage	Region
Sauvignon blanc	SAB1	2014	Marlborough, NZ
Sauvignon blanc	SAB2	2014	Marlborough, NZ
Sauvignon blanc	SAB3	2016	Marlborough, NZ
Sauvignon blanc	SAB4	2014	Adelaide Hills, SA
Sauvignon blanc	SAB5	2015	Adelaide Hills, SA
Sauvignon blanc	SAB6	2016	Reynella, SA
Sauvignon blanc	SAB7	2015	Orange, NSW
Sauvignon blanc	SAB8	2017	Riverina, NSW
Sauvignon blanc	SAB9	2016	Riverina, NSW
Sauvignon blanc	SAB10	2016	Sunraysia, VIC
Chardonnay	CH1	2013	Barossa, SA
Chardonnay	CH2	2014	Adelaide Hills, SA
White blend [a]	WB1	2014	McLaren Vale, SA
Semillon	SEM1	2014	Hunter Valley, NSW
Botrytised Semillon	BSEM1	2011	Riverina, NSW
Rosé [b]	R1	2016	Riverina, NSW
Rosé [b]	R2	2016	Surry Hills, NSW
Rosé (Shiraz)	R3	2017	Barossa, SA
Shiraz	SH1	2012	Pyrenees, VIC
Shiraz	SH2	2009	Yarra Valley, VIC
Shiraz	SH3	2013	Riverina, NSW
Shiraz	SH4	2011	Margaret River, WA
Cabernet Sauvignon	CS1	2013	Coonawarra, SA

[a] Riesling, Sauvignon blanc, Marsanne, Roussane.

[b] Variety not specified.

Table S2.

Chiral column screening conditions for 3-SH and 3-SHA analysed as their derivatives **3** and **4**.[a]

Column	3-SH enantiomers	Analysis time (min)	Mobile phase [b]	3-SHA enantiomers	Analysis time (min)	Mobile phase [b]
Amylose-1	baseline resolved	35	30:70	baseline resolved	15	40:60
Amylose-2	baseline resolved	15	60:40	baseline resolved	15	60:40
Cellulose-1	baseline resolved	15	60:40	partially resolved	15	50:50

[a] Data provided by Phenomenex Australia (Lane Cove, NSW, Australia).
[b] All tested chromatographic conditions were isocratic methods using 5 mM ammonium bicarbonate:MeCN mobile phase.

Table S3.

Endogenous concentrations (ng L^{-1}) of enantiomers of 3-SH and 3-SHA present in commercial bag-in-box wines used for method validation.

	SAB	Rosé	Red
(*S*)-3SH	1173	189	130
(*R*)-3SH	931	182	125
(*S*)-3SHA	38.8	7.6	3.7
(*R*)-3SHA	23.8	8.3	2.5

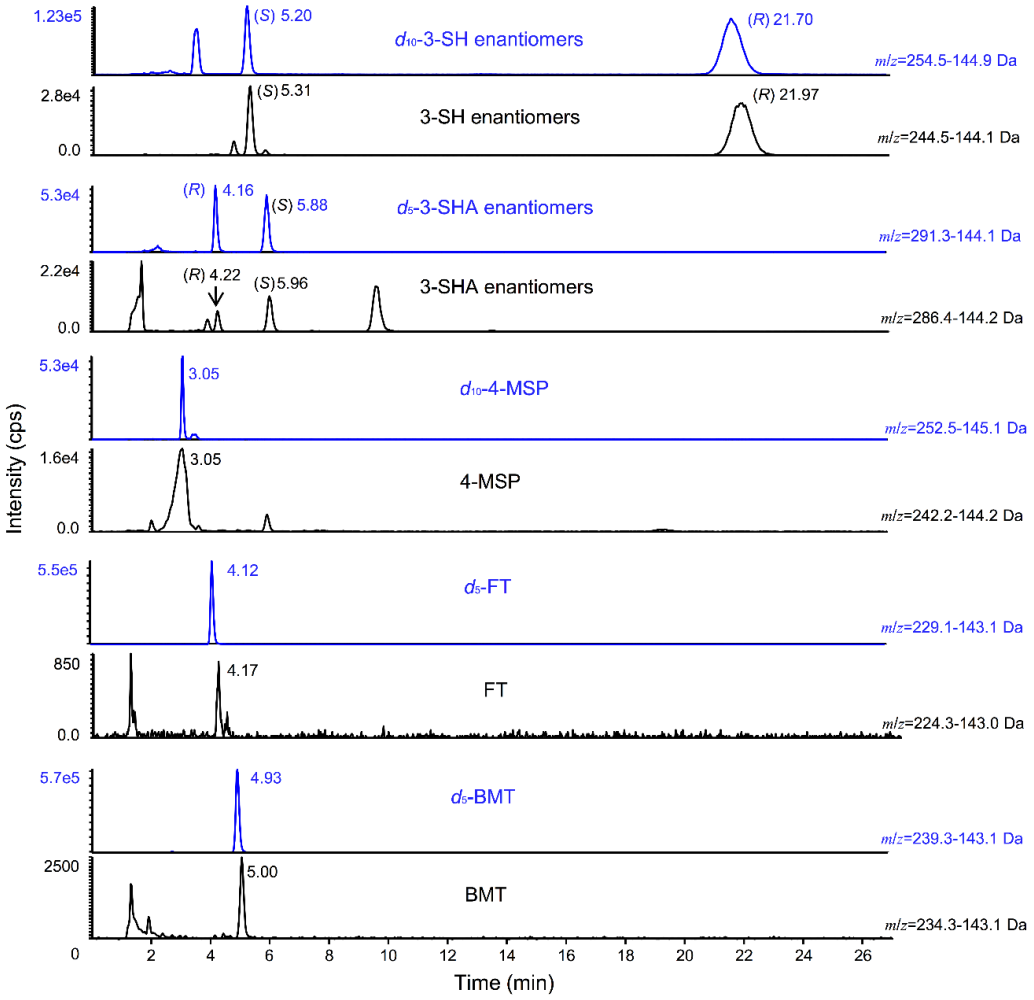

Fig. S1. Comparison of HPLC-MS/MS(ESI+) MRM chromatograms of enantiomers of 3-SH and 3-SHA, 4-MSP, FT, and BMT isolated as their derivatives from commercial Sauvignon blanc (3-SHA) and Chardonnay (3-SH, 4-MSP, FT, and BMT) wines, aligned with their respective deuterium labelled internal standards (d_{10}-3SH, d_5-3SHA, d_{10}-4MSP, d_5-FT, and d_5-BM). Blue line: MRM chromatogram of internal standard; black line: MRM chromatogram of analyte.

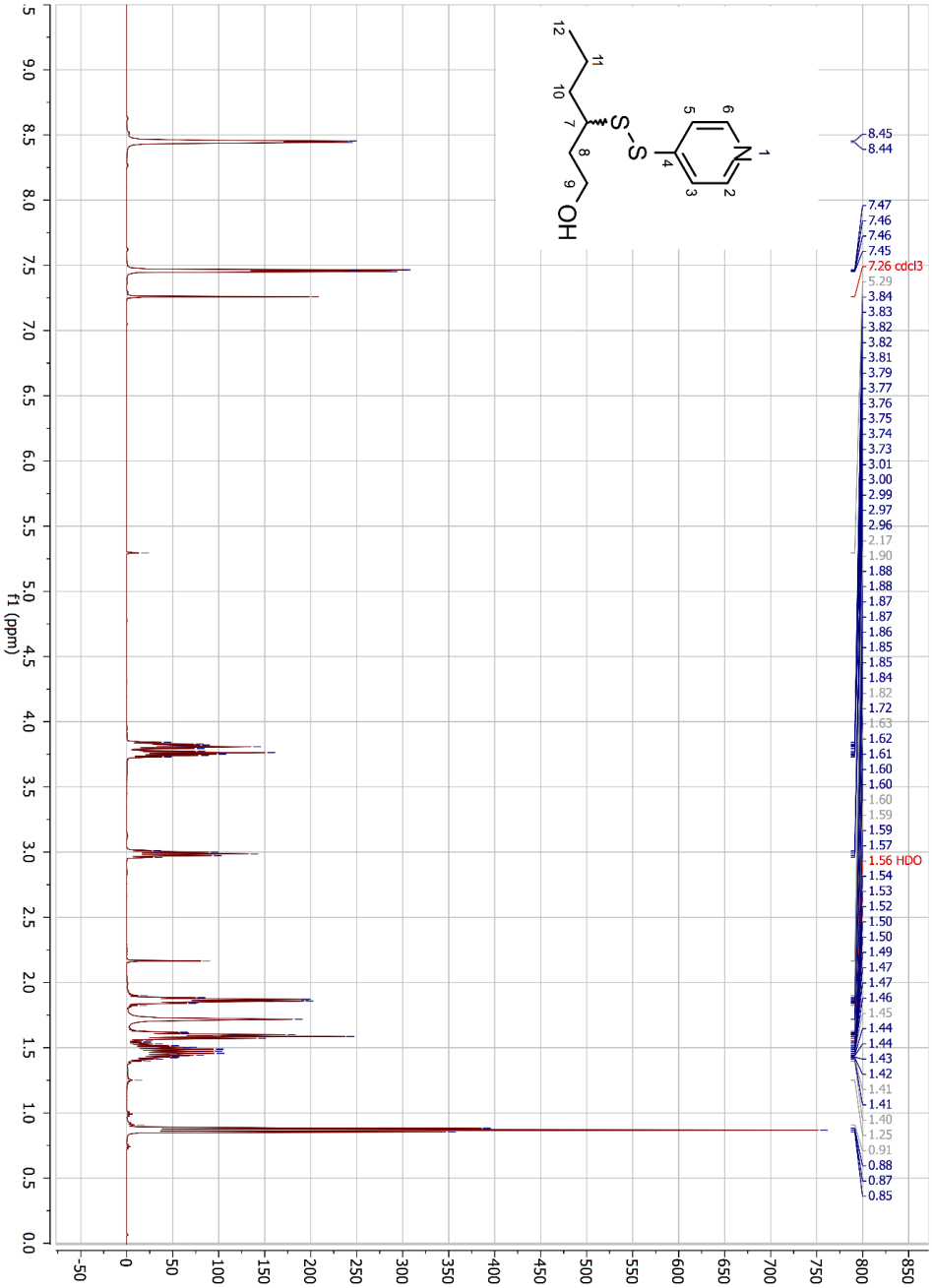

Fig. S2. ^{1}H NMR spectrum of synthesised 3-SH derivative **3**.

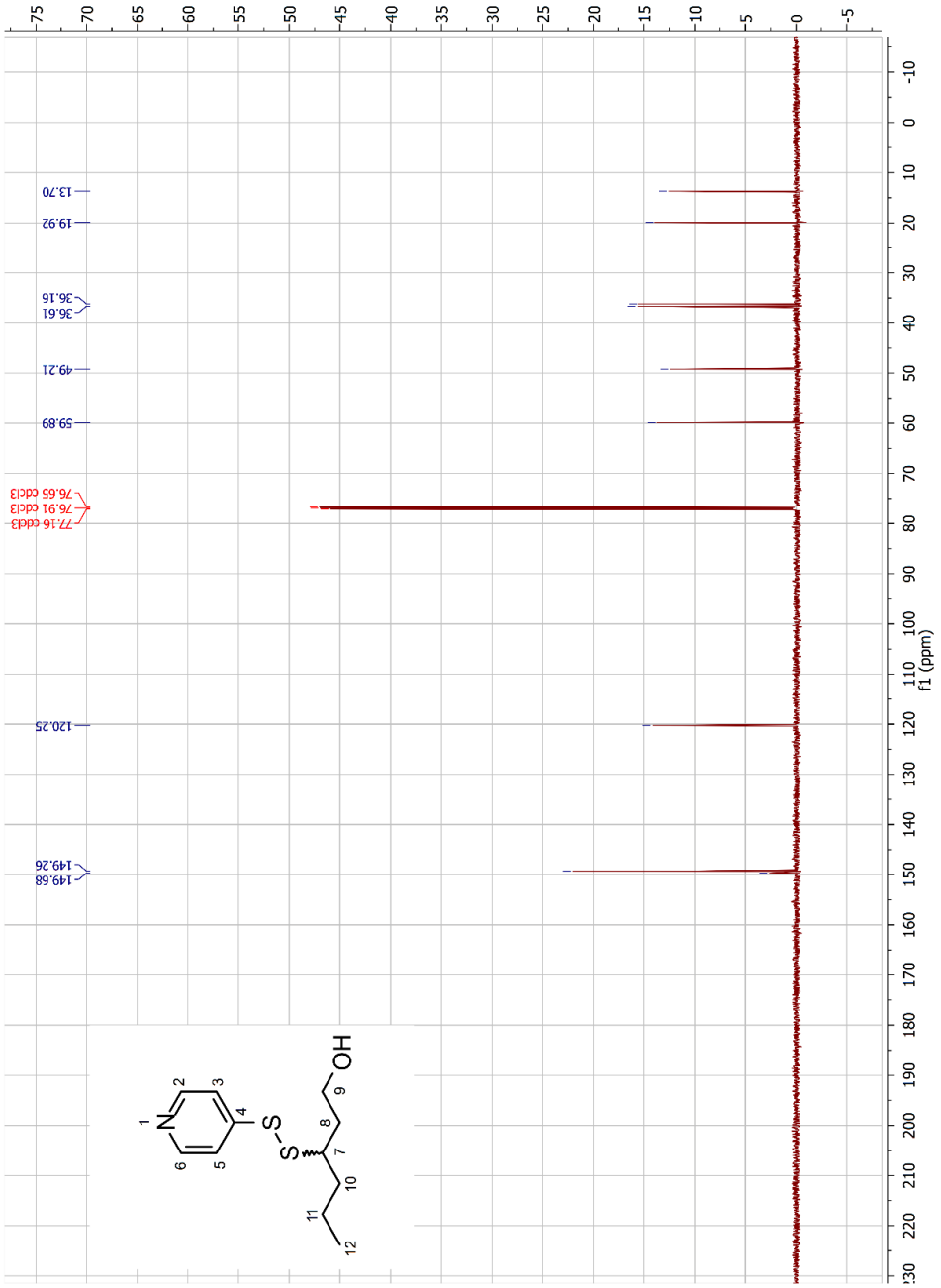

Fig. S3. ^{13}C NMR spectrum of synthesised 3-SH derivative **3**.

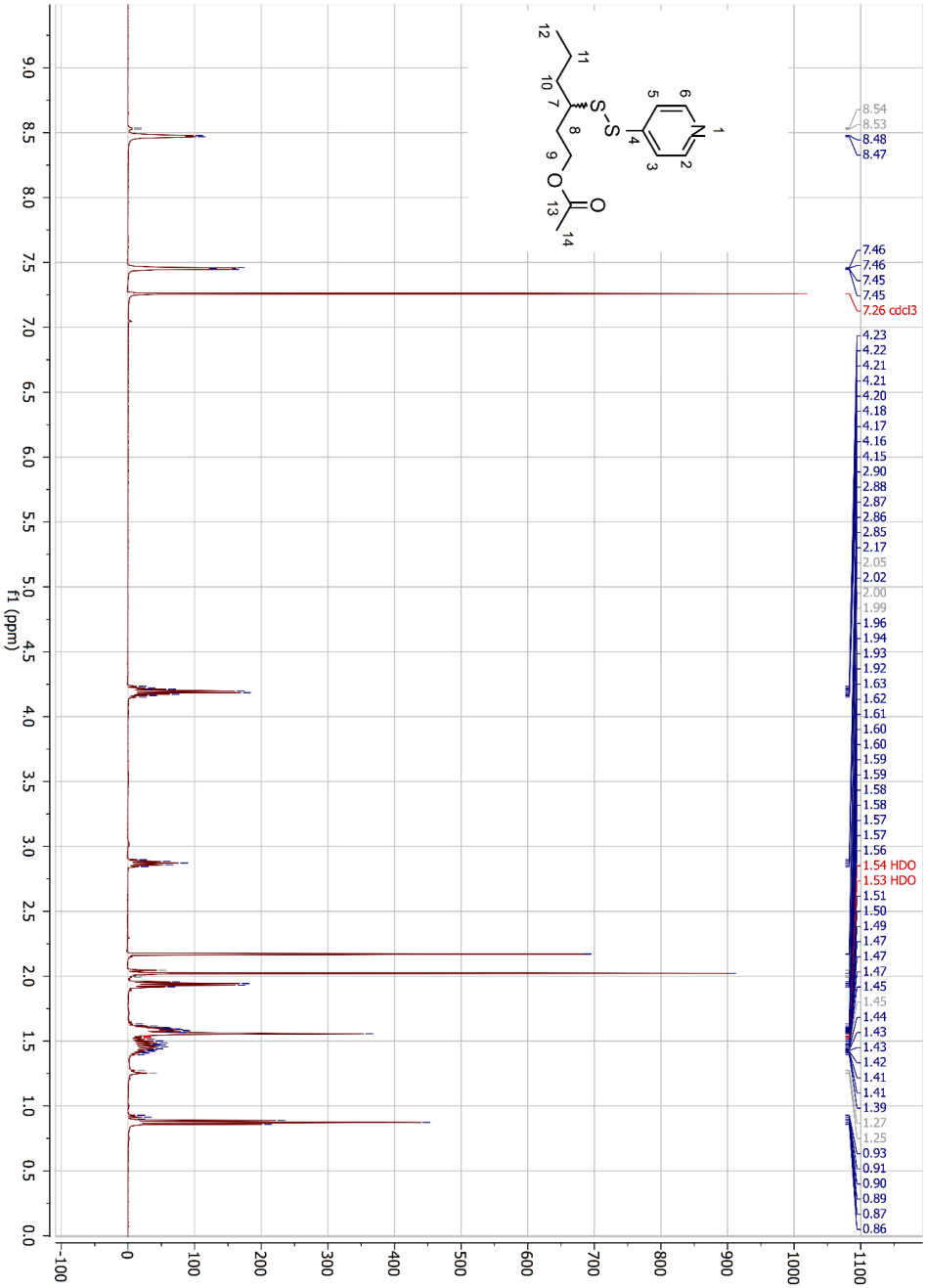

Fig. S4. ^{1}H NMR spectrum of synthesised 3-SHA derivative **4**.

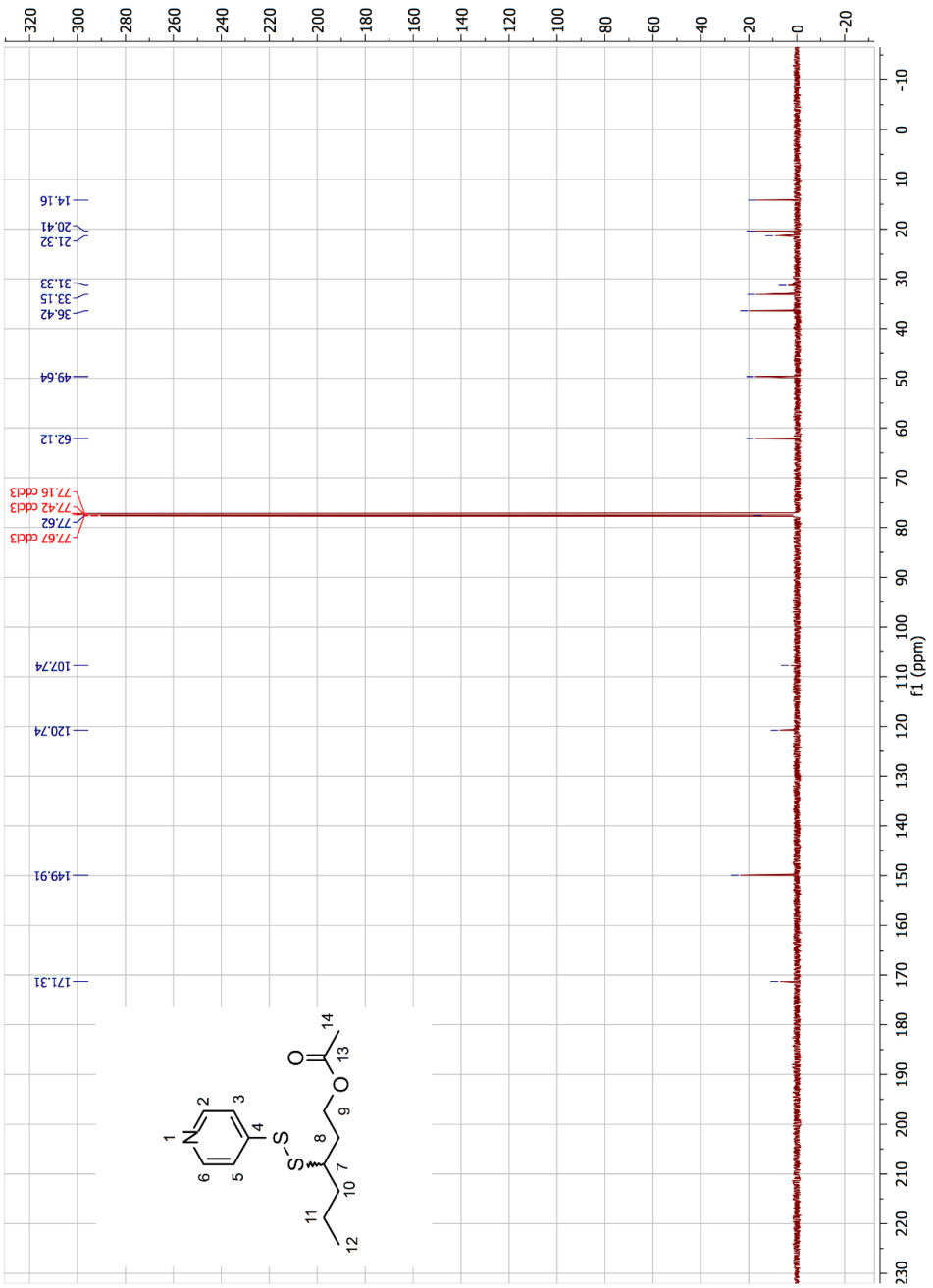

Fig. S5. ^{13}C NMR spectrum of synthesised 3-SHA derivative **4**.

Chapter 4

Chiral polyfunctional thiols and their conjugated precursors upon winemaking with five *Vitis vinifera* Sauvignon blanc clones.

Journal of Agricultural and Food Chemistry 2018, 66, 4674–4682. DOI.

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Q9720 5385 F4V6 **H5V10** F7V7

Statement of Authorship

Title of Paper	Chiral polyfunctional thiols and their conjugated precursors upon winemaking with five *Vitis vinifera* Sauvignon blanc clones
Publication Status	☑ Published ☐ Accepted for Publication ☐ Submitted for Publication ☐ Unpublished and Unsubmitted w ork w ritten in manuscript style
Publication Details	Chen, L.; Capone, D.L.; Tondini F.A.; Jeffery D.W. (**2018**) Chiral polyfunctional thiols and their conjugated precursors upon winemaking with five *Vitis vinifera* Sauvignon blanc clones. *Journal of Agriculture and Food Chemistry*, **66**, 4674-4682

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Contribution to the Paper	Provided conceptual suggestions on experimental design. Collected samples, performed fermentation experiment, prepared all samples, and carried out HPLC-MS/MS analysis. Collected, processed, and interpreted data. Wrote, edited, and produced a completed first draft. Revised the manuscript for publication.
Overall percentage (%)	70%
Certification:	This paper reports on original research I conducted during the period of my Higher Degree by Research candidature and is not subject to any obligations or contractual agreements with a third party that would constrain its inclusion in this thesis. I am the primary author of this paper.
Signature	Date 31-07-2018

Co-Author Contributions

By signing the Statement of Authorship, each author certifies that:

i. the candidate's stated contribution to the publication is accurate (as detailed above);

ii. permission is granted for the candidate in include the publication in the thesis; and

iii. the sum of all co-author contributions is equal to 100% less the candidate's stated contribution.

Name of Co-Author	Dimitra L. Capone
Contribution to the Paper	Supervised and designed the experiment. Collected, processed, and interpreted data. Prepared, edited, and revised the manuscript.
Signature	Date 31/07/18

Name of Co-Author	Federico A. Tondini
Contribution to the Paper	Performed fermentation experiment. Edited and revised the manuscript.
Signature	Date 31-07-2018

Name of Co-Author	David W. Jeffery
Contribution to the Paper	Conceived and designed the original concept of the study. Supervised the project. Wrote, edited, revised, and submitted the paper. Acted as corresponding author at all stages.
Signature	Date 31/07/2018

Please cut and paste additional co-author panels here as required.

JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY

Article

Cite This: J. Agric. Food Chem. 2018, 66, 4674−4682

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Chiral Polyfunctional Thiols and Their Conjugated Precursors upon Winemaking with Five *Vitis vinifera* Sauvignon blanc Clones

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Supporting Information

ABSTRACT: Five co-located clones of Sauvignon blanc grapes were fermented under controlled conditions at laboratory-scale to investigate the impact of yeast strain, commercial enzyme, or nutrient addition on the concentrations of enantiomers of 3-sulfanylhexan-1-ol (3-SH) and 3-sulfanylhexyl acetate (3-SHA) in resulting wines. The relationship of these enantiomers with the odorless 3-SH precursors present in diastereomeric forms in grape juice was also examined. Possible variations may have existed due to clone type, not only for the diastereomers of 3-SH precursors in juices but also for the enantiomers of 3-SH and 3-SHA in the resulting wines, although there was no obvious stereochemical relationship between precursors and free thiols. From a flavor enhancement perspective, the use of a commercial enzyme in the juice significantly enhanced 3-SH production for some clones. In contrast, less impact on the production of 3-SH and 3-SHA was seen as a result of yeast strain and nutrient regardless of clone type.

KEYWORDS: *3-sulfanylhexan-1-ol, 3-sulfanylhexyl acetate, stereochemistry, Vitis vinifera, winemaking*

INTRODUCTION

Chiral volatile compounds broadly distributed in fragrances, foods, and beverages are of great interest to chemists because of their importance to perceived aroma and flavor, and due to the fact that different olfactory thresholds and aroma qualities can exist for enantiomers.[1] Aroma compounds in wine provide an excellent example of this phenomenon, with potent chiral thiols, 3-sulfanylhexan-1-ol (3-SH) and related 3-sulfanylhexyl acetate (3-SHA), both existing as a pair of enantiomers that differ in odor intensity and quality. Found at ng/L levels[2] and mainly studied as racemic mixtures, these character impact compounds are among the polyfunctional thiols deemed important to the tropical aromas of certain varietal wines.[3] The olfactory properties and detection thresholds of enantiomer pairs for 3-SH ((*R*)-3-SH has a "grapefruit" or "citrus peel" aroma with a threshold of 50 ng/L; (*S*)-3-SH has a "passionfruit" aroma with threshold at 60 ng/L) and 3-SHA ((*R*)-3-SHA has a "passionfruit" aroma with a threshold of 9 ng/L; (*S*)-3-SHA has a "boxwood" aroma with a threshold of 2.5 ng/L) are distinguishable in model wine media,[2] and the sensory properties of wines can be affected by the (*R*):(*S*) ratios of enantiomers of 3-SH and 3-SHA.[4]

Despite the impact of 3-SH and 3-SHA on wine aroma, studies focusing on the chiral distribution of these key volatiles in wines are lacking, as is knowledge of factors that drive the chiral thiol profiles. One possible reason has been the lack of a sensitive analytical method for chiral determination of 3-SH and 3-SHA, which has been overcome with our recently reported approach using stable isotope dilution analysis (SIDA) with chiral high-performance liquid chromatography coupled with tandem mass spectrometry (HPLC-MS/MS).[3] This simple method has enabled us to begin inspecting chiral profiles of 3-SH and 3-SHA in wines on a broad scale,[3] but elucidating the pathways for the biogenesis of 3-SH in this complex natural system involving different biologies requires further research. Current understanding indicates that 3-SH in wine ostensibly derives mainly from odorless cysteine and glutathione (GSH) conjugated precursors (Cys-3-SH,[5] Glut-3-SH[6]) that are formed in grapes/musts and is released and partially acetylated during alcoholic fermentation by enzymes from *Saccharomyces cerevisiae*.[7,8] Cys-3-SH and Glut-3-SH are present as (*R*)- and (*S*)-diastereomers,[7−11] which are related to the enantiomers of 3-SH (and 3-SHA) in wine. However, the stereochemical relationship between diastereomeric precursors and enantiomeric free thiols has yet to be investigated, and the overall impact of formation of racemic 3-SH (thus racemic 3-SHA), via H_2S addition to (*E*)-2-hexenal during fermentation,[12] for example, is unknown.

From a winemaking perspective, the concentrations of 3-SH and 3-SHA in wines decisively depend on grape composition and winemaking operations.[7] Wines made from different grape varieties contain contrasting concentrations of such thiols,[13−15] although Sauvignon blanc wines usually have higher amounts.[14] Aside from the obvious varietal differences in thiol production, the concentrations of thiol precursors in grape juices[9] and the profile of free thiols and sensory properties of wines can also vary at the clone level.[9,16] In addition, the production of thiols during winemaking could potentially be affected by widely used

Received: April 8, 2018
Accepted: April 11, 2018
Published: April 27, 2018

DOI: 10.1021/acs.jafc.8b01806
J. Agric. Food Chem. 2018, 66, 4674−4682

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additives, such as enzymes[17−19] or nutrient supplements.[20] Despite the significance of thiol enantiomers to wine aroma and the intensive attention being paid to evaluate the effects of enological and viticultural applications on thiol production more generally,[7] scientific investigations have barely addressed thiol production from a stereochemical perspective.

We aimed to address knowledge gaps by exploring the stereochemical relationships between conjugated precursors and free thiols under the influence of several winemaking variables. Five clones of *Vitis vinifera* Sauvignon blanc grapes, two yeasts, one enzyme, and one nutrient were evaluated in a laboratory-scale fermentation trial using a high-throughput robot, and the diastereomers of Cys-3-SH and Glut-3-SH (and intermediate CysGly-3-SH) in juices and the enantiomers of 3-SH and 3-SHA in resulting wines were determined for the first time, to the best of our knowledge.

MATERIALS AND METHODS

Chemicals. Unlabeled standards and deuterium-labeled internal standards of thiols and thiol precursors were previously synthesized as their racemates (referring to the alkyl chain stereochemistry in the case of precursors) according to literature procedures: 3-SH and 3-SHA,[21] d_{10}-3-SH,[22] and d_5-3-SHA,[21] *S*-[(1*R*/*S*)-1-(2-hydroxyethyl)butyl]-L-cysteine (Cys-3-SH),[22] *S*-[(1*R*/*S*)-1-(2-hydroxyethyl)-1-butyl]-L-cysteinylglycine (CysGly-3-SH),[23] *S*-[(1*R*/*S*)-1-(2-hydroxyethyl)butyl]-L-cysteinylglycine (Glut-3-SH),[24] *S*-[(1*R*/*S*)-1-(2-hydroxyethyl)butyl-1,2,2,3,3,4,4,4-d_8]-L-cysteine (d_8-Cys-3-SH),[22] and γ-L-glutamyl-*S*-[(1*R*/*S*)-1-(2-hydroxyethyl-2-d_1)butyl-1,2,2,3,3,4,4,4-d_8]-L-cysteinylglycine (d_9-Glut-3-SH).[24] The following chemicals and consumables were purchased from commercial suppliers: 4,4′-dithioldipyridine (DTDP), acetaldehyde (>99%), ethylenediaminetetraacetic acid disodium salt (EDTA 2Na), ammonium bicarbonate, formic acid, and potassium hydrogen tartrate (Sigma-Aldrich, Castel Hills, NSW, Australia); Bond Elut C18 SPE cartridges (500 mg, 6 mL) (Agilent, Mulgrave, VIC, Australia); Strata SDB-L SPE cartridges (Phenomenex, Lane Cove, NSW, Australia); and Merck HPLC-grade ethanol, methanol, and acetonitrile (VWR International, Tingalpa, QLD, Australia). Water was obtained from a Milli-Q purification system (Millipore, North Ryde, NSW, Australia). All solutions were prepared volumetrically, and model wine was prepared with 10% (*v*/*v*) aqueous ethanol saturated with potassium hydrogen tartrate and pH adjusted to 3.4 with tartaric acid. DTDP derivatization reagent was prepared according to a previous procedure.[14] Stock solutions and DTDP reagent were stored at −20 °C. Working solutions were kept at 4 °C until required.

Grape Samples. Five samples of Sauvignon blanc grapes (clones H5V10, F4V6, F7V7, 538S, and Q9720), grown on own roots from vines of the same age, were collected from a commercial vineyard in the Adelaide Hills, South Australia, on 20 March 2017. The grapevines were grown adjacent to each other in the same block of the vineyard and collected from an area of about 0.6 ha. Harvesting was conducted in the morning 1 day prior to commercial harvest. Whole bunches of berries for each sample were randomly hand-picked from both sides of the canopy from multiple rows and stored in resealable plastic bags (approximately 8 kg total per clone). Grapes were immediately transported (<1 h) to the laboratory and stored at 4 °C overnight. Each bag of grapes (whole bunches) was dosed with 50 mg/kg SO_2 (added as aq. potassium metabisulfite solution), hand-crushed on a benchtop, and the juice obtained (approximately 5 L in total per clone) was passed through a coarse sieve and stored at −20 °C in sealed 5 L plastic containers until required. Juice handling was conducted in the presence of dry ice to minimize oxidation.

Basic Juice Parameters. Juices were measured for total soluble solids (TSS), pH, and titratable acidity (TA, expressed as g/L equivalents of tartaric acid), sugar content (glucose + fructose, g/L), and yeast assimilable nitrogen (YAN) in duplicate. TA and pH were measured using a T50 Autotitrator (Mettler Toledo, Port Melbourne, VIC, Australia). TSS was measured with a hand-held digital refractometer (PR-101, Atago, Tokyo, Japan). Sugar content and YAN were quantitated enzymatically using commercially available kits (Megazyme K-FRUGL for sugar, Megazyme K-AMIAR and K-PANOPA kits for YAN; VWR International) following the manufacturer's instructions.

Laboratory-Scale Fermentations. Frozen juices were thawed and settled at 4 °C for approximately 12 h. After siphoning into 5 L Schott bottles in the presence of dry ice, juices were homogenized prior to subsampling. For each clone, 5 L of juice was divided into another three Schott bottles for the treatments: one subsample acted as the control, and the remaining two were supplemented at the recommended doses with either a commercial nutrient (4 g/L NUTRICELL AA, Martin Vialatte, Grapeworks, Dingley Village, VIC, Australia) or a commercial enzyme (0.04 mL/L Endozym Thiol, AEB Oceania, Hanwood, NSW, Australia). Subsequently, for each treatment of the five clones, triplicate aliquots of 100 mL juice were transferred into 180 mL modified Schott bottles fitted with air locks, pending inoculation (1 mL of culture) with either VIN13 (Anchor, Cape Town, South Africa) or W28 (Sofralab, Grapeworks) cultured independently (from isolation, after plating) in liquid yeast extract−peptone−dextrose (YPD) medium overnight at 28 °C. Fermentations were conducted with a high-throughput fermentation robot (TEEBOT) at 16 °C (to minimize loss of volatiles), and without mixing except for gentle stirring for 5 min at 100 rpm prior to sampling for sugar measurements. Fermentation progress was monitored by enzymatic sugar analysis and wines were cold settled at 4 °C for 1 week once residual sugar content was <1 g/L. Wine (20 mL) was decanted from fermentation bottles for chiral thiol analysis immediately after bottles were opened. TA and pH of wines were also measured in triplicate. Wine samples have been abbreviated according to grape clone designation (e.g., H5V10 wines) in the following discussion.

Enantiomers of 3-SH and 3-SHA in Wine by SIDA Chiral HPLC-MS/MS. 3-SH and 3-SHA were extracted and analyzed according to our previous method.[3] An aliquot of 50 μL of combined internal standard solution containing d_{10}-3-SH and d_5-3-SHA in ethanol was spiked into 20 mL of wine, followed by the addition of 20 mg of EDTA 2Na, 80 μL of 50% aq. acetaldehyde solution, and 200 μL of DTDP reagent. After 30 min of derivatization, the wine sample was loaded onto a preconditioned (6 mL of methanol followed by 6 mL of water) Bond Elut C18 SPE cartridge. The cartridges were washed with 12 mL of 50% aq. methanol, dried under air for 5 min, and eluted with 3 mL of methanol. The eluted fractions were collected and dried under N_2 at 25 °C, dissolved in 200 μL of 10% aq. ethanol, and stored at −20 °C prior to analysis.

Quantitative analyses were performed on an Amylose-1 column (150 × 2.0 mm, 3 μm particle size, Phenomenex) protected by Amylose-1 guard cartridge (4 × 2.0 mm) fitted to a Sciex ExionLC AD system connected to a Sciex QTRAP 6500+ tandem mass spectrometer, which was equipped with an IonDrive source and TurboIonSpray probe. HPLC conditions, multiple reaction monitoring (MRM) transition pairs, and MS parameters were the same as previously reported.[3] Analyst software (Version 1.6.3) was used for instrument control, data acquisition and analysis.

Diastereomers of 3-SH Precursors in Juice by SIDA HPLC-MS/MS. Cys-3-SH, CysGly-3-SH, and Glut-3-SH in juices were extracted and analyzed according to the procedure detailed previously.[23] HPLC-MS/MS analysis was performed on the same instrument as used for chiral thiol determinations.

Statistical Analysis. Data reduction was performed using Excel (Version 2013 for Windows, Microsoft). Statistical treatments and figure constructions were performed using either XLSTAT (Version 2018.2 for Windows, Addinsoft) or Prism 7 (Version 7.02 for Windows, GraphPad Software Inc.). Where relevant, data are presented as mean values of replicates with uncertainty expressed as standard deviation (SD). Mean values were compared by unpaired *t* test ($p < 0.05$) or one-way analysis of variance (ANOVA) followed by Tukey's HSD multiple comparison test ($p < 0.05$). Principal component analysis (PCA, correlation type) was performed after data standardization to visualize the differences among treatments.

DOI: 10.1021/acs.jafc.8b01806
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Table 1. Basic Compositional Data for Juices from Five Clones of Sauvignon blanc[a]

clone	pH	TA (g/L)	TSS (°Brix)	YAN (mg N/L)
H5V10	2.88 ± 0.01 b	8.82 ± 0.33 a	20.0 ± 0.0 d	129 ± 4 c
F4V6	2.85 ± 0.01 b	8.79 ± 0.42 a	19.7 ± 0.1 c	111 ± 13 bc
Q9720	2.84 ± 0.02 b	8.94 ± 0.39 a	18.8 ± 0.1 b	125 ± 1 c
F7V7	2.85 ± 0.00 b	9.02 ± 0.25 a	18.1 ± 0.0 a	91 ± 1 ab
5385	2.79 ± 0.01 a	9.15 ± 0.16 a	18.1 ± 0.0 a	69 ± 1 a

[a]Data were derived from duplicate samples and presented as mean values ± standard deviations. Different letters in the same column indicate significant differences ($p < 0.05$) between the means.

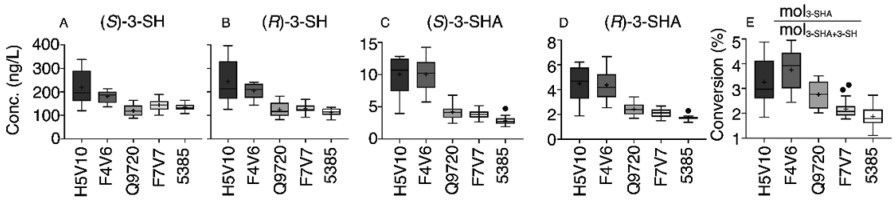

Figure 1. Tukey box-and-whisker plots showing concentrations of (A) (*S*)-3-SH, (B) (*R*)-3-SH, (C) (*S*)-3-SHA, (D) (*R*)-3-SHA, and (E) apparent molar conversions of 3-SH to 3-SHA in wines ($n = 90$) made from five clones of Sauvignon blanc. The "+" symbol indicates the mean value. Note the differences in the *y*-axis scales.

RESULTS AND DISCUSSION

Basic Grape Composition. Characterization of freshly obtained juices from the five Sauvignon blanc clones included the determination of pH, TA, TSS, and YAN (Table 1). Clone 5385 showed a pH (2.79) slightly lower than that of the other four clones but showed no significant differences in TA, which ranged from 8.82 to 9.15 g/L across the clones. TSS varied from 18.1 to 20.0 °Brix and was significantly higher in clones H5V10, followed by F4V6 and Q9720. Large variations in YAN were evident between the five samples, with almost 2-fold higher YAN in H5V10 (129 mg N/L) compared to 5385 (69 mg N/L). High variability of YAN, as seen among clones of other varieties, such as Albariño[25] and Cabernet franc,[26] could impact fermentation performance and consequently the quality of resulting wines.[27]

Fermentation. Two commercial yeast strains were put through trials in laboratory-scale fermentations: VIN13 acted as a reference strain due to its widely reported ability to release thiols,[21] and W28 was selected as a comparison yeast due to its apparent capability of releasing thiols.[28] Fermentations conducted at 16 °C were completed within 20 days or less, based on residual sugars of <1 g/L, although one sluggish control ferment for clone 5385 had 6.4 g/L. The fermentations were affected by clones, yeasts, and additives (Figure S1 of Supporting Information). In general, fermentations were in agreement with previous studies using the VIN13 strain,[29] but the fermentation rate and duration varied for different clones, which could be attributable to differences in YAN content (69 vs 129 mg N/L, Table 1).[27,30] Yeast W28 metabolized sugars equally or slightly faster than VIN13 for all clones. The inclusion of additive seemed to accelerate fermentation rates in most cases, with a greater effect from the addition of nutrient rather than enzyme, which could be related to greater nitrogen supplementation from the nutrient in these nitrogen deficient juices.[27]

3-SH and 3-SHA in Wines. The enantiomer profiles for 3-SH and 3-SHA in wines resulting from the five clones of Sauvignon blanc were assessed using a recently developed SIDA approach with chiral HPLC-MS/MS.[3] Total 3-SH (sum of enantiomers) ranged from 172 to 734 ng/L and covered a broader concentration range than determined previously for wines made from the same clones (313−419 ng/L, vintage 2011).[9] This was not surprising in light of probable differences due to vintage conditions, winemaking operations, and fermentation scales. Nonetheless, the pattern observed previously for wines obtained from the same clones, whereby the highest concentration of 3-SH was present in H5V10 wines, followed by F4V6 and F7V7, with the lowest in Q9720 and 5385,[9] was mirrored in the enantiomers found here (Figures 1A,B). The total amount of 3-SHA, not previously reported for these clones, was determined at 3−21 ng/L. The quantitative results for the summed enantiomers of 3-SH and 3-SHA in these laboratory-scale fermentations accorded somewhat with previous results from a commercial wine survey,[3] although the maximum concentrations were almost an order or magnitude lower in the present case. Other than technological or compositional differences during winemaking, scale effects[31] and greater losses due to volatilization or oxidation in microscale fermentations could be related to the disparity between the research and commercial wines.

Potential Influence of Clone Type on Chiral 3-SH and 3-SHA Profiles. Although the variation of racemic thiol production during winemaking across clones has been examined previously,[9,16] the determination of enantiomers of 3-SH and 3-SHA in wines made from different clones is reported here for what appears to be the first time (Table S1 of Supporting Information). The data were initially examined with a broad focus on the Sauvignon blanc clones. As shown in Figure 1A−D and Table S1 of Supporting Information, H5V10 and F4V6 wines had amounts of both pairs of enantiomers of 3-SH and 3-SHA significantly greater than those of wines from the other three clones (Table S2 of Supporting Information). This does not seem attributable to potential differences in grape ripeness, however, given the consistent pattern of 3-SH in the clones across the studies, as mentioned above (and considering the clones in 2011 were almost identical in TSS[9]). Respective average concentrations of (*S*)-3-SH in H5V10 and F4V6 wines were 219.3 and 180.7 ng/L, much higher than that in 5385 (135.4 ng/L), Q9720 (120.2 ng/L), and F7V7 (144.5 ng/L) wines (Figure 1A). The results for (*R*)-3-SH also showed that

DOI: 10.1021/acs.jafc.8b01806
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H5V10 had the highest average concentration (244.5 ng/L), followed by F4V6 (204.0 ng/L), F7V7 (130.3 ng/L), Q9720 (123.9 ng/L), and 5385 (113.3 ng/L) (Figure 1B). Calculated odor activity values (OAV = concentration/detection threshold, which may give some guide as to the importance of an odorant) for the 3-SH enantiomers all exceeded 1, indicating potentially important aroma contributions from these enantiomers to the overall aroma profiles of finished wines. According to the quantitative data, OAV variations among clones were evident, with H5V10 having the highest OAVs (3.7 and 4.9 for (*S*)- and (*R*)-3-SH, respectively), whereas the lowest OAVs were calculated for 5385 (2.2 and 2.3 for (*S*)- and (*R*)-3-SH, respectively).

In terms of 3-SHA enantiomers, respective average concentrations of (*R*)- and (*S*)-3-SHA in wines from five clones ranged from 1.7−4.5 ng/L and 3.8−10.0 ng/L. The distribution patterns (concentrations and OAVs) across clones were mostly in agreement with those of 3-SH, where H5V10 was the highest followed by F4V6, and 5385 was the lowest, but there were slightly higher concentrations in Q9720 than in F7V7 (Figure 1C,D). However, unlike the near equal OAVs between enantiomers of 3-SH, the OAVs for (*S*)-3-SHA (1.1−4.0) were higher than those of (*R*)-3-SHA (0.2−0.5), implying greater sensory effects from (*S*)-3-SHA ("boxwood") compared to (*R*)-3-SHA ("passionfruit").

Taking into account that 3-SHA is formed from 3-SH and assuming no losses, the molar conversion rates from 3-SH to 3-SHA ($mol_{3\text{-SHA}}/mol_{3\text{-SH+3-SHA}}$) were calculated and found to range from 1.1 to 4.7% in the wines (Figure 1E), which was close to but overall lower than that in the previous fermentation studies.[32] The highest apparent conversion occurred for F4V6 clone with an average of 3.6%, followed by H5V10 clone (average of 3.1%), which were both significantly higher than the other three clones ($p < 0.001$). In contrast, only about 1.8% of 3-SH, was seemingly converted to 3-SHA in clone 5385. The variable conversion rates indicated an effect of the clone on acetylation of 3-SH to 3-SHA during fermentation, possibly related to the expression of the yeast *ATF1* gene.[33]

Based on our previous investigations that included a small survey of commercial wines, enantiomers of 3-SH were roughly distributed equally in different varieties (with the exception of botrytized wines), and (*S*)-3-SHA was more abundant than its (*R*)-counterpart.[3] Similar patterns were evident in a total of 90 fermentations quantitated in the present study, although the ratios of pairs of enantiomers varied significantly across the clones regardless of treatment applied (Table S2 of Supporting Information). The (*S*):(*R*) ratio of 3-SH varied from 0.79 to 1.38 (Figure 2A), and 3-SHA varied from 1.34 to 2.71 (Figure 2B), which accorded well with previous data in dry commercial wines of different varieties.[3] Similarly, the Sauvignon blanc clone type may have affected the (*S*):(*R*) ratios of chiral 3-SH and 3-SHA to some extent. In terms of 3-SH, the (*S*)-enantiomer exceeded the (*R*)-form in clone F7V7 and 5385 (ratio >1.0), whereas H5V10 and F4V6 mostly contained less (*S*)-3-SH than (*R*)-3-SH. The amounts of (*S*)-3-SHA detected in clone H5V10 and F4V6 were more than 2-fold higher than that of (*R*)-3-SHA, in contrast to the ratios for Q9720, F7V7, and 5385, which were generally <2.0. Considering the concentrations of 3-SH and 3-SHA detected for each clone, in conjunction with the ratios between pairs of enantiomers, it appeared that (*S*)-3-SH tended to be less dominant when the combined concentrations of 3-SH were higher, but (*S*)-3-SHA was likely to be more prevalent when combined 3-SHA

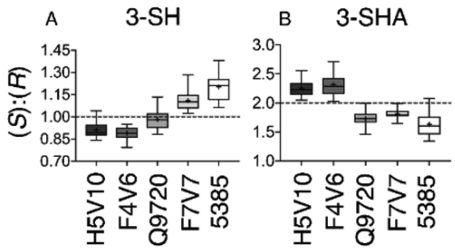

Figure 2. Tukey box-and-whiskers plot showing (A) ratios of (*S*):(*R*)-enantiomers of 3-SH and (B) 3-SHA in wines ($n = 90$) made from five clones of Sauvignon blanc. The "+" symbol indicates mean values. Note the differences in the *y*-axis scales.

amounts were higher (Figures 1 and 2). This apparent negative correlation for 3-SH and positive correlation for 3-SHA were verified by Pearson correlation analysis between the ratios of (*S*):(*R*) and combined enantiomer concentrations, yielding $r = -0.59$ for 3-SH and $r = 0.74$ for 3-SHA. These variations in the patterns of chirality for 3-SH and 3-SHA (concentrations and ratios) indicated the fundamental influences of grapes from different clones during fermentation, beyond the effects of yeast or additive that were tested.

Influence of Yeast, Enzyme, and Nutrient on Chiral Profiles of 3-SH and 3-SHA. The amounts of 3-SH and 3-SHA in wines could also be affected by various winemaking decisions.[7] Quantitative results of 3-SH and 3-SHA enantiomers from the treatments (five clones, two yeasts, plus enzyme or nutrient) are shown in Figures 3 and 4, and data were compared using an unpaired *t* test or a one-way ANOVA with a multiple comparison test.

Yeast. The formation of 3-SH during winemaking from its conjugated grape precursors is an enzymatic process requiring carbon−sulfur (C−S) lyase produced by yeast.[7] Acetyltransferase, also from yeast, then converts 3-SH to 3-SHA.[33] Numerous studies have investigated the impact of yeast on 3-SH and 3-SHA production[7] but have not evaluated the effect on the individual enantiomers, nor the effects of yeasts in combination with commercial additives. The comparison of two commercial yeasts on the final amounts of individual enantiomers of 3-SH and 3-SHA in finished wines (Table S1 of Supporting Information) is presented in Figure 3. In wines without any supplementation with additives (control in Figure 3), most fermentations showed no significant differences between the two yeasts, except for variations in F4V6 and 5385 wines. VIN13 yeast produced significantly more of each 3-SH enantiomer in 5385 wines compared to W28 (160.8 vs 122.5 ng/L for (*S*)-3-SH, $p = 0.0062$; 129.9 vs 93.2 ng/L for (*R*)-3-SH, $p = 0.0054$) but less (*S*)-3-SHA (2.1 vs 2.7 ng/L, $p = 0.0090$). A greater amount of (*R*)-3-SH was seen in F4V6 wines fermented with VIN13 as opposed to W28 (226.4 vs 167.7 ng/L, $p = 0.0181$).

Regarding treatments with commercial enzyme added prior to inoculation (enzyme in Figure 3), the two yeasts behaved very similarly apart from F7V7, in which wines fermented with W28 yeast showed significantly higher (*S*)-3-SH (27% increase relative to VIN13) and higher amounts of both enantiomers of 3-SHA, and 5385 wines, which had a higher concentration of (*R*)-3-SHA (Figure 3). When nutrient was added to juices, the concentrations of both pairs of enantiomers of 3-SH and 3-SHA were generally not different between yeasts, except in the case of H5V10 and F4V6, where VIN13 gave significantly

DOI: 10.1021/acs.jafc.8b01806
J. Agric. Food Chem. 2018, 66, 4674−4682

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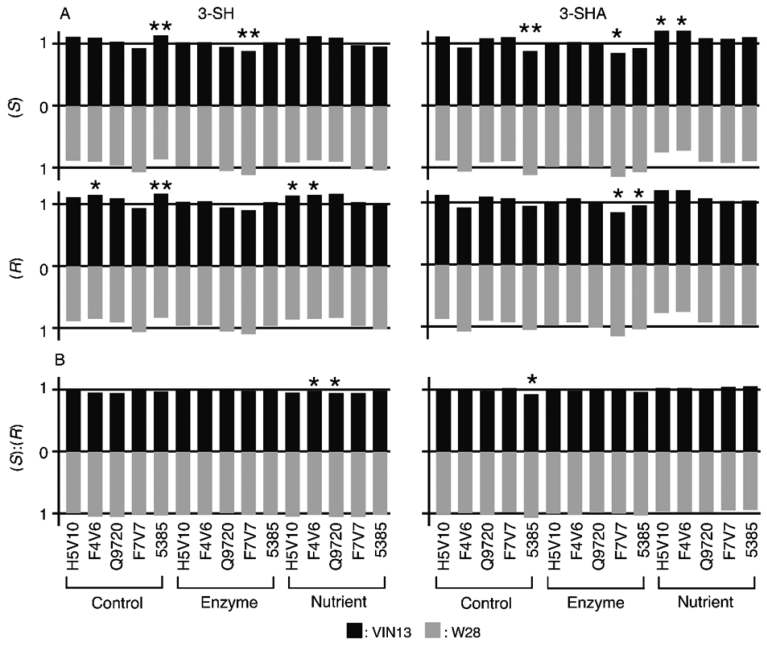

Figure 3. Comparison of *S. cerevisae* yeast strains (VIN13 and W28) on (A) the production of (*S*)- and (*R*)-enantiomers, and (B) the (*S*):(*R*) ratio of pairs of enantiomers of 3-SH and 3-SHA in wines from five clones of Sauvignon blanc. Results are presented as the standardized means of triplicate samples with yeast as the fixed variable compared with an unpaired *t* test ($p < 0.05$). * and ** indicate significant differences between the means at $p < 0.05$ and $p < 0.01$, respectively.

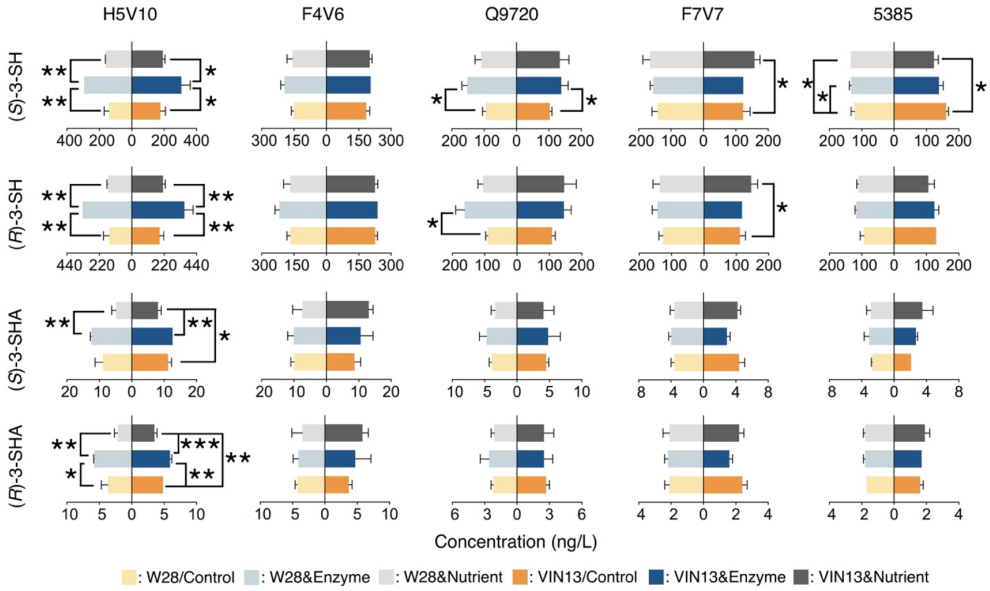

Figure 4. Comparison of the effects of winemaking additives on concentrations (ng/L) of pairs of enantiomers of 3-SH and 3-SHA in wines from five clones of Sauvignon blanc. Results represent mean values (bars) and SD (error bars) of triplicate samples compared with one-way ANOVA followed by Tukey HSD multiple comparison ($p < 0.05$). *, **, and *** indicate significant differences between the means at $p < 0.05$, $p < 0.01$, and $p < 0.001$, respectively. Note the differences in the *x*-axis scales.

higher amounts of (*R*)-3-SH and (*S*)-3-SHA. Similarly, there was minimal influence of the two yeasts on the (*S*):(*R*) ratios in most cases (Figure 3). The thiol production abilities of the two yeasts varied randomly between treatments, and overall there were no obvious consistent differences in thiol enantiomer production abilities in these juices. Changes that were evident with or without winemaking additives could possibly be a consequence of yeast metabolism related to thiol production, i.e., through the enzymes involved in uptake of thiol precursors and release of free thiols,[34−38] which could potentially depend on their stereochemical specificity.[3] Despite the inconsistent effects on enantiomers of the two yeasts that went through the

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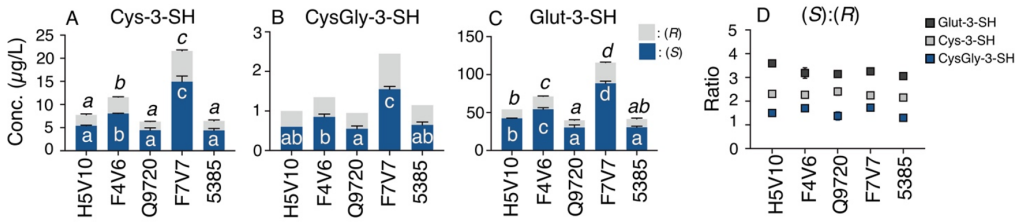

Figure 5. Concentrations (μg/L) of diastereomers of (A) Cys-3-SH, (B) CysGly-3-SH, and (C) Glut-3-SH, and (D) (*S*):(*R*) ratios of precursor diastereomers in juices from five clones of Sauvignon blanc. Data are presented stacked as diastereomer mean values (bars) and SD (error bars) for duplicate samples. Letters in the same format (normal or *italic*) indicate significant differences between the means for the particular precursor diastereomer ($p < 0.05$).

trials, the sensory profiles of wines from different yeasts could potentially be affected based on OAVs for these potent enantiomeric thiols. For instance, the OAV of (*R*)-3-SH in F4V6 wines with VIN13 yeast was 1.2-fold higher than that of W28 yeast (with no additive), which implied that the "grapefruit" and "citrus peel" aromas exhibited in F4V6 wines fermented with VIN13 may be stronger than those in W28. However, other aroma compounds, such as methoxypyrazines and esters in combination with thiol enantiomers, can be highly influential in the overall expression of wine aroma.[4]

Enzyme. Using enzymes in winemaking is a common practice that offers a number of benefits, including aroma enhancement,[39] but nothing detailed has been reported regarding the impact of exogenous enzymes on chiral thiols. Only scarce descriptions of the contributions (mixed or positive) of some commercial enzymes on racemic thiol production during winemaking have been reported previously.[17−19] To investigate the potential impact of winemaking enzymes on thiols, we selected a commercial enzyme (claimed to contain secondary enzymatic activities along with pectinase[40]) that had previously yielded mixed effects.[18] Our results clearly showed that this commercial enzyme could contribute positively to thiol production (Figure 4). The most remarkable example was an approximate 2-fold increase in concentration of 3-SH enantiomers in H5V10 wines after enzyme treatment compared with the control (Table S2 of Supporting Information) regardless of the yeast strain. Differences among the other clones were limited, and there were no significant changes in any enantiomers in F4V6 wines. In addition, it appeared that the chosen enzyme showed a more positive impact on 3-SH enantiomers than those of 3-SHA. There were no significant differences for 3-SHA enantiomers except for (*R*)-3-SHA in H5V10, whereas the effect on 3-SH enantiomers was seen in three out of five clones. This indicated that exogenous enzyme addition was more associated with enhancing the production of 3-SH rather than affecting its acetylation. From a sensory perspective, the enhancement of 3-SH enantiomers in the H5V10 wines upon enzyme treatment led to the OAVs increasing by factors of around 2 and 3 for the (*S*)- and (*R*)-enantiomers, respectively, indicating that the "tropical" aromas of enzyme-treated wines may be perceptibly more intense.

The use of pectinase during maceration of Muscadine grapes has been shown to produce greater amounts of thiols than traditional skin-contact, possibly due to the extraction of glutathione derivatives.[19] However, in the present study, the enzyme was added directly to the juice and additional extraction from the grapes could not occur, so perhaps the enzyme acted by breaking down juice substances (e.g., polysaccharides or proteins) to subsequently influence yeast metabolism and thiol production. Although the extent of enhancement of 3-SH and 3-SHA enantiomers depended on the possible interactions between grape clone and yeast, there appears to be good potential in boosting 3-SH in finished wines from exogenous enzyme addition.

Nutrient. Preferred nitrogen sources (e.g., ammonium, glutamine, asparagine) are vital for the metabolism of yeast[27] and can be another important factor affecting 3-SH and 3-SHA production.[20,36] Other than effects on H_2S production, alternation of these two thiols in wines as a result of yeast rehydration nutrient treatment has been demonstrated in a low nitrogen Riesling fermentation.[20] Amino acid composition has also been shown to affect the production of 3-SH and 3-SHA during fermentation.[36] The commercial nutrient used in our fermentations, consisting of inactive dry yeast rich in amino acids, was designed to provide favorable nitrogen sources for alcoholic fermentation, and especially for nitrogen-deficient circumstances.[41] Although the juices used in this study were deemed low in nitrogen content,[27] treating juices with nutrient showed less impacts than enzyme treatment, in terms of enantiomeric 3-SH and 3-SHA production, in comparison to untreated wines (Figure 4). Significant effects compared to the control were limited to the increases of (*S*)-3-SH (157.8 vs 122.8 ng/L, $p = 0.0219$) and (*R*)-3-SH (145.8 vs 111.3 ng/L, $p = 0.0168$) in F7V7 wines using VIN13 and of (*S*)-3-SH in 5385 wines (134.6 vs 122.5 ng/L, $p = 0.0132$) with W28 yeast. However, such enhancements may not be as significant in terms of sensory contributions (based on a difference in OAV < 1) compared to enzyme treatment. Significant decreases compared to the control were noted for (*S*)-3-SH in 5385 wine (123.1 vs 160.8 ng/L, $p = 0.0168$) and both enantiomers of 3-SHA in H5V10 wine when using VIN13 yeast. Moreover, the nutrient effects seemed to be more evident for VIN13 strain in comparison to W28 (Figure 4). These results suggested the use of nutrient alone was not a solution for thiol enhancement under the low nitrogen conditions tested, and it seems that consideration ideally needs to be given to juice amino acid composition as well.[36]

3-SH Precursors in Juices. Concentrations of the diastereomers of three 3-SH precursors (Cys-3-SH, CysGly-3-SH, and Glut-3-SH) were determined in juices from the five clones of Sauvignon blanc (Figure 5). The quantitative data for Cys-3-SH, CysGly-3-SH, and Glut-3-SH agreed with previous observations that Glut-3-SH always presented at higher concentrations followed by Cys-3-SH and CysGly-3-SH.[9,23] The sum of precursor diastereomers ranged from 40−116 μg/L for Glut-3-SH, 4−15 μg/L for Cys-3-SH, and 0.4−1.6 μg/L for CysGly-3-SH. Juice from F7V7 was found to contain

DOI: 10.1021/acs.jafc.8b01806
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significantly higher concentrations of precursors (up to twice as much) than the other four clones (Figure 5A–C), with this trend being consistent with previous studies.[9,23] Similarly, juice from F4V6 showed concentrations of 3-SH precursors relatively higher than that of H5V10, Q9720, and 5385, but on the whole, there was a lower abundance and larger variation in 3-SH precursor concentrations that was not seen in the previous studies on the same clones.[9,23] These inconsistencies may relate to differential responses due to variations in climate and vineyard management practices[7,42] between vintages, and the comparatively lower abundances could also be reflective of a gentler juice extraction process.

Stereochemically, (*S*)-diastereomers of Cys-3-SH, CysGly-3-SH, and Glut-3-SH were dominant over their (*R*)-diastereomers, which mirrored previous observations.[9,23,43] As shown in Figure 5D, the ratios of the (*S*):(*R*) diastereomers were highest for Glut-3-SH (3.0–3.6), followed by Cys-3-SH (2.1–2.5) and CysGly-3-SH (1.2–1.8). The ratios varied among precursor types for the different clones but showed no significant differences ($P > 0.05$), except for Glut-3-SH between 5383 and H5V10 clones. Any shift in diastereomer distributions could be a consequence of known and unknown formation and degradation pathways of these precursors in grapes or juice.[7,8]

Stereochemistry from Precursors to Thiols. Despite the relationships between precursors (and other metabolites) and free thiols having been investigated before,[9,44,45] a focus on the stereochemical perspective was lacking until now. The relationship between concentrations of thiol enantiomers (3-SH and 3-SHA, ng/L) in wines and diastereomeric precursors (Glut-3-SH, CysGly-3-SH, and Cys-3-SH, μg/L) in juices was examined by Pearson correlation analysis, but there were no obviously strong correlations (r = −0.34–0.06) among these quantitative data sets. The concentrations of 3-SH precursor diastereomers in grapes did not necessarily relate to levels of 3-SH enantiomers found in resulting wines, nor to the combined amounts of 3-SH and 3-SHA enantiomers with that of the precursor diasteromers (Glut-3-SH+CysGly-3-SH+Cys-3-SH). H5V10 had the highest concentrations of 3-SH enantiomers in wines (Figure 4) but was low in all precursors in juices (Figure 5). Following this, the correlation was checked between the ratios of (*S*):(*R*) enantiomers of 3-SH/3-SHA and concentrations of precursor diastereomers in juices, and again, the correlations were not evident or weak (r = −0.20–0.40). It appeared that no correlation existed between the concentrations (and ratios) of thiols in wines and that of the precursors in juices, which is in accordance with results for the racemic 3-SH in the previous studies.[9,45] However, correlations were more obvious when the (*S*):(*R*) ratios of thiols and precursors were compared. Weak to moderate negative correlations were seen for (*S*)-:(*R*)-3-SH with (*S*)-:(*R*)-precursors (r = −0.58, −0.32, −0.49, and −0.58 for Cys-3-SH, CysGly-3-SH, Glut-3-SH, and combined precursors, respectively), whereas positive correlations were found for (*S*):(*R*)-3-SHA (r = 0.16, 0.48, 0.55, and 0.59 for Cys-3-SH, CysGly-3-SH, Glut-3-SH, and combined precursors, respectively).

Principal Component Analysis. Quantitative chemical data on grapes and wines were subjected to PCA to visualize the differences among treatments (clone × enzyme or nutrient × yeast) and possible factors driving these differences. As shown in the biplot (Figure 6), the first two principal components (PC1+PC2) explained 84% of total variance, and wines were generally separated according to clone type. Among

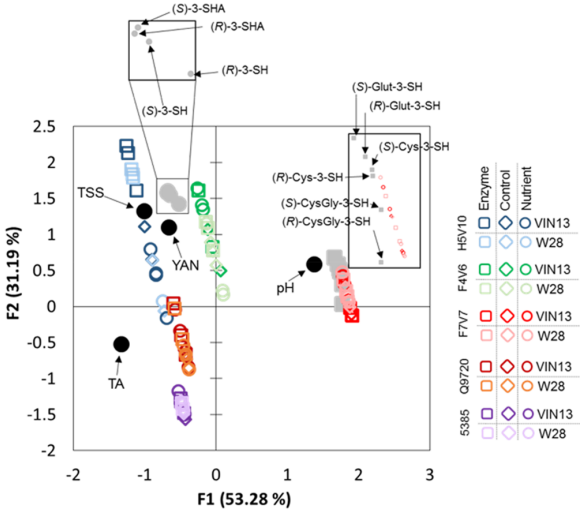

Figure 6. PCA biplot showing scores and loadings of the standardized means for juice and wine chemical data. The boxed insets show expanded regions of the plot to more clearly reveal those details.

them, F7V7 was well isolated from the rest and located on the right along PC1, which positively corresponded with all precursors and pH. In contrast, H5V10, Q9720, and 5385 wines were situated to the left along PC1, and F4V6 was positioned more centrally. The separation of H5V10, Q9720, and 5385 was obvious along PC2 and related to differences in free thiols, TSS, and YAN, which appeared in the top half, and TA located in the bottom half. Compared with the obvious separation of the clones, there was less evident impact of the different additives and yeasts within a clone, although slight separations were seen in H5V10 and F4V6, possibly due to an influence of thiol precursor profiles. When the basic juice data (TSS, pH, TA, and YAN) were omitted from the PCA, an almost identical biplot was obtained (data not shown), which suggested the differences present among treatments were less influenced by those basic parameters (i.e., measures of ripeness) and more by the interaction of grape precursor composition (which did not trend in parallel with ripeness) and winemaking in the release of free thiols, although the precise cause could not be pinpointed.

Future Directions. The prevalence of precursor stereochemistry in the (*S*)-form along with an almost equal distribution of 3-SH enantiomers, but greater abundance of (*S*)-3-SHA over (*R*)-3-SHA, suggested there was stereoselectivity of the enzymes involved in thiol production.[3] However, the relationship is potentially confounded because changes to thiol enantiomer profiles could also arise from the asymmetric production of precursors or free thiols. That is, the chemical formation of racemic Glut-3-SH from reaction of (*E*)-2-hexenal and GSH via 3-*S*-glutathionylhexanal as an intermediate,[10] or of thiols through the formation of racemic 3-SH from H_2S and (*E*)-2-hexenal[46] followed by enzymatic carbonyl reduction. Besides these (bio)chemical reactions that have been directly linked to thiol and precursor formation pathways, attention could also be paid to other compounds derived from thiols that are involved in their apparent consumption, such as the equilibrium between 3-SH and its disulfide,[47] and oxidative loss of thiols[48] during and/or after winemaking. Last but not the least, searching for unknown

DOI: 10.1021/acs.jafc.8b01806
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precursors[7,49] and better elucidating the pathways for thiol biogenesis would also be very useful. Given the dynamic and complex nature of the system and the incomplete picture of formation/consumption of precursors[8,9,50] and thiols,[3,7,50] the current lack of correlations regarding the stereochemistry of precursors and thiols raises more questions about the biogenesis of these potent volatile compounds that require further investigation.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.jafc.8b01806.

Tables containing data for pH, TA, and concentrations of enantiomers of 3-SH and 3-SHA in wines from five clones of Sauvignon blanc, and a matrix of ANOVA multiple comparisons of chiral thiol data between clones; and a figure showing the fermentation profiles of juices from five clones of Sauvignon blanc (PDF)

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Funding

L.C. was a recipient of the joint scholarship of UA and China Scholarship Council (CSC201506350012) and was also supported by an Australian Grape and Wine Authority supplementary scholarship (AGW Ph1512). This research was partly conducted by the Australian Research Council (ARC) Training Centre for Innovative Wine Production (www.adelaide.edu.au/tc-iwp/), which is funded by the Australian Government as a part of the ARC's Industrial Transformation Research Program (IC130100005), with support from Wine Australia and industry partners. AWRI is supported by Australia's grapegrowers and winemakers through their investment body, Wine Australia, with matching funds from the Australian government.

Notes

The authors declare no competing financial interest.

■ ACKNOWLEDGMENTS

We thank UA colleagues, Tommaso Watson, Nick van Holst, and Mandy Lin, for helping with small-scale fermentations and sugar measurements, and Ross Sanders for assistance with precursor analysis. We acknowledge the generous donation of Sauvignon blanc grapes arranged by David Coleman from Adelaide Hills Vine Improvement Inc. (AHVI), enzyme from AEB, and yeast (W28) and nutrient from Grapeworks. UA and AWRI are members of the Wine Innovation Cluster at the Waite Precinct in Adelaide.

■ ABBREVIATIONS USED

3-SH, 3-sulfanylhexan-1-ol; 3-SHA, 3-sulfanylhexyl acetate; ADY, active dry yeast; Cys-3-SH, 3-*S*-cysteinylhexan-1-ol; CysGly-3-SH, 3-*S*-cysteinylglycinehexan-1-ol; DTDP, 4,4′-dithiodipyridine; EDTA 2Na, ethylenediaminetetraacetic acid disodium salt; Glut-3-SH, 3-*S*-glutathionylhexan-1-ol; GSH, glutathione; HPLC-MS/MS, high-performance liquid chromatography-tandem mass spectrometry; MRM, multiple reaction monitoring; OAV, odor activity values; PC, principal component; PCA, principal component analysis; SD, standard deviation; SIDA, stable isotope dilution analysis; TA, titratable acidity; TSS, total soluble solids; YAN, yeast assimilable nitrogen; YPD, yeast extract-peptone-dextrose

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DOI: 10.1021/acs.jafc.8b01806
J. Agric. Food Chem. 2018, 66, 4674−4682

SUPPLEMENTARY INFORMATION FOR

Chiral Polyfunctional Thiols and Their Conjugated Precursors upon Winemaking with Five *Vitis vinifera* Sauvignon blanc Clones

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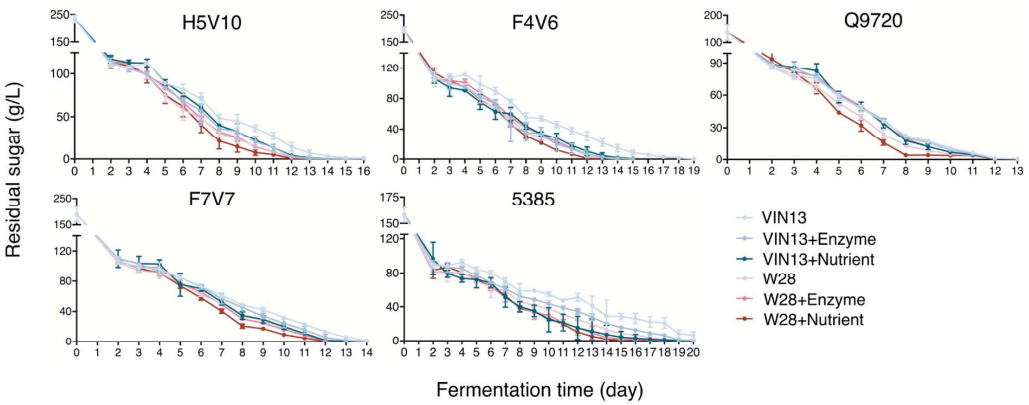


Figure S1. Fermentation profiles of juices from five clones of Sauvignon blanc. Treatment details are provided in Materials and Methods.

Table S1. Basic compositional data (pH, TA) and concentrations (ng/L) of enantiomers of 3-SH and 3-SHA in wines from five clones of Sauvignon blanc.[a]

clone	yeast	additive	pH	TA (g/L)	(*S*)-3-SH	(*R*)-3-SH	(*S*)-3-SHA	(*R*)-3-SHA
H5V10	VIN13	control	2.85±0.22	7.05±0.05	176.8±31.5	190.1±27.6	11.2±1.1	4.8±0.2
		enzyme	2.96±0.00	7.04±0.06	306.3±54.7	357.8±59.4	12.6±0.2	5.9±0.3
		nutrient	2.96±0.02	7.10±0.10	191.2±14.5	211.8±16.4	8.1±1.0	3.5±0.4
	W28	control	2.97±0.01	6.84±0.03	140.8±29.6	153.5±39.1	8.9±2.4	3.7±1.0
		enzyme	2.97±0.00	6.93±0.05	293.8±9.4	334.3±6.5	12.5±0.3	5.8±0.1
		nutrient	2.98±0.03	6.93±0.12	161.5±1.2	161.4±7.8	4.9±1.3	2.2±0.5
5385	VIN13	control	2.88±0.03	6.77±0.05	160.8±6.8	129.9±1.7	2.1±0.2	1.6±0.2
		enzyme	2.94±0.03	6.89±0.06	138.5±13.2	124.2±13.9	2.7±0.2	1.7±0.1
		nutrient	2.90±0.02	6.58±0.31	123.1±13.4	105.6±18.5	3.5±1.3	1.9±0.3
	W28	control	2.91±0.01	6.37±0.40	122.5±10.6	93.2±11.5	2.7±0.1	1.7±0.0
		enzyme	2.94±0.01	6.32±0.21	132.8±5.5	116.8±3.8	3.1±0.6	1.8±0.1
		nutrient	2.90±0.04	6.54±0.02	134.6±3.3	110.4±4.8	2.9±0.5	1.8±0.1
Q9720	VIN13	control	2.89±0.05	6.31±0.08	102.7±6.8	108.1±9.7	4.5±0.4	2.7±0.3
		enzyme	2.90±0.01	7.03±0.21	138.4±20.8	144.8±22.4	4.8±1.9	2.5±0.8
		nutrient	2.90±0.00	6.76±0.01	133.7±27.9	145.6±37.4	4.1±1.6	2.5±0.9
	W28	control	2.92±0.00	6.28±0.02	95.6 ±9.7	90.1±7.1	3.9±0.4	2.2±0.2
		enzyme	2.93±0.01	6.38±0.02	152.4±16.3	161.9±27.8	4.7±1.1	2.6±0.8
		nutrient	2.94±0.01	6.65±0.29	109.4±18.5	105.3±15.4	3.4±0.6	2.1±0.3
F4V6	VIN13	control	2.84±0.07	6.81±0.06	184.8±15.9	226.4±10.5	8.7±1.9	3.7±0.5
		enzyme	2.92±0.00	6.72±0.19	205.2±4.3	237.3±4.6	10.6±3.8	4.7±2.4
		nutrient	2.93±0.00	6.62±0.14	200.8±10.4	225.5±12.9	13.1±1.3	5.8±0.9
	W28	control	2.93±0.01	6.50±0.02	151.6±10.4	167.7±15	9.9±1.1	4.3±0.3
		enzyme	2.94±0.01	6.69±0.05	194.6±16.8	217.9±20.5	10.1±1.9	4.1±0.8
		nutrient	2.94±0.00	6.82±0.06	157.0±25.6	167.9±30.5	7.5±2.9	3.5±1.6
F7V7	VIN13	control	2.88±0.00	8.25±0.03	122.8±21.1	111.3±17.0	4.4±0.7	2.4±0.3
		enzyme	2.89±0.00	8.32±0.04	123.1±3.1	117.6±3.7	2.9±0.4	1.6±0.2
		nutrient	2.89±0.00	8.82±0.72	157.8±16.7	145.8±20.2	4.2±0.4	2.2±0.3
	W28	control	2.90±0.01	8.01±0.11	142.0±16.9	126.7±12.4	3.6±0.4	2.1±0.3
		enzyme	2.90±0.01	8.18±0.01	155.8±8.8	143.6±16.3	4.0±0.3	2.2±0.2
		nutrient	2.90±0.00	8.16±0.06	165.2±21.7	136.8±20.9	3.6±0.5	2.1±0.4

[a] Data indicate the mean ± standard deviation of triplicate samples. Results of the statistical analysis of quantitative chiral thiol data for clone type are presented in Table S2 of the Supporting Information.

Table S2. Matrix of ANOVA p-values from Tukey's HSD multiple comparison tests of the mean concentrations (ng/L) of enantiomers of 3-SH and 3-SHA, and the (*S*):(*R*) enantiomer ratios, in wines from five clones of Sauvignon blanc.

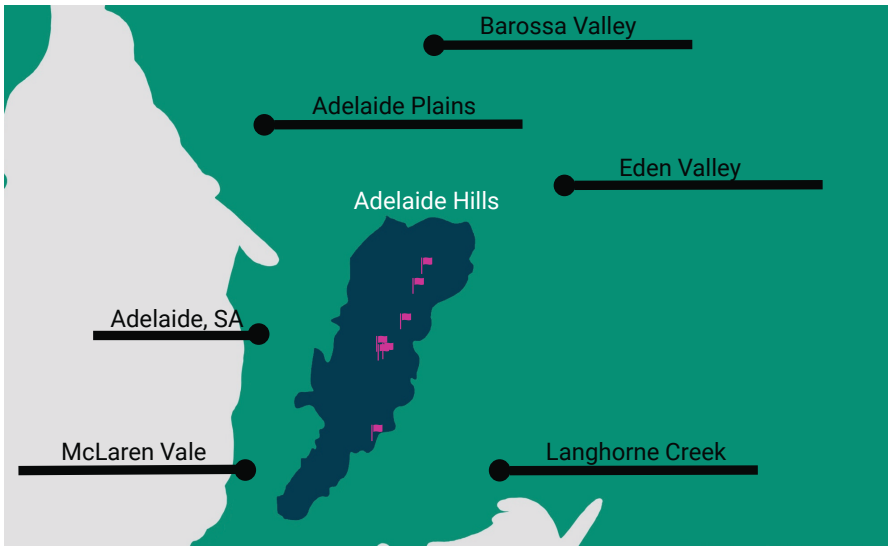
(*S*)-3-SH	F4V6	Q9720	F7V7	5385	**(*R*)-3-SH**	F4V6	Q9720	F7V7	5385
H5V10	0.0313	<0.0001	<0.0001	<0.0001	H5V10	0.0860	<0.0001	<0.0001	<0.0001
F4V6		<0.0001	0.0404	0.0050	F4V6		<0.0001	<0.0001	<0.0001
Q9720			0.3003	0.7398	Q9720			0.9930	0.9564
F7V7				0.9463	F7V7				0.7843
(*S*)-3-SHA	F4V6	Q9720	F7V7	5385	**(*R*)-3-SHA**	F4V6	Q9720	F7V7	5385
H5V10	>0.9999	<0.0001	<0.0001	<0.0001	H5V10	0.9944	<0.0001	<0.0001	<0.0001
F4V6		<0.0001	<0.0001	<0.0001	F4V6		<0.0001	<0.0001	<0.0001
Q9720			0.9620	0.1638	Q9720			0.7948	0.1157
F7V7				0.4845	F7V7				0.6695
(*S*)-:(*R*)-3-SH	F4V6	Q9720	F7V7	5385	**(*S*)-:(*R*)-3-SHA**	F4V6	Q9720	F7V7	5385
H5V10	0.9296	0.0162	<0.0001	<0.0001	H5V10	0.1875	0.1710	<0.0001	<0.0001
F4V6		0.0012	<0.0001	<0.0001	F4V6		0.0002	<0.0001	<0.0001
Q9720			<0.0001	<0.0001	Q9720			0.0536	0.0007
F7V7				0.0006	F7V7				0.6042

Chapter 5

Investigation of intraregional variation, grape amino acids, and pre-fermentation freezing on varietal thiols and their precursors for *Vitis vinifera* Sauvignon blanc.

Food Chemistry 2019, 295, 637–645. DOI.

Chen L., Capone D. L., Nicholson E. L., Jeffery D. W.



Statement of Authorship

Title of Paper	Investigation of intraregional variation, grape amino acids, and pre-fermentation freezing on varietal thiols and their precursors for *Vitis vinifera* Sauvignon blanc
Publication Status	☑ Published ☐ Accepted for Publication ☐ Submitted for Publication ☐ Unpublished and Unsubmitted work written in manuscript style
Publication Details	Chen, L.; Capone, D.L.; Nicholson, E.L.; Jeffery, D.W. (**2019**) Investigation of Intraregional Variation, Grape Amino Acids, and Pre-fermentation Freezing on Varietal Thiols and Their Precursors for *Vitis vinifera* Sauvignon blanc. *Food Chem.*, 295, 637–645.

Principal Author

Name of Principal Author (Candidate)	Liang Chen
Contribution to the Paper	Conceptual contribution to experiment design. Organised and harvested grapes. Prepared juices from grapes and performed winemaking trials. Performed chemical analysis (enzymatic assays for fermentation, basic physicochemical parameter measurements, SPE HPLC–MS/MS analysis for thiols and precursors). Collected raw instrument data, processed, and interpreted data for manuscript. Wrote the first draft of the manuscript. Edited and revised paper for publication.
Overall percentage (%)	70%
Certification:	This paper reports on original research I conducted during the period of my Higher Degree by Research candidature and is not subject to any obligations or contractual agreements with a third party that would constrain its inclusion in this thesis. I am the primary author of this paper.
Signature	Date 10/09/19

Co-Author Contributions

By signing the Statement of Authorship, each author certifies that:

i. the candidate's stated contribution to the publication is accurate (as detailed above);

ii. permission is granted for the candidate in include the publication in the thesis; and

iii. the sum of all co-author contributions is equal to 100% less the candidate's stated contribution.

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Signature	Date 10/09/19

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Contribution to the Paper	Conducted SPE HPLC analysis on grape amino acids. Collected raw instrument data and interpreted the data. Edited and revised the manuscript for publication.
Signature	Date 10/09/19

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Contribution to the Paper	Conceived the original experiment design. Supervised the entire project. Organised sample collection (grape harvest). Interpreted data for publication. Edited, revised, and submitted the manuscript for publication, acting as corresponding author at all stage.
Signature	Date 11/9/2019

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Food Chemistry 295 (2019) 637–645

Contents lists available at ScienceDirect

Food Chemistry

journal homepage: www.elsevier.com/locate/foodchem

Investigation of intraregional variation, grape amino acids, and pre-fermentation freezing on varietal thiols and their precursors for *Vitis vinifera* Sauvignon blanc

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ARTICLE INFO

Keywords:
3-Sulfanylhexan-1-ol
3-Sulfanylhexyl acetate
4-Methyl-4-sulfanylpentan-2-one
Aroma enhancement
Wine aroma
Winemaking

Chemical compounds studied in this article:
3-Sulfanylhexan-1-ol (PubChem CID 521348)
3-Sulfanylhexyl acetate (PubChem CID 518810)
4-Methyl-4-sulfanylpentan-2-one (PubChem CID 88290)
Arginine (PubChem CID 6322)
Proline (PubChem CID 145742)
Glutamic acid (PubChem CID 33032)
γ-Aminobutyric acid (PubChem CID 119)
α-Alanine (PubChem CID 5950)

ABSTRACT

Sauvignon blanc grape samples (n = 21) from across a single Geographical Indication of South Australia were analysed for thiol precursors and amino acids, and fermented in an identical laboratory-scale fermentation trial to investigate the intraregional pattern of varietal thiols in the wines. Precursors and thiols exhibited obvious intraregional diversity, and notably, stronger correlations were observed between a number of amino acids and thiol precursors (especially with glutamic acid, $r \leq -0.73$) rather than free thiols. Additionally, pre-fermentation freezing (−20 °C, 1 month) was applied to five selected fresh grape samples and their juices, followed by identical fermentation. In comparison to wines from fresh grapes or frozen juices, significant elevation of varietal thiols (up to 10-fold) occurred in the wines derived from frozen grapes, with parallel increases of precursors (up to 19-fold) in juices from frozen berries. These novel results may lead to new strategies for thiol enhancement during winemaking.

1. Introduction

Sauvignon blanc (*Vitis vinifera*) is one of the most widely cultivated grapevine varieties in all major wine-producing countries (OIV, 2017). According to the International Organisation of Vine and Wine, Sauvignon blanc is the only top white variety that had a significant increase (> 3%) in annual change of vineyard area worldwide from 2000 to 2015 (OIV, 2017). The success and popularity of Sauvignon blanc wine undoubtedly relate to its distinctive and characteristic "grassy", "citrus", and "tropical fruit" aromas, which are largely contributed by potent volatile compounds with odour thresholds in the nanogram-*per*-litre range, such as methoxypyrazines and varietal thiols (Coetzee & du Toit, 2012; Jeffery, 2016).

In relation to varietal thiols, 3-sulfanylhexan-1-ol (3-SH), 3-sulfanylhexyl acetate (3-SHA), and 4-methyl-4-sulfanylpentan-2-one (4-MSP) are well recognised as the fundamental volatile compounds imparting aromas of "passionfruit", "grapefruit", "guava", and "box tree" to Sauvignon blanc wine as well as wines made from several other *Vitis vinifera* grape varieties (Roland, Schneider, Razungles, & Cavelier, 2011). 3-SH and 4-MSP are formed through alcoholic fermentation by the action of yeast enzymes from their non-volatile precursors extracted from grapes, and 3-SHA is formed enzymatically from 3-SH (Roland

Abbreviations: 3-SH, 3-sulfanylhexan-1-ol; 3-SHA, 3-sulfanylhexyl acetate; 4-MSP, 4-methyl-4-sulfanylpentan-2-one; ANOVA, analysis of variance; Cys-3-SH, 3-*S*-cysteinylhexan-1-ol; DTDP, 4,4′-dithiodipyridine; GABA, γ-aminobutyric acid; GI, Geographical Indication; Glut-3-SH, 3-*S*-glutathionylhexan-1-ol; IS, internal standard; PC, principal component; PCA, principal component analysis; PFF, pre-fermentation freezing; SD, standard deviation; SIDA, stable isotope dilution assay; SPE, solid-phase extraction; TA, titratable acidity; TSS, total soluble solids

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https://doi.org/10.1016/j.foodchem.2019.05.126

Available online 21 May 2019

et al., 2011). However, precursors identified in grape juice so far, involving glutathione, dipeptide and cysteine conjugates, and α,β-unsaturated carbonyls, can only partially account for the amounts of the varietal thiols found in wine, primarily for 3-SH (Bonnaffoux et al., 2018; Roland et al., 2011). Furthermore, no consistent correlations have been seen between varietal thiols and their putative precursors (Chen, Capone, Tondini, & Jeffery, 2018; Jeffery, 2016; Pinu, Jouanneau, Nicolau, Gardner, & Villas-Boas, 2012), which suggests that other varietal thiol precursors or alternative biogenesis and fate pathways are still waiting to be revealed. Apart from precursor availability, varietal thiol production during fermentation also depends on grape composition (Pinu et al., 2019), such as the profile of amino acids and certain organic acids (Alegre, Culleré, Ferreira, & Hernández-Orte, 2017; Pinu et al., 2014). However, other than studies involving thiol precursors, literature linking grape composition to varietal thiol formation is limited, and although the enhancive or suppressive roles of amino acids (amounts and ratios) on varietal thiol production have been demonstrated as outlined already, such effects and relationships require further elucidation.

With the incomplete picture of biogenesis of varietal thiols and complex relationship to other grape metabolites, controllable management of the production of varietal thiols and the related sensory quality of a wine through viticultural or oenological practices is still not easy to achieve. In recent years, vineyard practices (application of nitrogen and sulfur), grapes (maturity, clones, grape metabolites), berry processing (harvest, crush, press etc.), and fermentation choices (yeast, commercial additives) have been investigated for their impacts on varietal thiols and/or their precursors (Chen et al., 2018; Jeffery, 2016; Roland et al., 2011; Santiago & Gardner, 2015). However, most of the practices exhibited mixed effects (grape-dependent or product-specific) and the modulation of precursors in grapes was not always reflected in the production of varietal thiols in wine. As such, vineyard and/or winemaking practices for enhancing thiol concentrations in wines are still required. Low temperature treatment of grapes maybe a useful option based on the use of cryogenic processing technology in the beverage industry (Brown, 1975; Pando Bedriñana, Picinelli Lobo, & Suárez Valles, 2019). The first indication of its potential utility for thiol management in Sauvignon blanc was revealed in a study of thiol precursors 3-*S*-cysteinylhexan-1-ol (Cys-3-SH) and 3-*S*-glutathionylhexan-1-ol (Glut-3-SH), whereby Glut-3-SH increased by around four times in frozen grapes stored at −20 °C for 2 months compared to frozen or fresh juices (Capone, Sefton, & Jeffery, 2011). In a subsequent study, pre-fermentative cryomaceration, undertaken by adding dry ice to crushed Sauvignon blanc grape must and leaving it to thaw over a 24-h period, was found to increase 3-SH and 3-SHA concentrations in the wine (Olejar, Fedrizzi, & Kilmartin, 2015). However, the effect of cryogenic storage on thiol production during fermentation remained to be further investigated, and influences of cryogenic treatments on grape precursors and wine thiols have never been shown in parallel.

The present work sought to investigate a number of hypotheses related to varietal thiols and precursors, which included: i) the presence of intraregional variation; ii) relationship with grape amino acids; iii) pre-fermentation freezing (PFF) as a tool to enhance thiols in wine. Parcels of Sauvignon blanc grapes (n = 21) were hand harvested from commercial vineyards within the Geographical Indication (GI) of the Adelaide Hills wine region. Amino acids and thiol precursors were measured in grape juices and laboratory-scale fermentation trials were conducted with a high throughput automated fermentation platform. Varietal thiols were analysed in the finished wines by HPLC–MS/MS after derivatisation. Intraregional variations of precursors in juices and varietal thiols in wines were examined and correlated with amino acids in grapes. To test the potential applicability for thiol enhancement during winemaking, PFF treatment (−20 °C, 1 month) was applied for the first time to the fermentation of a subset of fresh whole grape bunches and matched juices that were obtained from the fresh grapes.

2. Material and methods

2.1. Chemicals and solutions

The following chemicals and consumables were obtained from commercial suppliers: 4,4′-dithiodipyridine (DTDP), formic acid, acetaldehyde, and EDTA 2Na (Sigma-Aldrich, Castle Hill, NSW, Australia); Merck liquid chromatography-grade ethanol, methanol, and acetonitrile (VWR International, Tingalpa, QLD, Australia); Bond Elut C18 (500 mg, 6 mL) solid-phase extraction (SPE) cartridges (Agilent, Mulgrave, VIC, Australia); polymeric Strata-X-C (30 mg, 1 mL) and Strata SDB-L (500 mg, 6 mL) SPE cartridges (Phenomenex, Lane Cove, NSW, Australia); AccQ-Fluor amino acid reagent kit and AccQ-Tag eluent A (Waters, Rydalmere, NSW, Australia). Water used was purified through a Milli-Q purification system (Millipore, North Ryde, NSW, Australia). Thiol and precursor standards and internal standards (IS) were prepared as previously reported (Chen et al., 2018). Standard and IS solutions were prepared volumetrically either in Milli-Q water (for mixtures of precursors) or in absolute ethanol (for mixtures of thiols). Stock solutions were kept at −20 °C and working solutions were stored at 4 °C until required. DTDP solution (10 mM) was prepared as detailed previously (Capone, Ristic, Pardon, & Jeffery, 2015).

2.2. Grape and juice

Parcels of *Vitis vinifera* L. cv Sauvignon blanc grapes (n = 21, abbreviated in Table S1 of the Supporting Information) encompassing five clones were hand-picked from seven commercial vineyards located in the Adelaide Hills GI of South Australia (L1–L7, mapped in Fig. S1 of the Supporting Information) on 27th February (n = 9), 28th February (n = 7), and 7th March (n = 5) during the 2018 vintage. For each sample, ≈8 kg of whole grape bunches were collected from both sides of the vines across multiples rows within each vineyard, temporarily stored in food-grade resealable plastic bags (≈2 kg/bag), transported to the laboratory (< 2 h) and stored at 4 °C overnight. Grape bunches were then gently randomised in a plastic sample tray and divided into two subsets (≈5 kg + ≈3 kg).

The first subset of fresh grape bunches (≈5 kg) was sulfured (50 mg/kg SO_2 added as potassium metabisulfite) and crushed immediately under dry ice protection following a previously reported procedure (Chen et al., 2018). The resultant juices were collected in food-grade plastic storage bottles (1 L), cold settled at 4 °C for 12 h, and the clear juices were divided into two groups: the first group of juices (n = 21) was subjected to laboratory-scale fermentation immediately, acting as the Control wines (non-PFF); the other group of clear juices was stored in PET bottles (500 mL, protected by dry ice during filling) at −20 °C, and used as the frozen juice treatment (PFF-juice).

The second subset of fresh bunch grapes (≈3 kg) was carefully sealed in food-grade resealable plastic bags and wrapped in aluminium foil, and stored at −20 °C as the frozen grape treatment (PFF-grape). After 1 month, only the frozen juices and matching grape bunches from co-located Sauvignon blanc clones (L4, n = 5, Table S1 of the Supporting Information) were assessed to highlight this concept. Juices were thawed at 4 °C overnight, and defrosted grape bunches were crushed and the resultant juices were collected in the same manner as for non-PFF wine, undergoing cold settling at 4 °C overnight. Fermentation of the thawed juices and juices obtained from frozen grape bunches was conducted in an identical manner to the Control wines.

2.3. Fermentation

Laboratory-scale fermentations were performed in triplicate on an automated fermentation platform (TEE-BOT) as detailed previously (Chen et al., 2018). Yeast *Saccharomyces cerevisiae* strain VIN13 (after culturing in liquid YPD for 24 h at 28 °C) was used for inoculation (1 mL of culture). Fermentation temperature was set at 16 °C. Residual sugars

were sampled daily and measured using an enzymatic assay (Chen et al., 2018). Fermentation was considered to be completed when residual sugars were < 2.5 g/L. Finished ferments were cold settled at 4 °C for about 1 week before being opened for varietal thiol analysis.

2.4. Basic juice parameter measurement

Total soluble solids (TSS), pH, and titratable acidity (TA) were measured in freshly obtained juice samples in duplicate according to the previously reported methods (Chen et al., 2018).

2.5. High-performance liquid chromatography (HPLC) analysis for amino acids in juices

Freshly thawed juice obtained from fresh whole bunches (n = 21) was centrifuged at 14,462*g* for 10 min and 60 µL of supernatant was collected and mixed with 60 µL of α-aminobutyric acid (0.5 mM in MilliQ water). Mixed samples (100 µL) were loaded onto Strata-X-C cartridges preconditioned with 1 mL of methanol followed by 1 mL of water. After sample loading, the column was washed with 1 mL of 80% aq. methanol solution and eluted with 1 mL of freshly prepared 25% ammonium hydroxide:methanol (1:1) and the eluate was dried under nitrogen flow at room temperature using an Alltech drying lid attachment for a vacuum manifold (Grace Davison Discovery Sciences, Rowville, VIC, Australia). The dried extract was reconstituted with 1 mL of sodium borate buffer (0.2 M, pH = 8.8), derivatised according to the manufacturer's instructions using an AccQ-Fluor reagent kit, and analysed by HPLC with a fluorescence detector following a published procedure and using the same instrumentation and HPLC parameters (Culbert et al., 2017).

2.6. Stable isotope dilution assay (SIDA) using high-performance liquid chromatography and tandem mass spectrometry (HPLC–MS/MS) for thiol precursors in juices

Freshly thawed juice obtained from fresh whole bunches (n = 21) was cold settled at 4 °C for 2 h and aliquot was analysed for thiol precursors (Cys-3-SH, Glut-3-SH) in duplicate according to a previously reported method with modified reconstitution procedure (Capone & Jeffery, 2011). Analysis was performed on a Thermo Finnigan Surveyor HPLC fitted with an Alltima C18 HPLC column (250 × 2.1 mm i.d., 5 µm, 100 Å, Grace Davison Discovery Sciences, Rowville, VIC, Australia) connected to a Thermo Finnigan LCQ Deca XP Plus mass spectrometer using electrospray ionisation in positive ion mode. Chromatographic conditions and ion pairs were as described previously (Capone, Sefton, Hayasaka, & Jeffery, 2010) and helium was used as collision gas with the following source and mass spectrometer conditions: spray voltage of 4.5 kV, respective sheath and aux/sweep gas flow rates of 30 and 19, capillary voltage of 36 V, capillary temperature of 250 °C, single reaction monitoring mode with activation Q of 0.250, activation time of 30 ms, normalised collision energy of 35%, and isolation width m/z = 1.50. Xcalibur software (Thermo Finnigan, version 1.3) was used for instrument control and data acquisition. Cys-3-SH and Glut-3-SH concentrations were reported as the sum of the two respective diastereomers.

2.7. SIDA HPLC–MS/MS analysis for thiols in wines

Thiol extracts were prepared and analysed following a previously published method (Capone et al., 2015). After cold settling, ferment bottles were opened and an aliquot of wine (20 mL) was accurately pipetted into a 22 mL glass vial for sample preparation according to the previously reported derivatisation and isolation steps. Extracts were reconstituted with 10% aq. ethanol solution (200 µL) and stored at −20 °C pending analysis. A batch of calibration and quality control samples was prepared in the same manner with the wine samples for quantitation. HPLC–MS/MS analysis was performed with an Agilent 1200 Series HPLC connected to an Agilent 6410A Triple Quad MS (Agilent, Santa Clara, CA, USA) as reported previously (Chen, Capone, & Jeffery, 2018).

2.8. Statistics

Data reduction, mean values, standard deviation (SD), and Pearson correlation were performed with Microsoft Excel 2016. Unpaired *t*-test (two tailed) and one-way analysis of variance (ANOVA) was conducted with $\alpha = 0.05$ using Prism 7 (GraphPad Software, CA, USA). Principal component analysis (PCA) was undertaken on all significantly different variables after standardisation using The Unscrambler X (CAMO Software, Oslo, Norway).

3. Results and discussion

Regional investigations of varietal thiols and precursors have been previously reported in few instances and mostly focused on Sauvignon blanc from the world famous Marlborough region of New Zealand (Jouanneau et al., 2012; Pinu et al., 2012) although other regions and varieties have also been evaluated (Capone, Barker, Williamson, & Francis, 2017; Fracassetti et al., 2018). The cool climate Adelaide Hills wine region in South Australia was the focus for the present work, with a total of 21 Sauvignon blanc grape parcels sourced from vineyard blocks in seven locations (Fig. S1 of the Supporting Information) during the 2018 vintage to investigate the intraregional variations of precursors in juices and thiols in wines. Grape samples were harvested by hand at around the same maturity levels (Table S1 of the Supporting Information) and fermented in triplicate under identical winemaking conditions at laboratory-scale using an automated fermentation platform (Chen et al., 2018).

3.1. Basic juice parameters and fermentation

The results for TSS, pH, and TA for freshly obtained juices of the 21 Sauvignon blanc grape parcels are summarised in Table S1 of the Supporting Information. A TSS of around 20–21 °Brix was targeted but sampling had to occur within the constraints of the commercial vineyards. TSS values generally ranged from 19 to 22 °Brix (L1_1 and L3_2 were ≤ 17 °Brix), pH varied from 2.53 to 3.32, and TA was between 6.5 and 13.9 g/L. Except for the higher TA values in 2018, the basic juice parameters of L4 were similar to the data from the 2017 vintage for grapes from the same vines (Chen et al., 2018). Slight differences in ripeness within single locations (even for the same clones) and across the GI were considered to result from complex ecophysiological responses and/or viticulture practices (Dai et al., 2011). For all fermentation trials, cold-settled clear juices were fermented in triplicate in an identical manner without any adjustments to composition using commercial yeast strain VIN13 at 16 °C. Fermentations all proceeded to dryness (< 2.5 g/L) within 3 weeks and no obvious patterns of fermentation duration across grape samples were noticed.

3.2. Overview of intraregional variation on precursors in juices and thiols in wines

Data from quantitative analysis of juice precursors (Glut-3-SH and Cys-3-SH, sum of respective diastereomers) and wine varietal thiols (3-SH, 3-SHA, and 4-MSP) are presented in Fig. 1a–f and Table S2 of the Supporting Information. The two precursors were detected in all juice samples with Glut-3-SH (33.7–170.7 µg/L) dominating over Cys-3-SH (7.9–44.7 µg/L) (Fig. 1a, Table S2 of the Supporting Information). There was a strong positive correlation between Cys-3-SH and Glut-3-SH (r = 0.98, Fig. 1f). The higher abundance of Glut-3-SH and the strong correlation between precursors were in accord with previous studies (Capone et al., 2010; Fracassetti et al., 2018; Pinu et al., 2012),

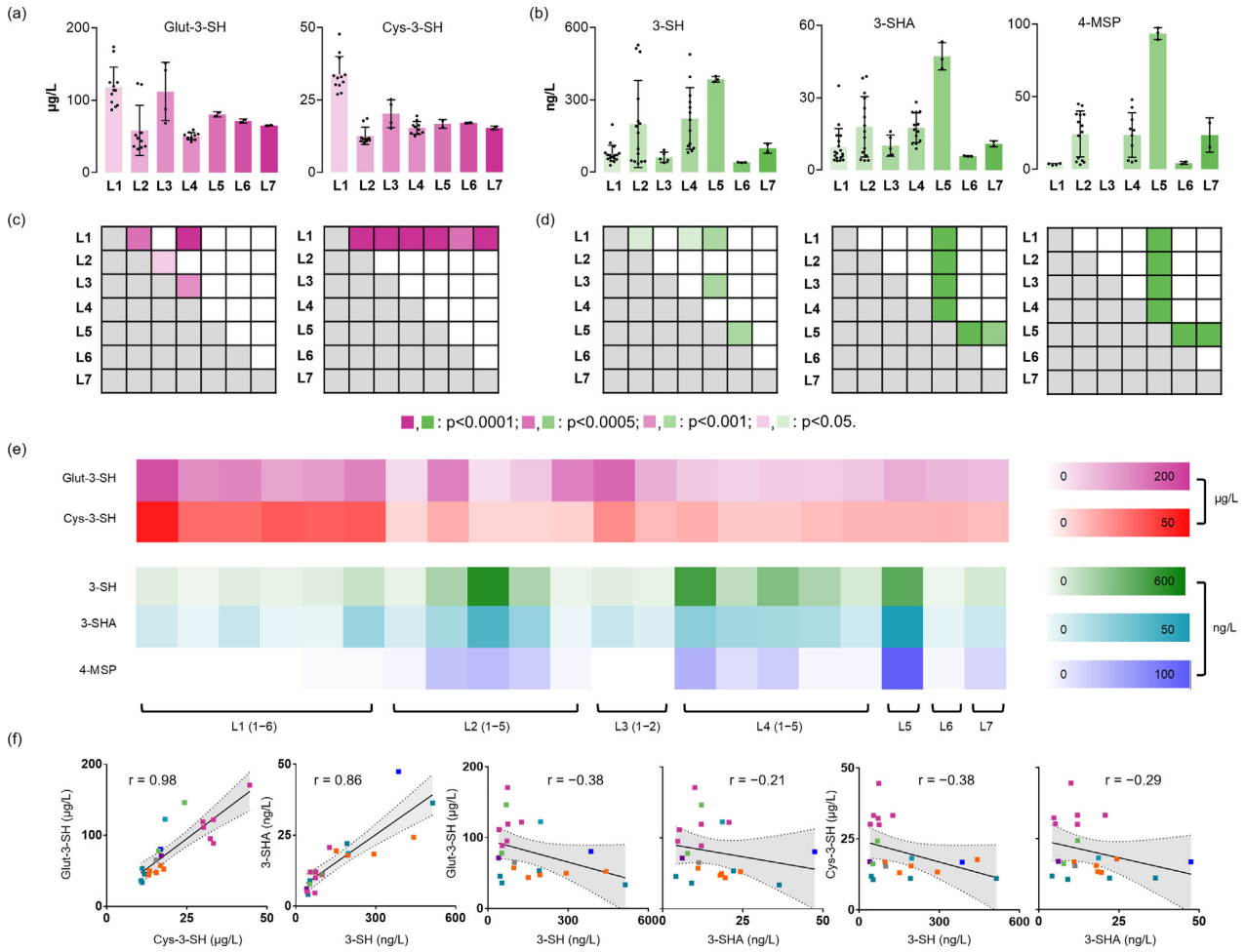

Fig. 1. Overview of the precursors (Glut-3-SH and Cys-3-SH) in juices and varietal thiols (3-SH, 3-SHA, and 4-MSP) in wines from 21 Sauvignon blanc grape parcels from seven locations (L1 to L7) within the Adelaide Hills wine region showing: mean concentrations of (a) precursors (µg/L) and (b) thiols (ng/L), where error bars represent the group SD and scattered dots in black indicate the measured value of analyte in individual samples; statistically significant differences (coloured) of (c) precursors and (d) thiols across locations, examined by one-way ANOVA ($\alpha = 0.05$); (e) heat maps showing the quantitative results of precursors and thiols by grape parcel; and (f) scatter plots (Glut-3-SH vs. Cys-3-SH, 3-SH vs. 3-SHA, precursors vs. varietal thiols) with shaded areas indicating 95% confidence bands and black lines showing the best-fit lines based on Pearson correlation analysis. For location (L) details, refer to Table S1 and Fig. S1 of the Supporting Information. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

and is reflective of an enzymatic degradation pathway of Glut-3-SH to Cys-3-SH as detailed previously (Jeffery, 2016). The overall concentrations of precursors were well in line with data from the previous vintage for grapes from the Adelaide Hills (samples from L4) (Chen et al., 2018).

Previous studies have demonstrated variations of precursors in Sauvignon blanc juices but they had either been assessed in a smaller sample set (n = 5) (Allen et al., 2011) or used commercial juices arising from standard practices (Pinu et al., 2012) that were unlikely to involve consistent grape processing (e.g., transport, maceration, and press cycle). In that latter report, variations of precursors in 55 commercial New Zealand Sauvignon blanc juices from different vintages but mainly from locations within Marlborough were up to 20-fold and 126-fold for Glut-3-SH and Cys-3-SH, respectively (Pinu et al., 2012). In the present study, 21 grape parcels and corresponding juices were obtained in an identical manner so the results may better reflect possible intraregional variations of precursors.

The concentrations of precursors in grapes from different locations were examined by one-way ANOVA ($\alpha = 0.05$), with results presented in Fig. 1c. In terms of Glut-3-SH, significant differences occurred between L1 (118.0 ± 27.8 µg/L) and L2 (58.3 ± 34.7 µg/L), L1 and L4 (50.3 ± 5.0 µg/L), L2 and L3 (112.2 ± 40.4 µg/L), and L3 and L4. For Cys-3-SH, a significant difference was only present between L1 samples (average 33.9 µg/L) and others (average 12.6–20.2 µg/L). Within the vineyard locations containing different blocks (and clones) that were sampled (i.e., L1 to L4), Cys-3-SH varied almost consistently, around 1.4-fold (L4) to 1.7-fold (L2), whereas Glut-3-SH fluctuated from 1.3-fold (L4) to 3.6-fold (L2), apparently independent of grape ripeness. This variation among grape parcels from within single locations may suggest that the biological accumulation of Glut-3-SH was more affected (e.g., by genetics and/or environment) than Cys-3-SH, as the post-harvest processing conditions were essentially identical.

3-SH, 3-SHA, and 4-MSP in the resulting wines also occurred at various concentrations (Fig. 1b, Table S2 of the Supporting Information), with 3-SH ranging from 29 to 528 ng/L (average 152 ng/L, 18-fold variation) and 3-SHA ranging from 4 to 53 ng/L (average 15 ng/L, 13-fold variation), in agreement with previous data reported for Adelaide Hills Sauvignon blanc wines (Capone et al., 2011; Chen et al., 2018). Wines high in 3-SH were usually high in 3-SHA, with the strong correlation ($r = 0.86$, Fig. 1f) being consistent with the yeast acetylation pathway linking 3-SHA to 3-SH (Roland et al., 2011). Concentrations of 4-MSP in the finished wines varied from undetectable

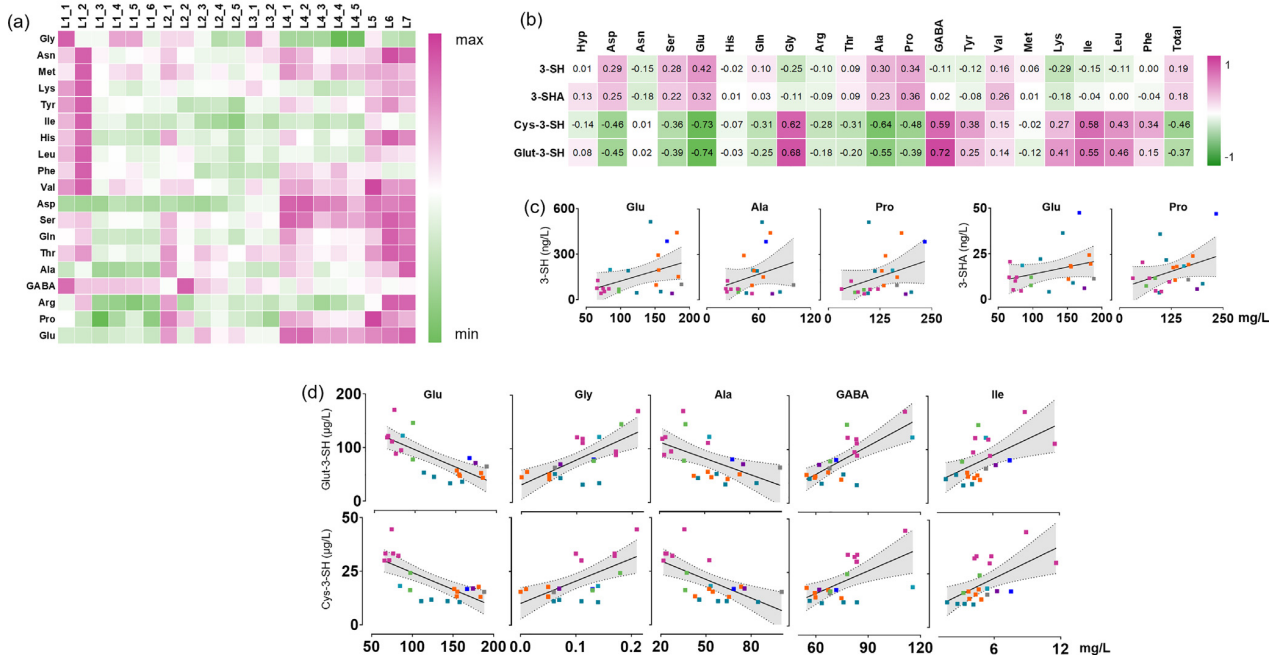

Fig. 2. (a) Relative quantity (%) of amino acids in 21 Sauvignon blanc juices from Adelaide Hills; (b) correlation values between thiols and precursors to amino acids; and (c, d) scatter plots (3-SH and 3-SHA, Glut-3-SH and Cys-3-SH vs. certain grape amino acids) with shaded areas indicating 95% confidence bands and black lines showing the best-fit lines based on Pearson correlation analysis. For location (L) details, refer to Fig. S1 and Table S1 of Supporting Information.

in six samples up to a notable high of 97 ng/L (Fig. 1b). Compared to reported odour detection thresholds of thiols (Roland et al., 2011), 15 out of 21 Sauvignon blanc wines contained 3-SH above its odour threshold (odour activity value, OAV: 1.1–8.8), 17 out of 21 wines had 3-SHA greater than its reported threshold (OAV: 1.0–13.3), and all wines containing 4-MSP had concentrations above its odour threshold (OAV: 3.4–121.7). The abundances of these thiols at concentrations well-above threshold means they would be expected to contribute perceivable "tropical fruit" aromas in these laboratory scale Adelaide Hills Sauvignon blanc wines.

Regarding intraregional variations, the patterns for 3-SH, and especially 3-SHA and 4-MSP, were similar (Fig. 1b), with L5 standing out with significantly higher thiol levels compared with others based on one-way ANOVA (Fig. 1d). In contrast, L3 and L6 showed lower amounts of all three thiols. In combination with precursor data, no obvious relationship from precursors to thiols was apparent in their patterns of variation. Juices with higher amounts of precursors did not necessarily lead to wines with greater levels of thiols, with L1 being a notable example (Fig. 1e). The opposite could also be said, as was the case for L5, with moderate juice precursor levels but high wine thiol concentrations. Quantitatively, 3-SH and 3-SHA in the wines were both negatively correlated to Glut-3-SH ($r = -0.38$ with 3-SH, $r = -0.21$ with 3-SHA) and Cys-3-SH ($r = -0.38$ with 3-SH, $r = -0.29$ with 3-SHA) in juices (Fig. 1f). These correlations between precursors and varietal thiols contrasted to previously reported correlation results for 55 Sauvignon blanc juices and wines, where little correlation was found for 3-SH and weak but positive correlations to Cys-3-SH, Glut-3-SH and total precursors were evident for 3-SHA (Pinu et al., 2012).

Due to the limited availability of results that examine correlations between precursors and thiols, several previously reported sets of quantitative data for Sauvignon blanc juice and wine (Allen et al., 2011; Capone et al., 2011; Chen et al., 2018) were selected and the correlation coefficients were calculated. Interestingly, the calculated correlations were 0.32 (Capone et al., 2011) and 0.40 (data from hand-picked grapes were selected) (Allen et al., 2011) for Glut-3-SH to 3-SH, indicating a weak to moderate positive relationship. The correlations between 3-SH to Cys-3-SH were negative but essentially absent (-0.05 and -0.11) (Allen et al., 2011; Capone et al., 2011; Chen et al., 2018) but 3-SHA was positively related to both Cys-3-SH ($r = 0.34$) and Glut-3-SH ($r = 0.61$) (Allen et al., 2011). The inconsistent correlations demonstrated in the present study and from the abovementioned literature indicate that the relationship between precursors and varietal thiols is even more complicated than perhaps is appreciated, and that ongoing work is required to resolve aspects of varietal thiol biogenesis during winemaking.

3.3. Potential relationship between grape amino acids with precursors and thiols

Grapes from *V. vinifera* cultivars are compositionally complex systems containing numerous chemical components of various categories. In relation to varietal thiols in wine, two major types of precursors to 3-SH and 4-MSP identified in grapes are conjugates of cysteine (Cys-3-SH and Cys-4-MSP) and glutathione (Glut-3-SH and Glut-4-MSP) (Roland et al., 2011). Interestingly, the conjugates all involve amino acid unit(s) (i.e., glycine, glutamic acid, cysteine), which also applies to some recently identified precursors (Bonnaffoux et al., 2018). As a key group of grape metabolites, amino acids have been intensively investigated for their relationship with aroma development during fermentation (Burin, Gomes, Caliari, Rosier, & Bordignon Luiz, 2015; Hernández-Orte, Ibarz, Cacho, & Ferreira, 2006; Park, Boulton, & Noble, 2000) but only a few publications have investigated their influences on varietal thiol production during fermentation (Alegre et al., 2017; Pinu et al., 2014, 2019). Since previous studies either involved synthetic media or a single Sauvignon blanc juice (Alegre et al., 2017), or showed inconsistent correlations between amino acids and thiols (Pinu et al., 2014, 2019), the profiles of amino acids in a range of Sauvignon blanc grapes from within a single GI were determined and compared with both precursor and varietal thiol concentrations.

The total amino acid concentrations of the 21 grape juices ranged from 390 to 1091 mg/L (L1_3 and L7, respectively). Compositionally, the major amino acids were arginine (146 ± 84 mg/L), proline

(124 ± 52 mg/L), glutamic acid (124 ± 44 mg/L), γ-amino butyric acid (GABA, 75 ± 16 mg/L), and α-alanine (52 ± 21 μg/L) in contrast to minor amino acids such as glycine, asparagine, methionine, lysine, tryptophan, and isoleucine (Table S3 of the Supporting Information), which accords with literature data on amino acids in Sauvignon blanc (Martin et al., 2016; Park et al., 2000; Spayd & Andersen-Bagge, 1996). The ratio of proline to arginine, a suggested cultivar-dependent index, varied widely from 0.36 to 2.78 in the 21 Sauvignon blanc juices, with such an inconsistency having been observed in a previous multi-cultivar survey (Spayd & Andersen-Bagge, 1996). Variation of individual amino acid concentrations between juices from different locations was apparent, as shown in the heatmap (Fig. 2a). Two samples from L1 (L1_1 and L1_2) and juices from L5 to L7 contained higher amounts of minor amino acids. Greater amounts of aspartic acid, serine, proline, and glutamic acid were seen in juices from L4 to L7. Various factors influence grape amino acid concentrations including fertilisation, irrigation and climatic conditions (Ortega-Heras et al., 2014).

Correlation analysis was performed to investigate the potential relationships between amino acids and both thiols and their precursors (Fig. 2b–d). As a whole, amino acids in juices were only weakly correlated to 3-SH and 3-SHA in the wines ($r < 0.2$, Fig. 2b). Individually, correlations (positive or negative) with 3-SH and 3-SHA ranged from absent to weak ($|r| \leq 0.30$, Fig. 2c) except for glutamic acid ($r = 0.42$ for 3-SH, $r = 0.32$ for 3-SHA) and proline ($r = 0.34$ for 3-SH, $r = 0.36$ for 3-SHA). Glutamic acid has previously been positively correlated to thiol concentrations in a metabolomic profiling study of Sauvignon blanc, along with GABA and glutamine (Pinu et al., 2014). As varietal thiol production is the result of yeast metabolism during fermentation, the observed correlations between amino acids and varietal thiols could indicate the impacts of amino acids (especially glutamic acid and proline in the present case) on thiol production or interactions between amino acids and thiol precursors during fermentation. The significant enhancing effects of glutamic acid on 3-SH and 3-SHA production were demonstrated previously (Pinu et al., 2014). Glutamic acid stands out perhaps because it is a preferred yeast nitrogen source for fermentation but the similar correlation results obtained for proline, a non-preferred nitrogen source, were somewhat intriguing.

In contrast to the results for the free thiols, precursors were more strongly correlated to a greater number of amino acids ($|r| \geq 0.30$ for thirteen amino acids) (Fig. 2b). Among these apparently novel findings, glutamic acid featured again and had the strongest correlation to both of the precursors ($r \leq -0.73$), followed by glycine ($r \geq 0.62$), GABA ($r \geq 0.59$), alanine ($r \leq -0.55$), and isoleucine ($r \geq 0.55$). The moderate to strong correlations were suggestive of the interaction between the biochemical accumulation/degradation outcomes of thiol precursors and amino acids during grape ripening. Glutamic acid and glycine are component amino acids of glutathione, which plants require to respond to environmental stress (Galant, Preuss, Cameron, & Jez, 2011), so the strong correlations likely relate to promotion (glycine) or inhibition (glutamic acid) of glutathione biosynthesis and thus of glutathione-conjugated thiol precursor Glut-3-SH, which in turn is linked to Cys-3SH formation. The moderate correlations between proline and thiol precursors ($r = -0.39$ for Glut-3-SH, $r = -0.48$ for Cys-3-SH) could also be related to glutamic acid production, which serves as a precursor to proline (Anjum et al., 2014). Nonetheless, the mechanisms underlying these correlations as well as those of precursors with GABA, alanine, and isoleucine are still unclear and require further investigations. Recent literature suggested that certain ratios of amino acids could also modify thiol production (Alegre et al., 2017) so the correlations of various amino acid combinations (Glu/GABA, Glu − GABA, Glu + GABA, Glu/Pro, Glu − Pro, Glu + Pro, GABA/Pro, GABA + Pro, GABA − Pro) with thiols and precursors were assessed but no notable correlations were observed (data not shown).

PCA analysis of quantitative data for varietal thiols, precursors, and amino acids is presented in Fig. 3. The first two principal components (PC) explained a total of 68% variance, with 43% and 25% of the total attributable to PC 1 and PC 2, respectively (Fig. 3a). Samples from L1 were located in the top quadrants of the figure and generally corresponded to higher concentrations of Cys-3-SH, Glut-3-SH, GABA, and glycine (Fig. 3b). Samples from L3 were relatively closely plotted to L1 samples, which indicated similarity between them. Three out of five L2 samples grouped together in the bottom left quadrant, close to the varietal thiols but far away from all amino acids. L4 samples were located together in the bottom quadrants and close to the free thiols (Fig. 3b), indicating relative higher amounts of 3-SH, 3-SHA, and 4-MSP (Fig. 1b). Notably, L5 wine contained the highest amounts of 3-SHA and 4-MSP and was clustered with L6 and almost inseparable from L7, which were dominated by the higher levels of amino acids, indicating the potential impact of amino acids on the variation of thiol metabolism.

3.4. Impact of pre-fermentation freezing (PFF) treatment on precursors and thiols

Cryomaceration (low temperature maceration with solid CO_2 for a period of time) or grape/must freezing can be employed to induce berry damage and enhance extraction of components (Sacchi, Bisson, & Adams, 2005), and has primarily been assessed for its impact on the non-volatile composition (e.g., phenolics or organic acids) of wines or on stability (Álvarez, Aleixandre, García, & Lizama, 2006; Baiano et al., 2012). Several studies have considered the impact of cryogenic treatment on volatile compounds in grape or wine (Moreno-Pérez, Vila-López, Fernández-Fernández, Martínez-Cutillas, & Gil-Muñoz, 2013; Ouellet & Pedneault, 2016; Peinado, Moreno, Bueno, Moreno, & Mauricio, 2004; Peng, Wen, Tao, & Lan, 2013) but only one report appeared to be available on the potential effect on varietal thiols (Olejar et al., 2015). This is despite the technique potentially offering a practical way to increase thiol concentrations in wine through greater extraction of components from grape skin or formation of precursors (Roland et al., 2011). Some further insight into the possible impact can be gained from a previous study, whereby frozen storage of fresh grapes increased the concentrations of Cys-3-SH (inconsistently) and Glut-3-SH (substantially) (Capone et al., 2011). However, the impact of PFF treatment on varietal thiols was not pursued in that work.

In the present study, a period of 30 days of frozen storage (–20 °C) was selected as the PFF treatment on freshly harvested whole grape bunches and their subsequently obtained fresh juices. The conditions for PFF were based on a previous study (Capone et al., 2011) and were also chosen for convenience, to accommodate other time-sensitive aspects of the experiments. Optimisation of PFF conditions (e.g., temperature, duration, thawing process) was not included but previous work has assessed some conditions and shown an effect on wine volatiles with as little as 6 h of freezing at −20 °C (Peng et al., 2013). The concentrations of Glut-3-SH and Cys-3-SH in juices obtained from grapes from L4 with/without PFF treatment and those of 3-SH, 3-SHA, and 4-MSP in subsequent wines from corresponding juices are demonstrated in Fig. 4. After PFF treatment of grape berries, concentrations of Glut-3-SH and Cys-3-SH were 724.3 ± 78.7 μg/L and 73.1 ± 11.7 μg/L, respectively. Compared to grapes without PFF (see L4 in Fig. 1, Glut-3-SH: 50.3 ± 5.0 μg/L, Cys-3-SH: 15.4 ± 2.2 μg/L), Glut-3-SH exhibited a significant 11–19 fold increase and Cys-3-SH increased about 4–6 fold, and with the exception of sample L4_5, all the increments were statistically significant (Fig. 4a). The enhancement of precursors after PFF was much higher than previously reported, in which Glut-3-SH increased by about 5-fold after 1 month of frozen storage but little change was observed for Cys-3-SH (Capone et al., 2011). The significant increase of Glut-3-SH appeared to be caused by *de novo* formation due to berry damage that occurred during PFF, as explained previously (Capone et al., 2011). Higher amounts of Cys-3-SH after PFF treatment in the present study suggested a similar formation mechanism might occur for Cys-3-SH, but potential degradation from Glut-3-SH to Cys-3-SH or improved extraction of Cys-3-SH from damaged cells (Sacchi

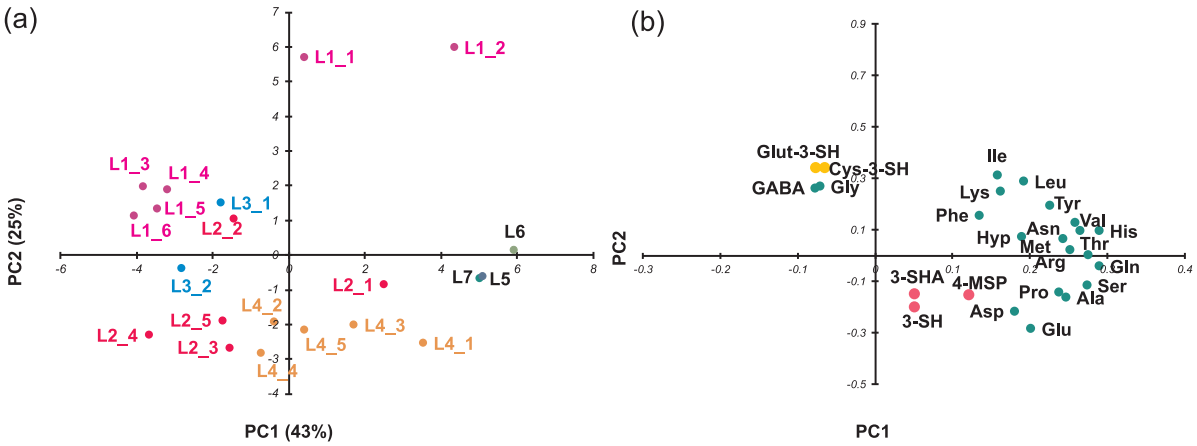

Fig. 3. PCA analysis showing (a) distribution of 21 Sauvignon blanc samples on PC1 vs PC2 and (b) loadings plot based on concentrations of varietal thiols in wines, and precursors and amino acids in juices. For sample codes, refer to Table S1 and Fig. S1 of the Supporting Information.

et al., 2005) during the freezing/thawing process could also contribute to the greater amounts of Cys-3-SH observed.

As with precursors in the juices, varietal thiol concentrations were also significantly enhanced in wines with PFF treatment (Fig. 4b) except for 3-SHA in L4_3 wine and 4-MSP in L4_1 wine. Overall, 3-SH concentrations of L4_1 to L4_5 were 1139.0 ± 412.1 and 526.0 ± 279.4 ng/L in wines from PFF treatment of grape bunches and juices, respectively, and both were higher than the average for wine derived from non-PFF treatment (222.8 ± 128.0 ng/L). Stronger increases of thiols were seen in wines from PFF of grapes bunches than PFF of juices and similar trends were observed for 3-SHA and 4-MSP. Compared to wines made from fresh grapes, 3-SH, 3-SHA, and 4-MSP increased by around 2–10, 3–7, and 2–8 times when PFF was applied to grapes. Although lower in magnitude, significant increases of varietal thiols were also noted when comparing wines from PFF grapes to wines arising from PFF juices (Fig. 4b). When considering production from fresh grapes versus frozen juices, significant differences were only observed for 3-SH production in L4_2 and L4_3 wines (approximate 4-fold increase). Notably, even though the increased concentrations from PFF treatments were evident for both precursors and free thiols, with the latter potentially being a reflection of elevated precursor levels, there were much greater relative increases for precursors. Consistent with the weak correlation between precursor and thiol concentrations after PFF treatments (data not shown), this outcome implied that only partial amounts of the enhanced precursor levels induced by PFF treatments were converted to varietal thiols. Nonetheless, whatever the precise mechanism (i.e., from known precursors or some other thiol biogenesis pathway), the significant effects of the freezing treatments showed that remarkable thiol augmentation in wine was possible, which complements the previous work involving dry ice cryomaceration of Sauvignon blanc grape musts (Olejar et al., 2015).

4. Conclusion

Intraregional variations of precursors in juice and varietal thiols in wine were characterised for 21 Sauvignon blanc samples from the Adelaide Hills wine region. Obvious intraregional variations were seen in the amounts of precursors in juices and thiols produced in wines. The mixed correlations, weak between grape amino acids and wine varietal thiols but moderate to strong between amino acids and precursors,

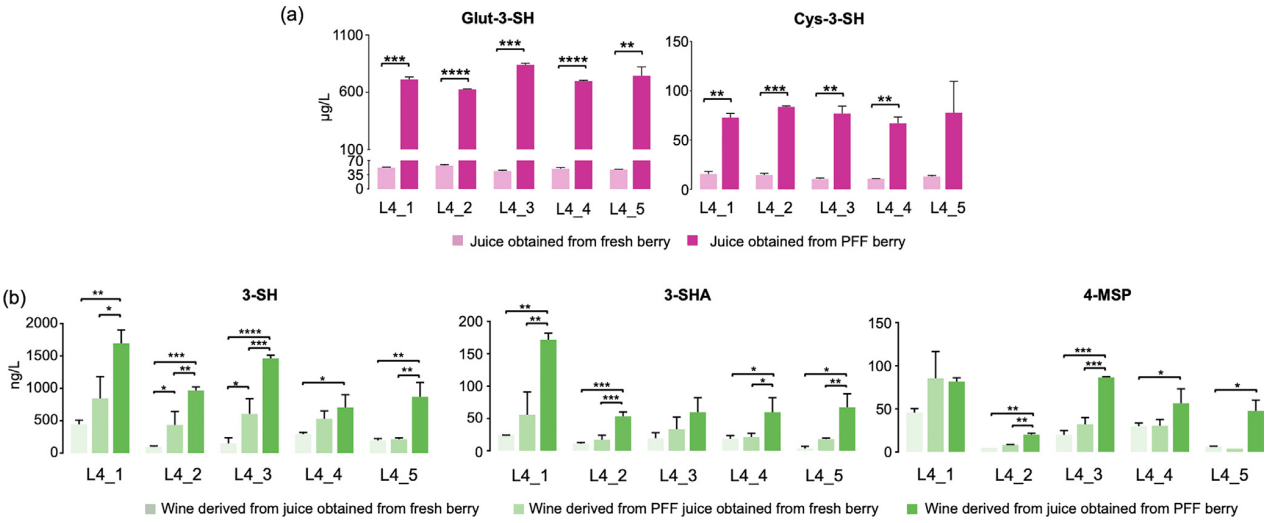

Fig. 4. Comparison of (a) concentrations (μg/L) of precursors (Glut-3-SH, Cys-3-SH) in juices from fresh and PFF treatment grapes, and (b) concentrations (ng/L) of varietal thiols (3-SH, 3-SHA, and 4-MSP) in wines made from juices from fresh grapes, PFF treatment juices, and PFF treatment grapes, sampled from Location 4. Error bars represent the SD derived from replicate analysis (n = 2 for precursors, n = 3 for varietal thiols). Precursor data were compared by unpaired *t*-test and thiol data were evaluated with one-way ANOVA. *: $p < 0.05$, **: $p < 0.001$, ***: $p < 0.0005$, ****: $p < 0.0001$. For sample codes, refer to Table S1 and Fig. S1 of the Supporting Information.

together with multivariate data analysis, indicated the potential interactions between amino acids and both precursor biosynthesis in grapes and thiol metabolism during fermentation. Notably, pre-fermentation freezing treatment of grape berry parcels induced significant increases in concentrations not only of precursors but also of free thiols, which was revealed for the first time on the same set of grape and wine samples. Pre-fermentation freezing could be a potential approach for winemakers to enhance the production of varietal thiols in wines and this warrants further investigation. In particular, experiments focusing on optimal PFF conditions, for instance, the duration of PFF, storage temperature, thawing process, and single/multiple PFF cycles, could be conducted.

Declaration of Competing Interest

The authors declare no conflict of interest.

Acknowledgements

We are immensely grateful to David Coleman from Adelaide Hills Vine Improvement Inc. (AHVI) for assistance of with grape harvesting and facilitating access to vineyards. We acknowledge the time contributions and generous donations of grapes provided by Peter Bird and David Kohl (Casella Family Brands), Chris Mein (Shaw + Smith), David Blows (Spring Dale), Spiro Papadopoulos (Australian Vintage Ltd), and James Thorpe (Nova Vita Wines). We are thankful to our UA colleagues, Michelle Walker and Nick Van Holst for help with fermentation, and Ross Sanders for laboratory assistance. Sue Maffei from CSIRO is acknowledged for providing assistance with HPLC–MS/MS analysis. L.C. was a recipient of the joint scholarship of UA and China Scholarship Council (CSC201506350012), and was also supported by a Wine Australia supplementary scholarship (AGW Ph1512). The Australian Research Council Training Centre for Innovative Wine Production (www.ARCwinecentre.org.au; project number IC170100008) is funded by the Australian Government with additional support from Wine Australia and industry partners. The University of Adelaide and CSIRO are members of the Wine Innovation Cluster.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.foodchem.2019.05.126.

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SUPPLEMENTARY INFORMATION FOR

Investigation of intraregional variation, grape amino acids, and pre-fermentation freezing on varietal thiols and their precursors for *Vitis vinifera* Sauvignon blanc

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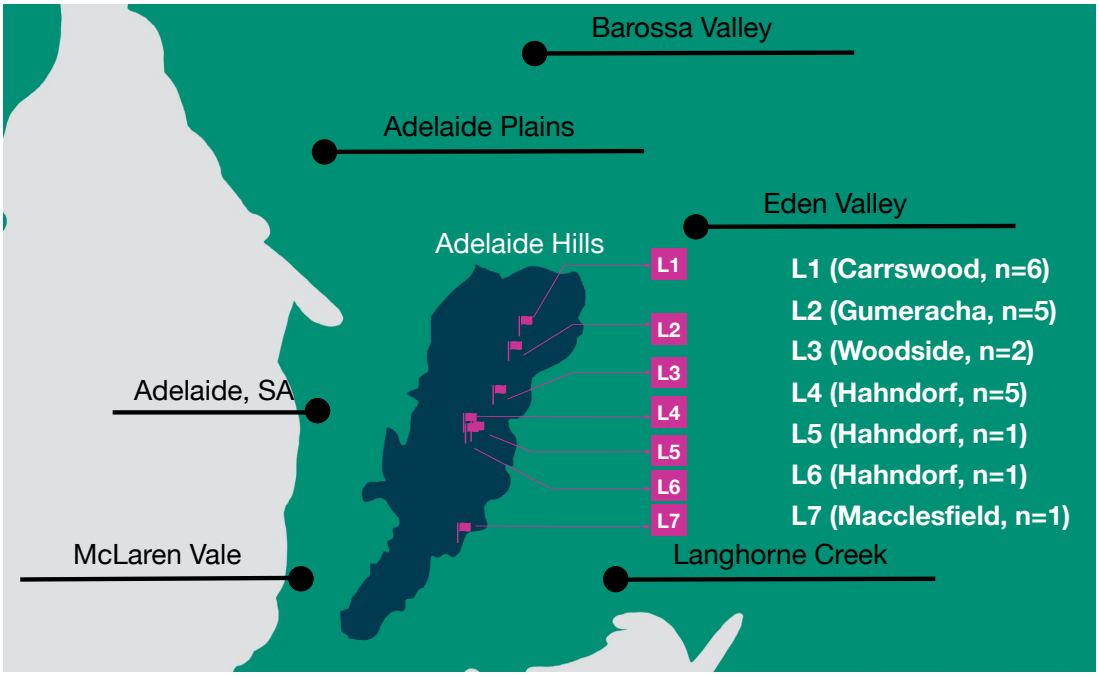

Fig. S1. Map of Adelaide Hills Geographical Indication (GI) and locations of sampled vineyards. GI map of Adelaide Hills derived from Wine Australia (https://www.adelaidehillswine.com.au/region/); pinned locations indicate the commercial vineyards (L1–7) sampled in this study; location information sourced from Google Map.

Table S1. Clone, harvest date and basic chemical parameters of 21 Sauvignon blanc grape samples from locations within the Adelaide Hills GI.

	Clone	Harvest date	pH[a]	TSS (°Brix)[a]	TA (g/L)[a]
L1_1	F4V6	27 Feb 2018	2.53 ± 0.02	16.0 ± 0.1	10.18 ± 0.01
L1_2	F4V6	27 Feb 2018	2.91 ± 0.01	20.1 ± 0.4	6.50 ± 0.03
L1_3	F4V6	27 Feb 2018	2.83 ± 0.01	18.8 ± 0.2	7.30 ± 0.01
L1_4	F4V6	27 Feb 2018	2.82 ± 0.01	20.7 ± 0.1	7.16 ± 0.02
L1_5	F4V6	27 Feb 2018	2.72 ± 0.01	19.3 ± 0.1	8.71 ± 0.02
L1_6	F4V6	27 Feb 2018	2.95 ± 0.01	21.6 ± 0.1	7.41 ± 0.05
average			***2.79 ± 0.14b***	***19.4 ± 1.9ab***	***7.87 ± 1.28bc***
L2_1	H5V10	7 Mar 2018	3.13 ± 0.02	21.8 ± 0.1	8.67 ± 0.01
L2_2	F4V6	7 Mar 2018	3.12 ± 0.02	22.3 ± 0.0	7.87 ± 0.01
L2_3	F7V7	7 Mar 2018	3.32 ± 0.06	21.0 ± 0.1	8.48 ± 0.08
L2_4	Q9720	7 Mar 2018	3.02 ± 0.03	21.1 ± 0.0	9.78 ± 0.01
L2_5	5385	7 Mar 2018	2.91 ± 0.01	19.5 ± 0.1	8.51 ± 0.01
average			***3.10 ± 0.14a***	***21.1 ± 1.1a***	***8.66 ± 0.65abc***
L3_1	F4V6	27 Feb 2018	2.71 ± 0.01	18.9 ± 0.0	8.70 ± 0.04
L3_2	F4V6	27 Feb 2018	2.76 ± 0.01	16.9 ± 0.2	8.45 ± 0.01
average			***2.73 ± 0.03b***	***17.9 ± 1.2b***	***8.58 ± 0.15abc***
L4_1	H5V10	28 Feb 2018	2.76 ± 0.04	21.5 ± 0.6	10.94 ± 0.01
L4_2	F4V6	28 Feb 2018	2.65 ± 0.01	20.7 ± 0.1	11.28 ± 0.23
L4_3	F7V7	28 Feb 2018	2.59 ± 0.04	19.4 ± 0.3	13.90 ± 0.06
L4_4	Q9720	28 Feb 2018	2.86 ± 0.02	21.4 ± 0.0	10.46 ± 0.02
L4_5	5385	28 Feb 2018	2.82 ± 0.01	20.7 ± 0.1	9.11 ± 0.01
average			***2.75 ± 0.12b***	***20.7 ± 0.8ab***	***11.14 ± 1.65a***
L5	F4V9	28 Feb 2018	2.94 ± 0.00ab	21.8 ± 0.1a	6.64 ± 0.02c
L6	F4V6	28 Feb 2018	2.74 ± 0.03b	19.8 ± 0.1ab	10.67 ± 0.05a
L7	F4V6	27 Feb 2018	2.64 ± 0.00b	19.8 ± 0.0ab	10.08 ± 0.05ab

[a] Data represent mean values ± standard deviations derived from duplicate samples. Different lower case letters within a column denote significant differences among the means (one-way ANOVA, $\alpha = 0.05$, Tukey multiple comparisons test).

Table S2. Concentrations of precursors (µg/L) in 21 Sauvignon blanc juices from the Adelaide Hills and varietal thiols (ng/L) in resulting wines.

	Glut-3-SH	Cys-3-SH	3-SH	3-SHA	4-MSP
L1_1	170.7 ± 4.3	44.6 ± 4.4	73.5 ± 4.2	10.0 ± 2.6	n.d.
L1_2	111.4 ± 3.9	30.2 ± 0.1	41.7 ± 12.2	5.2 ± 0.9	n.d.
L1_3	119.2 ± 7.7	30.0 ± 3.7	74.8 ± 30.5	12.1 ± 4.0	n.d.
L1_4	88.9 ± 3.1	33.3 ± 0.5	53.9 ± 6.5	5.5 ± 1.5	n.d.
L1_5	95.4 ± 2.9	32.3 ± 1.5	71.5 ± 7.9	4.7 ± 1.0	3.6 ± 0.8
L1_6	122.0 ± 3.7	33.3 ± 8.8	126.0 ± 69.4	20.7 ± 13.6	3.1[a]
L2_1	36.3 ± 0.3	10.7 ± 0.5	52.6 ± 21.3	9.0 ± 4.0	5.5 ± 3.2
L2_2	122.6 ± 11.3	18.2 ± 0.5	197.1 ± 104.0	18.6 ± 10.5	35.6 ± 11.5
L2_3	33.7 ± 1.5	11.1 ± 0.5	513.5 ± 14.0	36.4 ± 3.7	39.8 ± 3.5
L2_4	53.4 ± 2.4	11.1 ± 0.5	192.1 ± 25.6	22.1 ± 3.6	29.4 ± 3.5
L2_5	45.6 ± 2.6	11.8 ± 0.1	45.6 ± 2.7	4.1 ± 0.2	5.7 ± 1.1
L3_1	146.3 ± 6.8	24.3 ± 1.3	68.8 ± 27.0	12.1 ± 5.2	n.d.[b]
L3_2	78.2 ± 13.9	16.2 ± 1.4	52.0 ± 3.2	7.8 ± 1.6	n.d.
L4_1	52.5 ± 1.0	17.8 ± 2.4	442.2 ± 66.2	24.3 ± 0.1	45.8 ± 4.5
L4_2	43.9 ± 2.0	13.1 ± 0.9	151.3 ± 84.7	19.5 ± 8.3	19.8 ± 5.0
L4_3	49.9 ± 3.0	13.4 ± 0.1	292.7 ± 24.6	18.4 ± 5.2	29.8 ± 3.8
L4_4	47.5 ± 1.1	15.5 ± 1.0	194.6 ± 30.9	17.9 ±1.6	6.2 ± 0.6
L4_5	57.4 ± 2.4	16.8 ± 1.6	97.0 ± 14.2	11.0 ± 1.9	4.9[a]
L5	80.5 ± 3.5	16.8 ± 1.5	385.5 ± 11.8	47.4 ± 5.5	93.8 ± 4.1
L6	71.5 ± 2.8	17.0 ± 0.2	40.4 ± 1.4	6.1 ± 0.7	4.4 ± 1.2
L7	65.1 ± 0.6	15.4 ± 0.6	385.5 ± 11.8	11.3 ± 0.7	23.7 ± 12.2

[a] Only one replicate out of the three ferments was found with detectable amount of the analyte. [b] n.d., not detected (< 3 ng/L).

Table S3. Concentrations (mg/L) of amino acids in 21 Sauvignon blanc grape juices from the Adelaide Hills.[a]

	Aspartate	Asparagine	Serine	Glutamate	Histidine	Glutamine	Glycine	Arginine	Threonine
RT (min)[b]	20.813	22.189	22.567	22.810	23.794	24.223	24.403	26.469	28.096
L1_1	10.1 ± 0.2	1.4 ± 0.0	22.8 ± 0.3	74.7 ± 1.7	9.4 ± 0.0	31.8 ± 0.4	0.21 ± 0.02	162.7 ± 2.9	46.8 ± 0.8
L1_2	8.5 ± 0.5	1.9 ± 0.0	24.2 ± 0.	71.6 ± 0.9	12.4 ± 0.1	39.2 ± 0.2	0.11 ± 0.01	242.4 ± 1.0	55.9 ± 0.1
L1_3	8.3 ± 0.0	1.1 ± 0.0	15.4 ± 0.2	65.7 ± 0.9	4.7 ± 0.1	20.7 ± 0.1	0.11 ± 0.00	67.6 ± 0.3	29.8 ± 0.1
L1_4	12.9 ± 0.4	1.0 ± 0.0	18.6 ± 0.2	76.5 ± 0.6	5.2 ± 0.1	21.5 ± 0.1	0.17 ± 0.02	57.4 ± 1.8	35.2 ± 0.4
L1_5	11.3 ± 0.2	1.0 ± 0.0	18.5 ± 0.3	82.9 ± 0.6	4.9 ± 0.1	20.4 ± 0.2	0.17 ± 0.01	36.3 ± 1.3	30.6 ± 0.5
L1_6	10.0 ± 0.1	1.1 ± 0.0	16.2 ± 0.0	67.0 ± 0.8	4.3 ± 0.0	21.0 ± 0.1	0.10 ± 0.02	60.4 ± 0.8	31.9 ± 0.5
L2_1	11.8 ± 0.5	1.4 ± 0.1	25.6 ± 0.2	158.1 ± 9.2	10.3 ± 0.1	42.1 ± 0.4	0.14 ± 0.01	271.1 ± 1.7	56.1 ± 0.5
L2_2	10.1 ± 0.3	1.0 ± 0.0	19.5 ± 0.5	84.8 ± 5.3	6.0 ± 0.2	30.0 ± 1.2	0.14 ± 0.02	87.2 ± 3.5	37.8 ± 1.3
L2_3	8.8 ± 0.1	1.0 ± 0.0	21.0 ± 0.8	143.3 ± 2.4	5.6 ± 0.1	24.9 ± 0.3	0.11 ± 0.01	146.4 ± 0.9	48.4 ± 0.0
L2_4	11.5 ± 0.3	0.9 ± 0.0	18.9 ± 0.3	110.9 ± 0.8	6.5 ± 0.0	27.5 ± 0.2	0.06 ± 0.00	160.4 ± 1.1	41.8 ± 0.9
L2_5	16.4 ± 0.1	0.9 ± 0.0	16.9 ± 0.1	123.3 ± 4.2	4.4 ± 0.0	20.1 ± 0.4	0.07 ± 0.00	123.5 ± 0.9	36.9 ± 0.4
L3_1	20.4 ± 0.2	1.2 ± 0.0	20.7 ± 0.0	97.5 ± 2.5	6.3 ± 0.0	28.6 ± 0.1	0.18 ± 0.01	135.0 ± 0.7	45.9 ± 0.5
L3_2	20.1 ± 0.3	1.3 ± 0.0	18.7 ± 0.1	97.3 ± 0.4	5.9 ± 0.1	26.5 ± 0.0	0.13 ± 0.00	110.4 ± 1.0	43.0 ± 0.4
L4_1	39.1 ± 0.9	1.6 ± 0.0	33.3 ± 0.4	181.4 ± 9.1	9.2 ± 0.1	47.2 ± 0.4	0.05 ± 0.02	149.1 ± 1.	48.8 ± 0.8
L4_2	39.5 ± 0.0	1.4 ± 0.0	31.6 ± 0.8	183.6 ± 2.4	7.3 ± 0.2	37.0 ± 0.8	0.05 ± 0.02	113.5 ± 1.6	43.3 ± 0.4
L4_3	36.4 ± 0.2	1.1 ± 0.0	24.6 ± 0.0	154.5 ± 3.5	5.5 ± 0.0	31.3 ± 0.2	0.05 ± 0.01	71.9 ± 0.4	38.8 ± 0.2
L4_4	35.3 ± 0.1	1.2 ± 0.0	27.7 ± 0.1	155.2 ± 4.5	7.1 ± 0.1	33.2 ± 0.4	0.003 ± 0.002	95.8 ± 0.3	42.3 ± 0.3
L4_5	29.7 ± 0.1	1.2 ± 0.0	26.9 ± 0.3	151.5 ± 3.6	6.1 ± 0.1	33.4 ± 0.4	0.008 ± 0.005	124.8 ± 0.1	39.2 ± 0.4
L5	37.1 ± 0.2	1.4 ± 0.0	30.2 ± 0.1	167.1 ± 2.4	10.9 ± 0.1	39.0 ± 0.0	0.13 ± 0.01	189.0 ± 0.5	55.5 ± 0.2
L6	35.9 ± 0.2	2.0 ± 0.0	33.7 ± 0.2	174.5 ± 5.1	12.9 ± 0.1	56.0 ± 0.6	0.07 ± 0.01	329.7 ± 1.0	66.1 ± 0.5
L7	36.8 ± 0.2	1.8 ± 0.0	31.7 ± 0.4	188.2 ± 4.0	11.4 ± 0.2	51.2 ± 0.3	0.06 ± 0.01	324.5 ± 6.3	63.8 ± 0.3

Table S3. Contd.

	Alanine	Proline	GABA	Tyrosine	Valine	Methionine	Lysine	Isoleucine	Leucine	Phenylalanine
RT (min)[b]	29.346	31.534	32.186	37.121	39.187	40.183	42.819	44.868	45.618	46.820
L1_1	35.7 ± 0.9	120.2 ± 3.0	111.2 ± 3.5	4.5 ± 0.0	14.5 ± 0.3	2.8 ± 0.0	3.8 ± 0.1	8.9 ± 0.2	10.7 ± 0.2	12.1 ± 0.1
L1_2	52.2 ± 0.8	87.1 ± 0.2	83.5 ± 0.3	5.5 ± 0.2	17.0 ± 0.1	4.4 ± 0.3	4.6 ± 0.3	11.6 ± 0.6	14.0 ± 0.3	17.6 ± 0.2
L1_3	21.8 ± 0.4	32.2 ± 0.4	83.3 ± 1.7	2.8 ± 0.0	8.6 ± 0.0	2.5 ± 0.2	2.6 ± 0.2	5.6 ± 0.1	7.1 ± 0.1	9.8 ± 0.0
L1_4	23.0 ± 0.1	71.9 ± 1.1	83.7 ± 1.1	3.0 ± 0.0	11.7 ± 0.2	2.9 ± 0.1	2.1 ± 0.0	5.8 ± 0.1	7.8 ± 0.1	9.7 ± 0.0
L1_5	27.6 ± 0.1	101.0 ± 1.7	82.2 ± 2.3	3.1 ± 0.0	10.5 ± 0.2	3.1 ± 0.1	1.9 ± 0.0	4.2 ± 0.1	6.5 ± 0.1	10.7 ± 0.0
L1_6	23.8 ± 0.0	55.4 ± 0.4	78.3 ± 1.7	2.7 ± 0.0	9.3 ± 0.0	2.3 ± 0.1	2.3 ± 0.0	4.4 ± 0.0	6.6 ± 0.0	8.6 ± 0.1
L2_1	84.5 ± 1.7	200.4 ± 1.9	63.4 ± 5.3	2.7 ± 0.0	11.3 ± 0.1	3.4 ± 0.1	2.8 ± 0.1	4.1 ± 0.0	7.6 ± 0.1	9.6 ± 0.0
L2_2	53.0 ± 1.9	155.2 ± 5.9	115.5 ± 7.4	1.8 ± 0.0	11.5 ± 0.3	3.0 ± 0.1	2.3 ± 0.1	5.4 ± 0.2	8.4 ± 0.2	10.2 ± 0.2
L2_3	63.7 ± 0.6	98.0 ± 1.6	83.5 ± 4.5	2.0 ± 0.0	9.6 ± 0.1	2.3 ± 0.1	2.5 ± 0.0	3.3 ± 0.2	6.4 ± 0.1	5.8 ± 0.1
L2_4	58.0 ± 0.6	112.7 ± 1.6	75.8 ± 0.7	2.1 ± 0.0	8.9 ± 0.0	2.7 ± 0.0	2.5 ± 0.0	2.7 ± 0.0	6.1 ± 0.0	7.0 ± 0.1
L2_5	45.5 ± 0.3	96.0 ± 1.7	56.4 ± 3.3	1.3 ± 0.0	6.9 ± 0.1	2.1 ± 0.1	2.8 ± 0.0	1.8 ± 0.0	4.5 ± 0.0	5.8 ± 0.0
L3_1	37.2 ± 0.6	86.1 ± 0.9	77.9 ± 4.0	2.8 ± 0.0	11.2 ± 0.1	2.6 ± 0.0	3.1 ± 0.0	4.7 ± 0.1	7.2 ± 0.1	6.4 ± 0.0
L3_2	36.2 ± 0.3	64.8 ± 0.194	68.3 ± 1.4	2.6 ± 0.0	8.4 ± 0.0	2.2 ± 0.1	2.6 ± 0.1	3.2 ± 0.1	5.8 ± 0.1	6.0 ± 0.1
L4_1	73.3 ± 0.6	176.8 ± 0.5	54.7 ± 4.4	3.9 ± 0.1	13.6 ± 0.1	3.8 ± 0.6	2.2 ± 0.1	4.6 ± 0.0	7.2 ± 0.1	12.5 ± 0.2
L4_2	65.2 ± 0.1	166.3 ± 3.7	74.6 ± 9.2	3.5 ± 0.1	13.7 ± 0.0	3.4 ± 0.5	2.1 ± 0.1	4.8 ± 0.0	7.2 ± 0.0	12.1 ± 0.1
L4_3	42.7 ± 0.1	138.0 ± 0.8	59.7 ± 2.9	2.7 ± 0.0	11.7 ± 0.0	2.8 ± 0.2	1.5 ± 0.0	3.8 ± 0.0	5.9 ± 0.0	10.9 ± 0.0
L4_4	54.4 ± 0.1	129.3 ± 0.9	59.4 ± 2.6	3.4 ± 0.0	12.1 ± 0.1	3.5 ± 0.1	1.8 ± 0.1	4.3 ± 0.0	6.4 ± 0.0	13.2 ± 0.0
L4_5	52.1 ± 0.0	134.0 ± 0.4	67.1 ± 0.9	3.1 ± 0.0	10.9 ± 0.1	3.3 ± 0.0	2.0 ± 0.0	3.7 ± 0.4	5.8 ± 0.0	10.7 ± 0.0
L5	68.3 ± 0.3	233.0 ± 0.2	71.7 ± 1.1	4.1 ± 0.0	18.1 ± 0.0	3.5 ± 0.1	3.3 ± 0.0	7.5 ± 0.0	10.2 ± 0.0	11.0 ± 0.1
L6	75.6 ± 0.1	187.1 ± 0.1	61.9 ± 1.7	4.7 ± 0.0	14.4 ± 0.1	3.5 ± 0.2	3.6 ± 0.0	6.3 ± 0.3	9.2 ± 0.0	8.2 ± 0.0
L7	100.2 ± 0.3	165.9 ± 0.9	67.9 ± 2.0	3.9 ± 0.0	14.5 ± 0.0	3.2 ± 0.1	3.6 ± 0.1	5.4 ± 0.1	8.8 ± 0.1	8.6 ± 0.1

[a] Quantitative data for amino acids represent mean values ± standard deviations derived from duplicate analyses. [b] RT: retention time.

Chapter 6

Identification and quantitative analysis of 2-methyl-4-propyl-1,3-oxathiane in wine.

Journal of Agricultural and Food Chemistry 2018, 66, 10808–10815. DOI.

Chen L., Capone D. L., Jeffery D. W.

Statement of Authorship

Title of Paper	Identification and quantitative analysis of 2-methyl-4-propyl-1,3-oxathiane in wine
Publication Status	☑ Published ☐ Accepted for Publication ☐ Submitted for Publication ☐ Unpublished and Unsubmitted work written in manuscript style
Publication Details	Chen, L.; Capone, D.L.; Jeffery D.W. (**2018**) Identification and quantitative analysis of 2-methyl-4-propyl-1,3-oxathiane in wine. *Journal of Agriculture and Food Chemistry*, **66**, 10808-10815

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Overall percentage (%)	75%
Certification:	This paper reports on original research I conducted during the period of my Higher Degree by Research candidature and is not subject to any obligations or contractual agreements with a third party that would constrain its inclusion in this thesis. I am the primary author of this paper.
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Co-Author Contributions

By signing the Statement of Authorship, each author certifies that:

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iii. the sum of all co-author contributions is equal to 100% less the candidate's stated contribution.

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Contribution to the Paper	Supervised and directed the entire analytical method development and validation process. Sourced samples used in this work. Collected, processed, and interpreted instrument data. Prepared, edited, and revised the manuscript.
Signature	Date 22/10/18

Name of Co-Author	David W. Jeffery
Contribution to the Paper	Conceived the original concept of the study. Designed and supervised this project on all aspects. Wrote, edited, revised, and submitted the paper. Acted as corresponding author at all stages.
Signature	Date 19/10/18

Please cut and paste additional co-author panels here as required.

JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY

Article

Cite This: J. Agric. Food Chem. 2018, 66, 10808−10815

pubs.acs.org/JAFC

Identification and Quantitative Analysis of 2-Methyl-4-propyl-1,3-oxathiane in Wine

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Supporting Information

ABSTRACT: On the basis of the chemistry of wine and the co-occurrence of 3-sulfanylhexan-1-ol (3-SH) and acetaldehyde, we investigated the existence of 2-methyl-4-propyl-1,3-oxathiane (**1**) and identified the presence of a single detectable geometric isomer, *cis*-**1**, in wines for the first time. A stable isotope dilution assay (SIDA) using headspace−solid-phase microextraction (HS−SPME) and gas chromatography−mass spectrometry (GC−MS) was developed and validated, and used to quantitate *cis*-**1** in a survey of wines, revealing a range from undetectable (limit of detection = 2.6 ng/L) to 460 ng/L. The odor detection threshold of **1** (using a standard comprising 85% *cis*-**1** and 15% *trans*-**1**) in neutral white wine was determined to be 7.1 μg/L. Despite *cis*-**1** not appearing above the determined sensory threshold in the studied wines, the findings demonstrated the presence of a new volatile sulfur compound with a strong correlation to 3-SH concentration (r = 0.72), showing that *cis*-**1** has potential implications for the fate of the important wine aroma compound 3-SH.

KEYWORDS: *3-sulfanylhexan-1-ol, polyfunctional thiol, stable isotope dilution assay, tropical fruit, wine aroma*

INTRODUCTION

Extensive knowledge of the volatiles associated with the aroma of foods and beverages has been progressively achieved in recent decades through instrumental and sensory analysis. Typically, key aroma volatiles are first noticed for their unique olfactory attributes, which, in turn, leads to studies involving their identification and quantitative analysis to explore the relevance of the new odorants.

As a prime example, the first mention of polyfunctional thiols in wine was raised in a sensory study focusing on "guava" aroma in white wines.[1] Naturally, this led to the development of analytical methods, and ultimately a number of polyfunctional thiols were identified[2−5] as odorants that could impart profound organoleptic impacts in wine.[5] Polyfunctional thiols including 3-sulfanylhexan-1-ol (3-SH), 3-sulfanylhexyl acetate (3-SHA), and 4-methyl-4-sulfanylpentan-2-one (4-MSP) possess extremely low olfactory thresholds (nanogram per liter levels) and are regarded as key volatile sulfur compounds (VSCs) responsible for distinctive citrus and tropical fruit aromas, usually described as "grapefruit", "box hedge", and "passionfruit", in many wine varieties and particularly white wines made from Sauvignon blanc.[6−8] Indeed, these same polyfunctional thiols occur in grapefruit[9] and passionfruit,[10,11] so the high resemblance of the aromas of such fruits and wines should not be surprising.

Among the VSCs identified in foods and beverages, 2-methyl-4-propyl-1,3-oxathiane (**1**, Figure 1, potentially existing as pairs of enantiomers of the geometric isomers) is another odorant that significantly contributes to a characteristic "tropical" aroma.[10−13] Found in passionfruit,[10−13] oxathiane **1** has a strong "fruity" aroma with "green" and "slightly burnt" descriptors,[11] with reported olfactory thresholds in water for *cis*-**1** enantiomers of 2 and 4 μg/L for (2*S*,4*R*)-**1** and (2*R*,4*S*)-**1**, respectively.[14] As a high-impact volatile, **1** has been used as an important ingredient in the fragrance industry.[15]

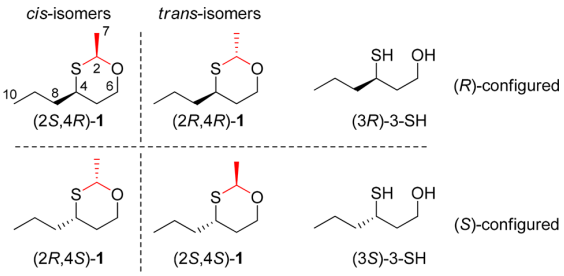

Figure 1. Structures of the possible stereoisomers of 2-methyl-4-propyl-1,3-oxathiane (**1**) and the enantiomers of 3-sulfanylhexan-1-ol (3-SH).

The occurrence of **1** has been reported in passionfruit at trace levels as (4*S*)-**1** (1 μg/L of *cis*-(2*R*,4*S*)-**1** and <0.5 μg/L of *trans*-(2*S*,4*S*)-**1**),[12] and apart from being a powerful VSC itself, **1** was suggested to be related to the extremely potent polyfunctional thiol, 3-SH (Figure 1).[12] However, studies of **1** in foods and beverages are very limited, and according to available reports, **1** has only been occasionally found in passionfruit[11−13,16] and has apparently not been revealed in other foodstuffs, including wine. This was peculiar to us, given the distinct possibility that **1** could be formed through the coupling of 3-SH with acetaldehyde naturally present in an acidic wine matrix. Indeed, acid-catalyzed reactions between 3-

Received: July 27, 2018
Revised: September 21, 2018
Accepted: September 22, 2018
Published: October 5, 2018

Journal of Agricultural and Food Chemistry Article

Table 1. Details of Commercial Wines Used for Quantitation of 1

	variety[a]	vintage	region[b]		variety[a]	vintage	region[b]
1	SAB	2007	Frankland River, WA	22	CH	2013	Yenda, NSW
2	SAB	2007	Marlborough, NZ	23	CH	2013	Margaret River, WA
3	SAB	2013	Marlborough, NZ	24	CH	2013	Coonawarra, SA
4	SAB	2014	Adelaide Hills, SA	25	CH	2013	Adelaide Hills, SA
5	SAB	2014	Adelaide Hills, SA	26	CH	2012	Adelaide Hills, SA
6	SAB	2014	Adelaide Hills, SA	27	CH	2011	Margaret River, WA
7	SAB	2014	Marlborough, NZ	28	CH	2014	Margaret River, WA
8	SAB	2013	Marlborough, NZ	29	CH	2011	Yarra Valley, VIC
9	SAB	2014	Sancerre, FR	30	CH	2013	Barossa Valley, SA
10	SAB	2014	Sancerre, FR	31	CH	2013	Southern WA
11	WB[c]	2015	Bordeaux, FR	32	CH	2013	Barossa Valley, SA
12	WB[d]	2015	Bordeaux, FR	33	CH	2011	Eden Valley, SA
13	SAB	2015	Vendée-Poitou, FR	34	CH	2011	Beechworth, VIC
14	SAB	2015	Cabardès, FR	35	CH	2013	Margaret River, WA
15	CH	2013	Hunter Valley, NSW	36	PG	2007	Pokolbin, NSW
16	CH	2012	Bicheno, TAS	37	RIES	2007	Rowella, TAS
17	CH	2012	Eden Valley, SA	38	WB[e]	2014	Caversham, WA
18	CH	2012	Piccadilly Valley, SA	39	M	2013	Milawa, VIC
19	CH	2012	Margaret River, WA	40	PN	2012	Lake George, NSW
20	CH	2013	Mornington Peninsula, VIC	41	SAN	2012	Whitfield, VIC
21	CH	2012	Pipers River, TAS	42	RO[e]	2012	Yarra Valley, VIC

[a]Variety abbreviated as SAB, Sauvignon blanc; WB, white blend; CH, Chardonnay; PG, Pinot Grigio; RIES, Riesling; M, Muscat; PN, Pinot noir; SAN, Sangiovese; RO, rosé. [b]Regions abbreviated as WA, Western Australia; SA, South Australia; TAS, Tasmania; VIC, Victoria; NSW, New South Wales; FR, France; NZ, New Zealand. [c]85% SAB. [d]70% SAB, 20% Muscadelle, 10% Sauvignon gris. [e]Variety not specified.

SH and acetaldehyde have already been applied for the chemical synthesis of **1**.[11,14,17] Sensory interactions between 3-SH and acetaldehyde reported in a recent sensory study further prompted our interest, wherein acetaldehyde influenced the intensity of "grapefruit", "guava", and "passionfruit" aromas when coexisting with 3-SH in model wine.[18] Enhancive and suppressive effects were used to rationalize this sensorial phenomenon between 3-SH and acetaldehyde,[18] but nothing has been addressed at the molecular level from an analytical perspective.

On the basis of the aforementioned chemical and sensory evidence, and given the potential relationship with 3-SH, we aimed to investigate the presence of 2-methyl-4-propyl-1,3-oxathiane (**1**) in wine. Headspace−solid-phase microextraction (HS−SPME) coupled to gas chromatography−mass spectrometry (GC−MS) was applied to the identification of **1** in wine for the first time, and upon the synthesis of deuterium-labeled **1** (d_4-**1**), a stable isotope dilution assay (SIDA) was developed and validated. The new method was applied to a selection of commercial wines to assess the occurrence of *cis*-**1**, which was correlated with 3-SH and 3-SHA concentrations in those wines. In addition, the odor detection threshold of **1** was determined in a neutral white wine for the first time.

MATERIALS AND METHODS

Chemicals and Solutions. The following chemicals were obtained from commercial suppliers: 2-methyl-4-propyl-1,3-oxathiane (**1**) (≥98% purity, mixture of *cis*- and *trans*-**1**), d_8-naphthalene, C_7−C_{40} alkanes, EDTA 2Na, formic acid, 4,4′-dithiodipyridine (DTDP), acetaldehyde, and silica gel (Sigma-Aldrich, Castle Hill, NSW, Australia); AR-grade sodium chloride (Chem-Supply, Gillman, SA, Australia); d_4-acetaldehyde (≥99 atom % D, ≥98% chemical purity) (Cambridge Isotope Laboratories, Tewksbury, MA); AR-grade dichloromethane and ethanol and HPLC-grade ethanol, acetonitrile, and methanol (VWR International, Tingalpa, QLD, Australia); Bond Elut C18 cartridges (500 mg, 6 mL) (Agilent, Mulgrave, VIC, Australia). Water was obtained from a Milli-Q purification system (Millipore, North Ryde, NSW, Australia). Standard and internal standard (IS) syntheses were reported in previous studies (3-SH and 3-SHA,[19] d_{10}-3-SH,[20] d_5-3SHA[19]) and in the present work (d_4-**1**, see later). Standard and IS solutions were prepared volumetrically in absolute ethanol. EDTA was added to solutions of thiol standards to prevent oxidation. Solutions of **1** and d_4-**1** were prepared freshly before use. Model wine and DTDP reagent were prepared according to a previously published procedure.[7] Stock solutions and DTDP reagent were kept at −20 °C, and working solutions were stored at 4 °C until required.

Nuclear Magnetic Resonance (NMR) Spectroscopy. Proton (^{1}H) and carbon (^{13}C) spectra were recorded with a Varian 500 instrument (Agilent, Santa Clara, CA) operating at 500 and 125 MHz, respectively. Chemical shifts were recorded as δ values in parts per million (ppm). Spectra were acquired in chloroform-*d* at 26 °C. The isomeric purity of commercial **1** was determined to be 85% *cis*-**1** and 15% *trans*-**1** by ^{1}H NMR using a relaxation delay of 10 s and integration of the methine proton on C-2.

High-Resolution Mass Spectrometry (HRMS). Spectra were obtained on an Agilent 1290 Infinity II HPLC coupled to an Agilent 6530 Accurate-Mass Q-TOF LC−MS system with electrospray ionization (ESI) in positive mode using a solution prepared in 50% v/v aqueous ethanol at a concentration of ∼1 mg/L.

Synthesis of 2-($^2H_3$)methyl-4-propyl-1,3-(2-^{2}H)oxathiane (d_4-1). This IS was prepared by adapting the method reported for the synthesis of unlabeled **1**[14] using 3-SH and d_4-acetaldehyde. In brief, to a stirred solution of d_4-acetaldehyde (511 mg, 10.6 mmol) in dry CH_2Cl_2 (5 mL) containing a catalytic amount of *p*-TsOH (25 mg) and activated 4 Å sieves (0.96 g) under nitrogen was added 3-SH (400 μL, 391 mg, 2.4 mmol), and the reaction was stirred for 30 min. The solution was diluted with CH_2Cl_2 (5 mL), and the organic layer was washed with $NaHCO_3$ (5 mL) and brine (5 mL), dried over anhydrous $MgSO_4$, and concentrated by short-path distillation. The crude product, isolated as a colorless oil, was purified by flash column chromatography using silica gel (60 Å, 230−400 mesh) with CH_2Cl_2 (R_f = 0.45), and the removal of solvent in vacuo afforded d_4-**1** (306 mg, 1.91 mmol, 80% based on 3-SH) as a colorless oil with a purity of >93% and a *cis*/*trans* ratio of 85:15 by ^{1}H NMR and GC−MS

DOI: 10.1021/acs.jafc.8b04027
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(method details provided in the caption of Figure S1 of the Supporting Information). Spectroscopic data were in full accord with those reported for the unlabeled compound,[11] apart from the absence of signals corresponding to the labeled positions in the ^{1}H NMR spectrum. ESI−HRMS (m/z): [M+H$^+$] calcd for $C_8H_{13}{}^2H_4OS^+$ 165.1173; found 165.1239. EI−MS, m/z (%) 164 (M^+, 90), 146 (100), 114 (20), 101 (65), 87 (75), 73 (55), 55 (67).

Wine Samples. Wines (n = 42) of various vintages (Table 1) were obtained from commercial retailers and screened for the occurrence of **1**.

Analytical Method Development. *Sample Preparation.* Sample (5 mL), sodium chloride (2 g), and an aliquot of IS solution were added to a 20 mL clear HS−SPME vial, and the vial was sealed with a PTFE-lined screwcap (Agilent). For early preliminary method development, 10 μL of d_8-naphthalene ethanolic solution was spiked as IS, affording a final concentration of 2 μg/L. For the final SIDA HS−SPME−GC−MS method validation and quantitative analysis (commercial wine survey and sensory verification), 40 μL of d_4-**1** ethanolic solution was used as IS, giving a final concentration of 100 ng/L.

HS−SPME. Evaluation of SPME fibers included divinylbenzene/carboxen/polydimethylsiloxane (1 or 2 cm DVB/CAR/PDMS, 50/30 μm), PDMS/DVB (65 μm), CAR/PDMS (75 μm), and polyacrylate (PA, 85 μm) (Supelco, Sigma-Aldrich). The PDMS/DVB fiber was used for subsequent method development and quantitative analysis. Fibers were preconditioned according to the manufacturer's recommendation. Sample vials were incubated at 35 °C for 0.1 min with agitation speed of 500 rpm and extracted for 30 min (agitator on time 10 s and off time 1 s) at the same speed and temperature. The fiber was then desorbed in the inlet for 900 s at 250 °C. HS−SPME was performed using a Gerstel MPS autosampler (Lasersan Australasia, Robina, QLD, Australia).

Preliminary GC−MS Method Development. *Instrumentation for Preliminary GC−MS Method.* Preliminary GC−MS method development was performed using an Agilent 7890 GC (Santa Clara, CA) coupled to an Agilent 5897 mass spectrometer fitted with a DB-WAXetr capillary GC column (60 m × 0.25 mm, 0.25 μm, Agilent J&W). Ultrapure helium (BOC, North Ryde, NSW, Australia) was used as carrier gas at a constant flow rate of 1.5 mL/min. Splitless injection mode was used with a desorption temperature of 250 °C into an inlet fitted with an ultra inert SPME liner (0.75 mm i.d., Agilent). The oven program started at 40 °C for 3 min, then increased at 5 °C/min to 250 °C and was held for 10 min at that temperature. The transfer line was maintained at 250 °C. Positive ion electron impact spectra at 70 eV were recorded. Full-scan mode (m/z 35−350) and selective ion monitoring (SIM) mode were used for method development. The ions subsequently monitored in SIM runs were m/z 160, 145, 101, and 87 for **1** and 136 and 108 for d_8-naphthalene. A dwell time of 25 ms was used for all ions. The underlined ions were used for quantitation, and the other ions were used as qualifiers. Instrument control and data acquisition were performed using MSD ChemStation software (version E02, Agilent).

Matrix Effects with Preliminary GC−MS Method. For the investigation of matrix effects when using model wine, three sets of check samples (control, nitrogen headspace, acetaldehyde-spiked) were prepared with seven replicates in each set for consecutive analysis. Control samples were prepared according to the above-mentioned sample preparation procedure. Nitrogen headspace and acetaldehyde-spiked samples were prepared in the same manner, except vials were either carefully flushed with nitrogen or spiked at 100 mg/L with pure acetaldehyde prior to being sealed.

Validation of Preliminary GC−MS Method. Method validation was conducted in model wine and a commercial dry white wine using d_8-naphthalene as IS. In duplicate, a series of standard additions of authentic **1** spiked into model or white wine led to nine calibration levels (0, 0.1, 0.25, 0.5, 1, 2.5, 5, 10, 15 μg/L). Seven replicates of samples spiked with 0.5 and 5 μg/L of **1** were prepared along with the calibration samples to check precision and accuracy. The limit of detection (LOD) and limit of quantitation (LOQ) were estimated as three and ten times the standard error of the y intercept divided by the slope of the calibration equation.

Verification of 1 in Wine. *Linear Retention Index (LRI).* Values were calculated on a DB-WAXetr column (60 m × 0.25 mm, 0.25 μm, J&W Agilent) and a DB-5MS UI column (60 m × 0.25 mm, 0.25 μm, J&W Agilent) using C_7−C_{40} n-alkanes and the preliminary GC−MS method.

Co-Injection Experiments. Co-injection experiments were performed on a DB-WAXetr column (with preliminary GC−MS method) and an HP-INNOWax column (with SIDA GC−MS method). Increased amounts of **1** (containing 85% *cis*-**1** and 15% *trans*-**1**) spiked into a random wine selected from a laboratory-scale Sauvignon blanc fermentation trial[21] (containing 5 ng/L of *cis*-**1**, affording an extra 8.5 and 17 ng/L) and a commercial Sauvignon blanc wine (containing 80 ng/L of *cis*-**1**, to yield an extra 100, 200, and 300 ng/L) were used for co-injection experiments.

SIDA GC−MS Method Development. *Instrumentation for SIDA GC−MS Method.* SIDA GC−MS method development, validation, and quantitative analysis were performed with an Agilent 6890 GC coupled to an Agilent 5793N mass spectrometer (Santa Clara, CA). Ultrapure helium (BOC, North Ryde, NSW, Australia) was used as carrier gas at a constant flow rate of 1.4 mL/min. Splitless injection mode was used with a desorption temperature of 250 °C into an inlet fitted with an ultra inert SPME liner (0.75 mm i.d., Agilent). The transfer line was maintained at 250 °C. Positive ion electron impact spectra at 70 eV were recorded. The ions monitored in SIM runs were m/z 160, 145, 101, and 87 for **1** and 164, 146, 101, and 87 for d_4-**1**. A dwell time of 30 ms was used for all ions. The underlined ions were used for quantitation, and the other ions were used as qualifiers. Instrument control and data acquisition were performed using MSD ChemStation software (version E02, Agilent).

Initially, GC capillary column evaluation was undertaken with the same oven program as the preliminary GC−MS method, and DB5-MS UI (60 m × 0.25 mm, 0.25 μm, Agilent J&W), Solgel-WAX (30 m × 0.25 mm, 0.25 μm, SGE, Ringwood, VIC, Australia), VF-200 ms (30 m × 0.25 mm, 0.25 μm, Agilent J&W), and HP-INNOWax (60 m × 0.25 mm, 0.25 μm, Agilent J&W) were compared for their sensitivity and selectivity for **1**.

Finally, an HP-INNOWax column (60 m × 0.25 mm, 0.25 μm, Agilent J&W) was selected for the optimized method validation and quantitative analysis using an improved oven program that began at 40 °C for 3 min, increased at 5 °C/min to 150 °C, then at 15 °C/min to 250 °C, and was held at this temperature for 10 min.

Validation of SIDA GC−MS Method. In brief, a series of duplicate standard additions of unlabeled **1** (giving 0, 2.5, 5, 7.5, 10, 25, 50, 75, 100, 250, 500, and 1000 ng/L of *cis*-**1**) and d_4-**1** (100 ng/L) were prepared in a commercial Sauvignon blanc wine. Seven replicates of samples spiked with low and high levels of **1** (7.5 and 75 ng/L of *cis*-**1**) were prepared for repeatability (using the same Sauvignon blanc wine). Recovery and matrix effect were evaluated in duplicate in a commercial Chardonnay, a rosé, and a red wine at low (7.5 ng/L) and high (75 ng/L) spiked levels of *cis*-**1**. LOD and LOQ were determined as previously outlined for the preliminary GC-MS method.

Quantitation of *cis*-1 in Commercial Wines by SIDA GC−MS. Fresh calibration and QC samples prepared in duplicate were included when analyzing a batch of commercial wine samples. Calibration levels were spiked at 0, 10, 25, 50, 100, 250, and 500 ng/L of *cis*-**1** in a commercial Sauvignon blanc wine, and QC samples were spiked at 7.5 and 75 ng/L in a commercial Chardonnay wine. All samples were prepared and analyzed according to the optimized conditions.

Quantitation of 3-SH and 3-SHA by SIDA HPLC−MS/MS. 3-SH and 3-SHA were analyzed by HPLC−MS/MS after derivatization with DTDP according to a previously reported method.[7] HPLC−MS/MS analysis was performed using an Agilent 1200 Series HPLC connected to an Agilent 6410A Triple Quad MS (Agilent, Santa Clara, CA). The HPLC was fitted with an Alltima C18 column (250 mm × 2.1 mm i.d., 5 μm, 100 Å) protected by a C18 guard cartridge (7.5 mm × 2.1 mm i.d.). Chromatographic and mass spectrometric

DOI: 10.1021/acs.jafc.8b04027
J. Agric. Food Chem. 2018, 66, 10808−10815

parameters were the same as previously reported.[7] Instrument control and data acquisition were performed using Agilent MassHunter Workstation software (B.03.01).

Odor Detection Threshold Determination. The odor detection threshold of **1** in white wine was determined according to the forced-choice ascending concentration series method of limit (E679-04, Reapproved 2011) by ASTM International. This sensory study was approved by the Human Research Ethics Committee of The University of Adelaide (Ethics approval number: H-2018-152). The panelists (n = 18) were recruited from the School of Agriculture, Food and Wine, The University of Adelaide, aged from 24 to 51 years old, with 11 males and 7 females, and most panelists had previous experience in sensory evaluation of wine. A commercial Chardonnay wine (vintage 2013, unoaked) was selected as the base wine for threshold determinations because of its neutral aroma character. The employed ascending concentrations of **1** were decided based on quantitative data from our commercial wine survey and further refined by informal benchtop sensory testing with six researchers. Bottles of the commercial wine were carefully blended in a 20 L glass demi-john to obtain a sufficient quantity of homogeneous base wine, which was then divided into seven lots, with one to be used as a control and the other six for spiking.

Wines with ascending concentrations (0.486, 1.46, 4.40, 13.1, 39.4, and 118 μg/L of *cis*-**1** using 85:15 *cis*-**1**/*trans*-**1** standard) were prepared by spiking 500 μL of ethanolic working solutions of **1**, whereas an equal amount of ethanol was spiked into the control wine. Wines to be evaluated were prepared on the same day as the sensory session, and samples (20 mL) were presented in clear ISO XL 5 wine tasting glasses labeled with four-digit codes and covered with clear glass lids. The sensory session was conducted in sensory booths at ambient temperature (22−24 °C) under orange lights to mask color. The panelists were asked to sniff the wine only and were forced to take a short break between each sample. The best-estimate threshold (BET) for each panelist was calculated as the geometric mean of the highest concentration missed and next higher concentration detected. The panel threshold was the geometric mean of BETs of all panelists. Sample randomization and data collection were performed with RedJade software (Redwood City, CA).

Statistics. Data reduction, mean values, standard deviations, linear regressions, and Pearson correlation were calculated in Microsoft Excel (Microsoft Office Professional Plus 2013 for Windows). Normality testing of residuals (α = 0.05) was performed in GraphPad Prism 7 (GraphPad Software, version 7.02) to verify the linearity of calibration curves.

RESULTS AND DISCUSSION

Preliminary GC−MS Method Development. A suitable analytical method was required to verify our hypothesis that **1** existed in wine (likely as a result of the reaction of 3-SH with acetaldehyde). Historically, **1** was isolated from passionfruit using liquid−liquid extraction[12,22] and simultaneous distillation−extraction,[10,11] followed by GC with flame ionization detection, while more recently, **1** was tentatively identified in an aroma profiling study of passionfruit using HS−SPME and GC−MS.[16] Applying HS−SPME to the analysis of **1** in wine-like medium was an obvious approach and was first investigated with a PDMS/DVB/CAR fiber to extract **1** after being spiked into Milli-Q water or model wine. GC−MS analysis was conducted in scan mode with separation on a DB-WAXetr column. One major and one minor peak appeared for **1** at respective retention times (RTs) of 23.622 and 24.525 min (Figure S2A of the Supporting Information), and the mass spectra (with ions at m/z 160 (M^+), 145, 133 (relatively more intense for the peak that was later confirmed as *trans*-**1**), 114, 101, 87, and 73) (Figure S2B,C) were matched with the NIST05 MS library and previously reported spectra of **1**.[11] Ions obtained in this stage (m/z 160, 145, 101, and 87) were considered as ions to monitor in the later stages of method development. The two peaks had a peak area ratio of approximately 85:15 in favor of the first peak (Figure S2A), which corresponded to the proportion of *cis*/*trans* in commercial **1**, as verified by ^{1}H NMR. The elution order of *cis*-**1** prior to *trans*-**1** agreed with that reported for passionfruit juice extracts using a polar column.[11]

HS−SPME. After obtaining chromatographic and spectral data of authentic **1**, four SPME fibers (PDMS/DVB/CAR, PDMS/DVB, PDMS, and PA) were compared for their extraction efficiency of **1** spiked in model wine. Deuterated **1** was not commercially available, and d_8-naphthalene was selected as the IS for preliminary method development. d_8-Naphthalene eluted about 5 min after *cis*-**1** and *trans*-**1** at 29.190 min and showed no interfering ions (abundant ions at m/z 136 and 108 were selected as SIM ions for d_8-napthalene). Compared with other fibers, PDMS/DVB of 1 cm length demonstrated comparably better extraction and exhibited no carryover of **1** (data not shown) and was selected for further experimentation.

Preliminary GC−MS Method Validation. Preliminary GC−MS method development proceeded with d_8-naphthalene as IS in model wine and white wine to assess the calibration range, linearity, precision (evaluated as repeatability, % RSD), accuracy (evaluated as recovery), and sensitivity. Duplicate samples spiked with **1** (0, 0.1, 0.25, 0.5, 1, 2.5, 5, 10, and 15 μg/L) and d_8-naphthalene (2 μg/L) were analyzed in SIM mode. The standard curve of **1** obtained in a commercial Sauvignon blanc wine was linear across the calibration range (0−15 μg/L), having a coefficient of determination (R^2) of 0.9987. Precision values at spiking levels of 0.5 and 5 μg/L of seven replicates were 5.6 and 6.6%, respectively. Accuracy values were 92−104% and 93−110% at respective spiking levels of 0.5 and 5 μg/L. The calculated LOD and LOQ for this white wine matrix were 0.085 and 0.28 μg/L, respectively. However, a deterioration of method performance was noted when method validation was attempted in model wine.

Matrix Effects with Preliminary GC−MS Method. Upon reviewing the GC−MS chromatograms of model wine repeatability samples spiked with **1**, a decrease in peak intensity over time was noticed (data not shown), and it seemed that **1** was decomposing while awaiting analysis. Compared with the successful method performance in Sauvignon blanc wine, the aberrant results in model wine indicated that the wine matrix (possibly the equilibrium between ordinary wine matrix compounds, i.e., 3-SH, acetaldehyde, and **1**) was crucial for stabilizing **1**. In other words, a lack of 3-SH or acetaldehyde in model wine may lead to an equilibrium shift toward the reactants and away from **1**. This was investigated by controlling the headspace compositions of the vials. Three sets of check samples (seven replicates) were prepared with model wine spiked with **1** at 5 μg/L. Before capping the vials, one set was gently flushed with N_2, the second set was spiked at 100 mg/L acetaldehyde, and a control set was prepared without any alterations. Serial HS−SPME−GC−MS analysis of the replicates was performed to ascertain the peak ratios of **1** versus d_8-naphthalene (Figure 2).

Notably, the peak areas of **1** in control samples (air as headspace) decreased dramatically over time (Figure 2). Samples flushed with N_2 also showed a decline in peak area ratio but at a slower rate compared with the controls. Relatively consistent peak area ratios were only observed in model wine containing acetaldehyde, which evidently played

DOI: 10.1021/acs.jafc.8b04027
J. Agric. Food Chem. 2018, 66, 10808−10815

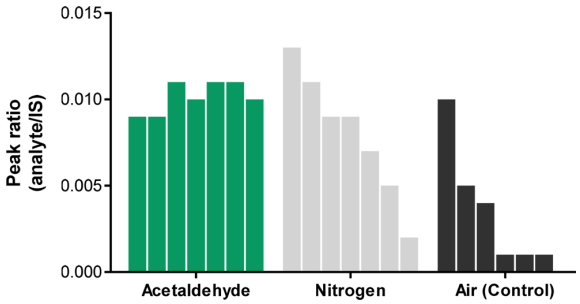

Figure 2. Grouped bar chart of serial HS−SPME−GC−MS analysis on three sets of replicate samples (n = 7, with bars from left to right in each group representing samples in the serial injection sequence) prepared in model wine with acetaldehyde, nitrogen, or air as headspace (control).

an important role in stabilizing **1**. As such, validation of a preliminary method in model wine was performed by adding acetaldehyde (final concentration at 100 mg/L) when preparing calibration solutions. Method performance was improved compared with the initial attempt in model wine and yielded a standard curve for **1** that was linear (R^2 = 0.9957) throughout the calibration range (0−15 μg/L), with good precision (RSD < 5% for 0.5 and 5 μg/L levels) and accuracy (92−110% for 0.5 μg/L and 99−108% for 5 μg/L) and good sensitivity (0.05 and 0.13 μg/L for calculated LOD and LOQ, respectively).

VERIFICATION OF 1 IN WINE BY GC−MS

With a workable HS−SPME−GC−MS method using commercially available d_8-naphthalene, a range of wines was submitted to a preliminary search for the presence of **1**; wines comprised samples from two independent projects that were active at the time. One set consisted of commercial red, white, and rosé wines (n = 23) obtained from retail outlets, as detailed elsewhere;[23] another set (n = 90) was from a laboratory-scale Sauvignon blanc fermentation trial.[21] The commercial wines (including 10 Sauvignon blanc wines) did not appear to contain any **1**, whereas wines[21] from the laboratory-scale fermentation trial had detectable levels (data not shown). The identification of **1** was based on the comparison of mass spectra with authentic **1**, LRIs of naturally present **1** on two GC phases, and co-injection experiments. The observed peaks at 23.700 min of four ions (m/z 160, 145, 101, and 87) aligned at almost the same retention time as authentic *cis*-**1** (23.622 min) and had the same ratio of qualifier ions to quantifier ion. The measured LRIs were 1261 on a DB-5MS UI column and 1538 on a DB-WAXetr column, with the latter according well with the reported LRI of 1530 on a Carbowax column for the *cis*-isomer of **1**.[11]

Co-injection experiments were performed using a fermentation-trial Sauvignon blanc wine[21] (that was deemed to contain 5 ng/L of *cis*-**1**) to verify the peak identity. Spiking increased amounts of authentic **1** yielded corresponding peak enhancements in the selected ion chromatograms and ion ratios within the expected range (data not shown). Together these data ensured the positive identification of *cis*-2-methyl-4-propyl-1,3-oxathiane (*cis*-**1**) in wine for the first time. The co-injection experiment was repeated in a commercial Sauvignon blanc wine using the final SIDA GC−MS method described later to confirm the identification: Wine no. 1 (Table 1), which was found to contain *cis*-**1** at 80 ng/L (Table S1 of the Supporting Information), was spiked with *cis*-**1** at 100, 200, and 300 ng/L, yielding the expected increases in peak intensity of all selected qualifier ions (Figure 3). The ion ratios were also taken into account for confirming the identity of *cis*-**1** in the wine. In contrast, although spiking with **1** also increased *trans*-**1** in the correct proportions (Figure S3A of the Supporting Information), the endogenous presence of *trans*-**1** was not detectable based on signal-to-noise ratio for the m/z 160 ion (Figure S3B), and the four SIM ions did not line up at the correct retention time in the unspiked wine sample (Figure S3C).

Development of SIDA HS−SPME−GC−MS Method for Quantitation of *cis*-1 in Wines. From the identification of *cis*-**1** in wine, we noticed that its concentration tended to be below the μg/L level (≈ ng/L), so the calibration range was adjusted from 0−15 μg/L to 0−1000 ng/L. Upon further application of the HS−SPME−GC−MS method to a broader range of commercial wines (Table 1), interferences were observed in a proportion of the wines, which hampered the positive identification of **1**. Modification of the temperature program was trialed (data not shown) but no improvement was seen for separation of *cis*-**1** from the coeluter(s) that gave similar ions. At this point, we revisited the method develop-

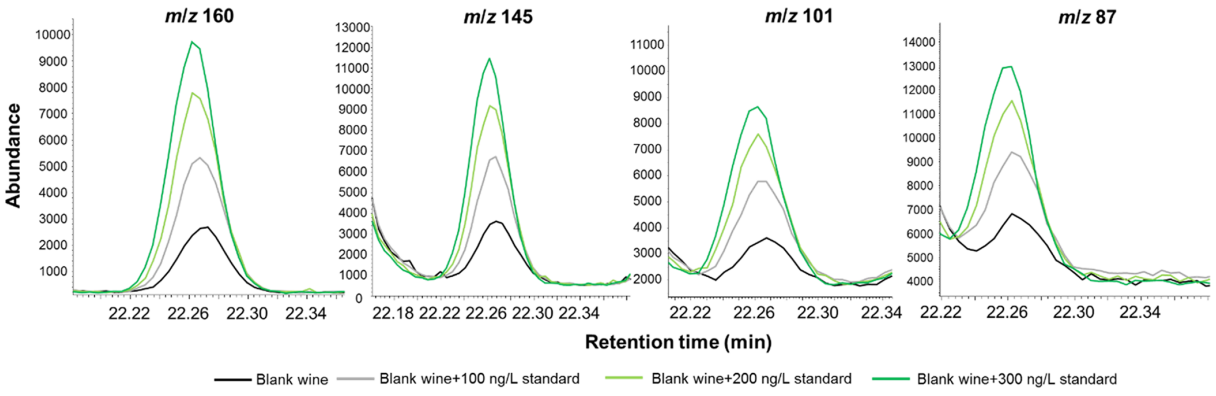

Figure 3. Overlaid selected ion chromatograms (obtained with the optimized SIDA HS−SPME−GC−MS method) of co-injection experiments using a commercial Sauvignon blanc wine (wine no. 1 in Table 1) found to contain 80 ng/L of *cis*-**1**, with further addition of 100, 200, and 300 ng/L of *cis*-**1**.

DOI: 10.1021/acs.jafc.8b04027
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ment and tested additional column phases with the aim of eliminating interferences, while also developing a SIDA approach after synthesizing d_4-**1** as IS (from 3-SH and d_4-acetaldehyde) to compensate for possible changes to the concentration of *cis*-**1** during analysis. Four GC capillary columns of various stationary phases were tested for their ability to resolve *cis*-**1** from interfering components. Milli-Q water spiked with 200 ng/L of **1** was first run on the different columns to obtain RTs, and a wine that previously showed interferences was tested on the candidate columns. In the end, an HP-INNOWax column was selected due to being best able to eliminate possible interferences.

Validation of SIDA Method Using HS−SPME−GC−MS for Quantitation of *cis*-1 in Wine. Synthesized d_4-**1** (85:15 mixture of *cis* and *trans*) was used as the IS at a concentration of 100 ng/L (i.e., 85 ng/L of d_4-*cis*-**1**), and d_4-*cis*-**1** eluted slightly earlier than *cis*-**1** (Figure S1C,D of the Supporting Information). Because of the previously observed matrix effects when developing a method in model wine (despite this being overcome by the addition of acetaldehyde), the validation of the SIDA GC−MS method was only performed in a commercial Sauvignon blanc, which encompassed the determination of linearity, precision (repeatability), accuracy (recovery in commercial Chardonnay, rosé, and red wines), and sensitivity (LOD and LOQ). The calibration levels of 0−1000 ng/L, being the most likely range that could be expected in real wines, showed linearity (R^2 = 0.9997) throughout the range. Values for repeatability (RSD < 4% at 7.5 and 75 ng/L spiking levels) and recovery (94−106 and 95−106% for 7.5 and 75 ng/L, respectively, in three wine matrices) showed the precision and accuracy of the method. The estimated LOD and LOQ were 2.6 and 8.6 ng/L, respectively, which were entirely appropriate for this analytical method.

Survey of *cis*-1 in Commercial Wines by SIDA with HS−SPME−GC−MS. In our preliminary screening, *cis*-**1** (present as an unresolved pair of enantiomers, Figure 1) was quantitated at nanogram per liter concentrations in wines[21] from a laboratory-scale fermentation trial (data not shown). After the analytical approach was upgraded to a SIDA method, we screened a new set of commercial wines (n = 42) of various vintages, which included Chardonnay (n = 21), Sauvignon blanc (n = 14), and a few other varieties (Table 1). *cis*-**1** was present in 35 out of the 42 wines and ranged from undetectable (LOD = 2.6 ng/L) to 460 ng/L. Notably, *trans*-**1** (LOD = 3.4 ng/L) was not found in any of the samples analyzed throughout this entire study, with the lack of *trans*-**1**, as demonstrated in Figure S3C of the Supporting Information, being representative of the studied wines. The predominant occurrence of *cis*-**1** could be explained on the basis that *cis*-**1** is thermodynamically more stable due to having the methyl and propyl groups in equatorial positions in the oxathiane ring.[11] However, the possibility of the presence of *trans*-**1** in wine could not be excluded given that only a small number of wines was surveyed in this study.

Wines found to contain *cis*-**1** included Sauvignon blanc, Chardonnay, Riesling, Pinot Grigio, Muscat, Pinot noir, and Sangiovese (Table 2). This broad presence of *cis*-**1** across different varieties indicated its formation was general rather than exclusive to certain varieties. The high likelihood that *cis*-**1** derives from 3-SH means its presence in different wines could be expected due to the widespread occurrence of 3-SH itself.[7] Even so, the highest concentration of *cis*-**1** was seen in a Sauvignon blanc wine (460 ng/L), and this variety generally

Table 2. Summary of Concentrations (ng/L) of *cis*-1 in Commercial Wines Selected for the Study

			concentration[b]			
wine		occurrence[a]	min	max	mean	SD
variety	Sauvignon blanc	10/12	14	460	119	155
	Chardonnay	17/21	7	69	27	22
	white blend	2/3	16	33	16	16
	Pinot Grigio	1/1	–	–	15	–
	Riesling	1/1	–	–	367	–
	Muscat	1/1	–	–	22	–
	Sangiovese	1/1	–	–	14	–
	Pinot noir	1/1	–	–	14	–
	rosé	1/1	–	–	35	–
origin[c]	New Zealand	4/4	131	460	287	171
	Australia	3/4	14	80	36	36
	France	4/6	33	62	28	24

[a]Number of wines found containing *cis*-**1**/number of wines analyzed for that variety. [b]–, not applicable (only one sample). [c]Sauvignon blanc wines (France includes Sauvignon blanc-dominant white blend wines 11 and 12).

contained higher amounts of *cis*-**1** than others that were analyzed (Table S1 of the Supporting Information). This fits well with the fact that Sauvignon blanc wines tend to have higher abundances of 3-SH than other varieties.[24] Apart from Sauvignon blanc, a Riesling (367 ng/L) had relatively high amounts of *cis*-**1**, and, notably, 17 out of 21 Chardonnay wines contained *cis*-**1** ranging in concentration from 7 to 69 ng/L. Besides the suspected influence of grape variety, the potential impact of wine origin was also evident when comparing Sauvignon blanc wines made in New Zealand, Australia, and France. Sauvignon blanc wines from the Marlborough region of New Zealand had considerably greater amounts of *cis*-**1** than those from Australia or France, which potentially reflects the fact that Marlborough Sauvignon blanc wines are well known for their high 3-SH concentrations.[25,26]

To further investigate the possible relationship between *cis*-**1** and 3-SH (and 3-SHA), 3-SH and 3-SHA were measured by HPLC−MS/MS analysis[7] in the commercial wines in parallel to compare against the concentrations of *cis*-**1** in the same wines (Table S1 of the Supporting Information). 3-SH and 3-SHA were present in all 42 wines at various concentrations: 3-SH ranged from 459−7923 ng/L (wine no. 7 in Table 1 was outside the calibration range and was extrapolated), and 3-SHA varied from 4−20 ng/L. The concentrations of 3-SH in this selection of wines were similar to data reported elsewhere,[23,25] whereas the relatively low levels of 3-SHA in these wines were not too surprising given the vintages of the wines (2007 to 2015, Table 1) and that 3-SHA can hydrolyze during storage.[27] Sauvignon blanc wines contained higher amounts of 3-SH (516−7923 ng/L) than other wines, followed by Chardonnay (459−3347 ng/L), which mirrored the previous data reported for these two varieties.[7,23,25,28] The Pearson correlation analysis between concentrations of *cis*-**1** and 3-SH was used to evaluate the relationship among the data, revealing a strong positive correlation (r = 0.72) between *cis*-**1** and 3-SH when all wines (n = 42) were considered, which was highly suggestive that *cis*-**1** could originate from 3-SH in the proposed manner (i.e., reaction with acetaldehyde under acidic wine conditions). Even stronger positive correlations were observed when correlation analysis was conducted with single varieties (r = 0.81 for Sauvignon blanc wines, n = 14; r =

DOI: 10.1021/acs.jafc.8b04027
J. Agric. Food Chem. 2018, 66, 10808−10815

0.84 for Chardonnay wines, $n = 21$). A very weak but positive correlation ($r = 0.20$, all wine data) was seen between 3-SHA and *cis*-**1**, but we hesitate to indicate any possible correlation with 3-SHA and *cis*-**1** in this case because the concentrations of 3-SHA were relatively low (with some being close to LOQ). Another point worth mentioning was that all 42 wines contained detectable amounts of 3-SH, but *cis*-**1** was not detected in seven wines (three Sauvignon blanc and four Chardonnay) in which the concentrations of 3-SH were far from negligible (467−1596 ng/L). This suggested that grape and wine components other than 3-SH or biological or chemical factors (such as yeast strains, postfermentation conditions, etc.) could play a significant role in the formation of *cis*-**1** in wines.

Odor Detection Threshold of 1 in White Wine. The odor detection threshold of **1** was determined to be 7.1 μg/L using a commercially available mixture of 85:15 *cis*/*trans* (assuming the presence of 15% *trans*-**1** did not impact the odor threshold of *cis*-**1**) in a neutral Australian Chardonnay wine. Although this appeared to constitute the first report of a threshold for **1** in wine, the value was in general agreement (but several times higher) with those measured in water (2 μg/L for (2*S*,4*R*)-**1** and 4 μg/L for (2*R*,4*S*)-**1**[14]). Such a difference was to be expected given that aroma thresholds of odorants determined in water are normally lower than those in wine.[29] Combining the quantitative results and odor detection threshold, the calculated odor activity value (OAV) of *cis*-**1** (based on the threshold of **1** as described above) in wines was ≤0.06 (Table S1 of the Supporting Information). On the basis of OAV, it seemed that the contribution of *cis*-**1** to the aroma of the studied wines was not significant, although volatiles with low OAV can still play a crucial role in wine aroma.[30] In addition, taking the odor detection thresholds of **1**, 3-SH (60 ng/L[31]), and acetaldehyde (500 μg/L[32]) into consideration, it is anticipated that the production of several hundred nanograms per liter of *cis*-**1** may provoke a considerable sensory impact through the consumption of similar amounts of 3-SH, as opposed to affecting wine aroma due to a direct contribution of *cis*-**1** or a decrease in acetaldehyde. Notably, a previous study had already reported the sensory interactions between acetaldehyde and 3-SH,[18] but with our new identification of *cis*-**1** it seems necessary to conduct further studies to better elucidate the sensory impact of *cis*-**1** on the aroma profile of wines, especially in conjunction with changes in concentration of 3-SH.

Taking all results into account, this work presents a detailed study that arose by considering the potential presence of **1** in wine based on the chemistry of 3-SH and acetaldehyde that are present in a wine matrix. This led to the first identification of *cis*-**1** as a new VSC in wine and the development of a targeted HS−SPME−GC−MS method. Quantitation of *cis*-**1** in wines was achieved by SIDA using synthesized d_4-**1** as an IS. The concentrations of *cis*-**1** in a small set of commercial wines ranged from undetectable to 460 ng/L, with the highest concentration being below the odor detection threshold of 7.1 μg/L determined in a neutral white wine. Nonetheless, *cis*-**1** could still play a significant indirect role in wine aroma as a source or sink of potent odorant 3-SH, and our findings expand the knowledge of the potential fate of 3-SH during winemaking and storage. More research is required to better understand the role of *cis*-**1** in wine in terms of formation and sensory impacts (including studies investigating chirality), which could provide opportunities to optimize the production and/or preservation of 3-SH in wines to improve or retain desirable sensory qualities.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.jafc.8b04027.

Figures showing mass spectra and selected ion chromatograms of unlabeled and labeled *cis*-**1** (Figure S1), chromatographic and mass spectrometric data of *cis*-**1** and *trans*-**1** (Figure S2), and detection of *cis*-**1** and absence of *trans*-**1** in wine (Figure S3); table containing data for *cis*-**1** (as well as calculated OAVs), 3-SH, and 3-SHA concentrations in commercial wines (Table S1). (PDF)

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Funding

L.C. was a recipient of the joint scholarship of UA and China Scholarship Council (CSC201506350012) and was also supported by a Wine Australia supplementary scholarship (AGW Ph1512). AWRI is supported by Australia's grape-growers and winemakers through their investment body, Wine Australia, with matching funds from the Australian government. UA and AWRI are the members of the Wine Innovation Cluster at the Waite Precinct in Adelaide.

Notes

The authors declare no competing financial interest.

■ ACKNOWLEDGMENTS

We thank Phil Clements (UA) for undertaking NMR analyses, Sue Bastian and Lukas Danner (UA) for advice on aspects of the sensory study, Sue Maffei, Christine Böttcher, and Paul Boss (CSIRO) for assistance with accurate mass measurements and access to HPLC−MS/MS instrumentation, Remi Schneider (IFV) for providing the French wines, and sensory panelists for participating in the threshold study.

■ ABBREVIATIONS USED

3-SH, 3-sulfanylhexan-1-ol; 3-SHA, 3-sulfanylhexyl acetate; 4-MSP, 4-methyl-4-sulfanylpentan-2-one; BET, best-estimate threshold; DTDP, 4,4′-dithiodipyridine; ESI, electrospray ionization; GC−MS, gas chromatography−mass spectrometry; HPLC−MS/MS, high-performance liquid chromatography with tandem mass spectrometry; HS−SPME, headspace−solid-phase microextraction; HRMS, high-resolution mass spectrometry; IS, internal standard; NMR, nuclear magnetic resonance; LRI, linear retention index; OAV, odor activity value; SIDA, stable isotope dilution assay; SIM, selected ion monitoring; VSC, volatile sulfur compound.

DOI: 10.1021/acs.jafc.8b04027
J. Agric. Food Chem. 2018, 66, 10808−10815

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DOI: 10.1021/acs.jafc.8b04027
J. Agric. Food Chem. 2018, 66, 10808−10815

SUPPLEMENTARY INFORMATION FOR

Identification and Quantitative Analysis of 2-Methyl-4-propyl-1,3-oxathiane in Wine

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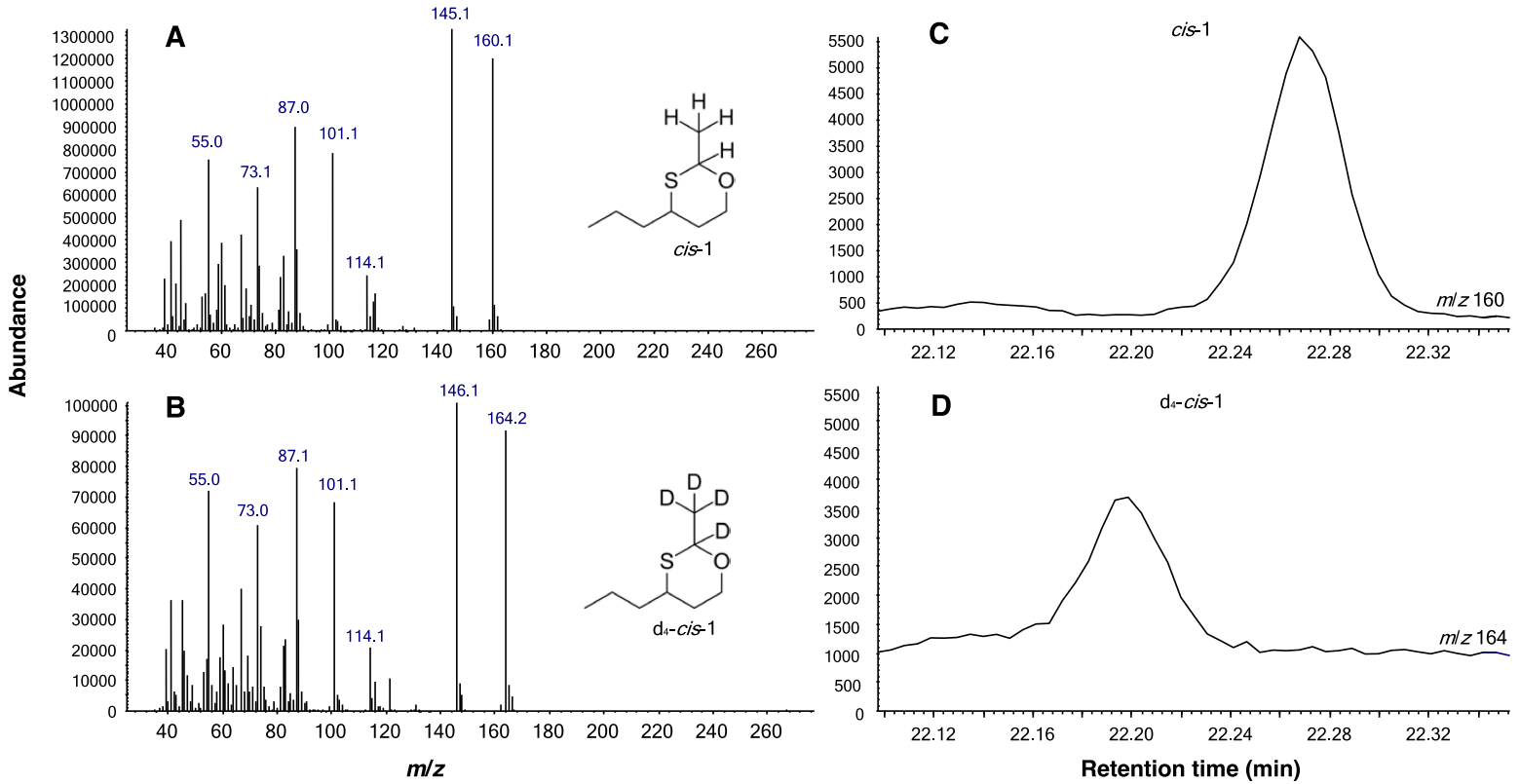


Figure S1. Background subtracted mass spectra of standards showing (**A**) commercial *cis*-**1** (850 ng/L in water, preliminary HS-SPME-GC-MS method) and (**B**) synthesized *d*4-*cis*-**1** (approximately 100 µg/L in dichloromethane, liquid injection, HP-INNOWax column, 40 °C for 1 min, 5 °C/min to 250 °C and held for 10 min, 1.4 mL/min He, inlet 220 °C, MS source 250 °C, auxiliary 250 °C, *m*/*z* 35–350), and selected ion chromatograms of (**C**) naturally present *cis*-**1** (131 ng/L) and (**D**) *d*4-*cis*-**1** (85 ng/L) (both obtained using final SIDA HS-SPME GC-MS method) spiked in a Sauvignon blanc wine (wine no. 3 in **Table S1**).

Table S1. Concentrations (ng/L) and Calculated Odor Activity Values (OAVs) of *cis*-1, and Concentrations (ng/L) of 3-SH and 3-SHA in Commercial Wines Selected for the Study (see Table 1).

wine	*cis*-1	OAV	3-SH	3-SHA	wine	*cis*-1	OAV	3-SH	3-SHA
1	80 ± 1[a]	0.01	1064 ± 47	4.1 ± 0.2	**22**	7 ± 0	0.00	461 ± 1	7.4 ± 0.2
2	460 ± 1	0.06	4523 ± 96	5.4 ± 0.0	**23**	25 ± 0	0.00	1210 ± 10	7.1 ± 0.9
3	131 ± 1	0.02	4470 ± 28	20.2 ± 0.3	**24**	18 ± 1	0.00	940 ± 1	6.6 ± 0.4
4	14 ± 0	0.00	1489 ± 13	10.3 ± 2.1	**25**	15 ± 0	0.00	953 ± 4	11.9 ± 0.4
5	–[b]	–	853 ± 15	7.1 ± 0.3	**26**	–	–	932 ± 15	8.4 ± 0.9
6	50 ± 2	0.01	2420 ± 46	9.5 ± 0.3	**27**	36 ± 1	0.00	1631 ± 75	8.1 ± 0.5
7	407 ± 10	0.06	7923 ± 153[c]	18.1 ± 1.1	**28**	83 ± 3	0.01	2888 ± 50	17.9 ± 1.4
8	150 ± 5	0.02	4948 ± 51	10.2 ± 0.0	**29**	17 ± 1	0.00	1249 ± 8	11.5 ± 0.3
9	62 ± 2	0.01	1156 ± 22	6.2 ± 0.2	**30**	14 ± 0	0.00	682 ± 14	6.3 ± 0.1
10	–	–	1334 ± 1	7.1 ± 0.1	**31**	20 ± 1	0.00	969 ± 9	8.4 ± 0.4
11	–	–	516 ± 9	3.9 ± 0.5	**32**	12 ± 1	0.00	718 ± 65	10.7 ± 4.4
12	33 ± 1	0.01	1464 ± 22	14.2 ± 0.1	**33**	44 ± 3	0.01	825 ± 21	6.0 ± 0.3
13	33 ± 3	0.01	1179 ± 2	7.1 ± 0.4	**34**	–	–	657 ± 1	7.0 ± 1.1
14	40 ± 0	0.01	1132 ± 11	5.3 ± 0.4	**35**	69 ± 1	0.01	3347 ± 1	9.5 ± 0.4
15	7 ± 0	0.00	459 ± 11	5.6 ± 0.2	**36**	15 ± 0	0.00	729 ± 9	4.5 ± 0.3
16	–	–	1596 ± 42	6.4 ± 0.2	**37**	367 ± 5	0.05	1116 ± 37	4.7 ± 0.2
17	–	–	467 ± 2	4.6 ± 0.3	**38**	16 ± 0	0.00	722 ± 1	5.9 ± 0.3
18	54 ± 0	0.01	882 ± 47	5.8 ± 0.2	**39**	22 ± 0	0.00	670 ± 15	5.5 ± 0.1
19	19 ± 1	0.00	791 ± 43	6.8 ± 0.6	**40**	14 ± 0	0.00	1127 ± 5	7.3 ± 0.3
20	9 ± 0	0.01	717 ± 3	6.7 ± 0.2	**41**	14 ± 1	0.00	514 ± 1	4.9 ± 0.3
21	17 ± 0	0.00	654 ± 24	7.0 ± 0.6	**42**	35 ± 2	0.01	1128 ± 6	4.1 ± 0.2

[a] Data presented as the mean ± standard deviation of duplicate analyses. See **Table 1** of the paper for details about the wines. [b]–, not detected (LOD = 2.6 ng/L). [c]Extrapolated, outside the calibration range (0–5000 ng/L).

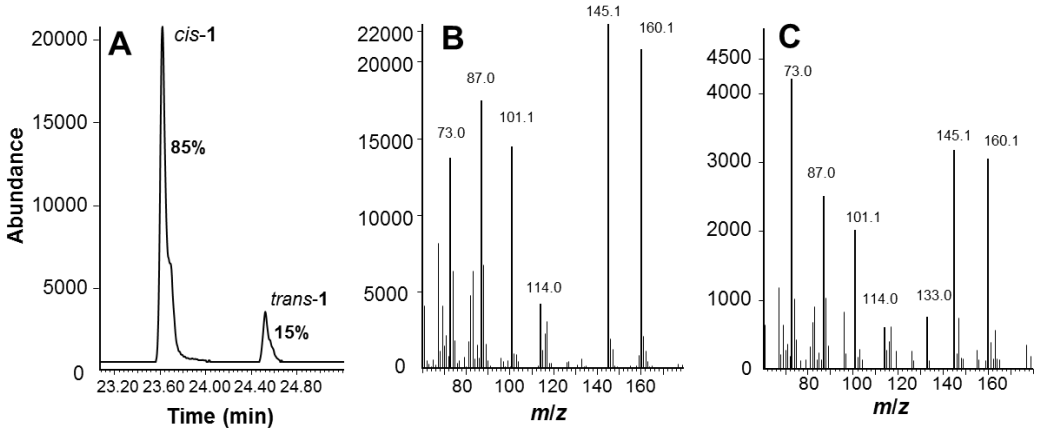


Figure S2. Total ion chromatogram of commercial standard **1** (10 µg/L in water) showing (**A**) separation of *cis*-**1** and *trans*-**1**, and the corresponding mass spectra of (**B**) *cis*-**1** and (**C**) *trans*-**1**. Chromatograms and spectra data obtained using the preliminary GC-MS method.

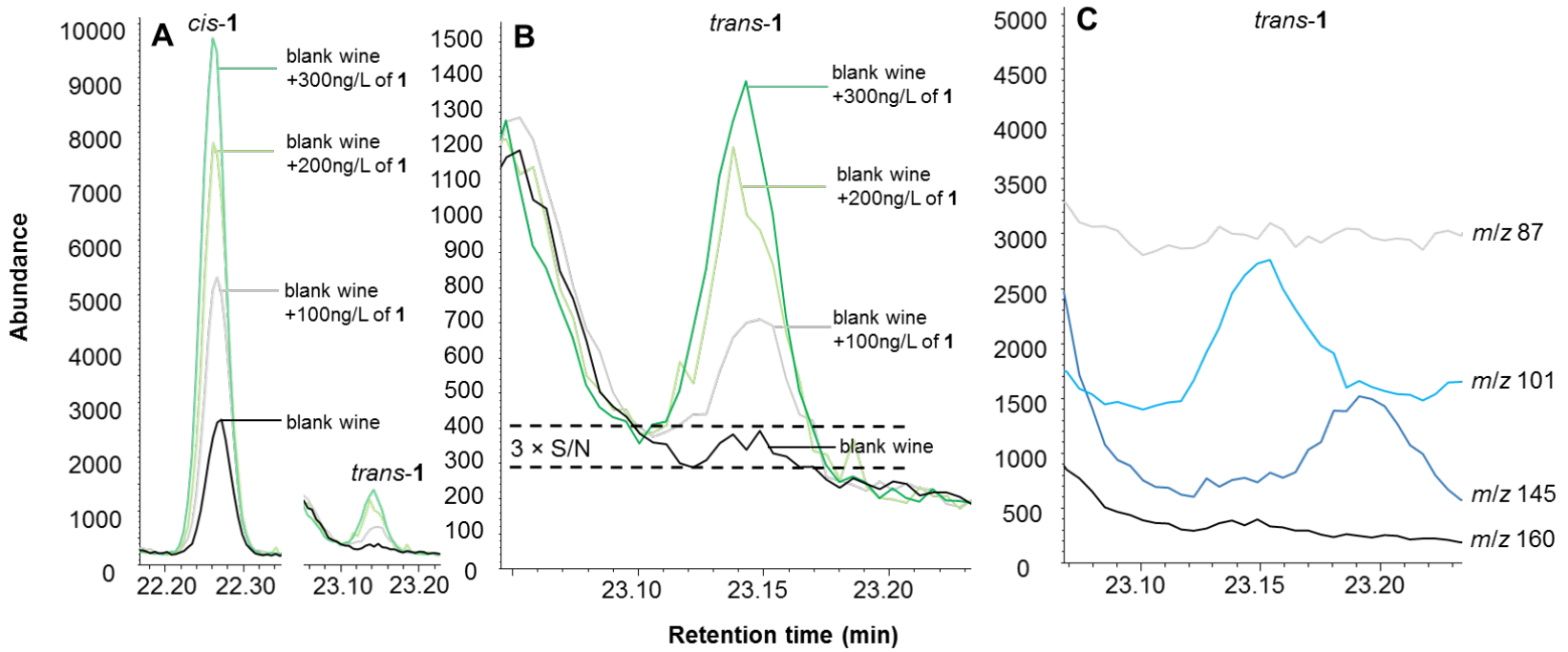
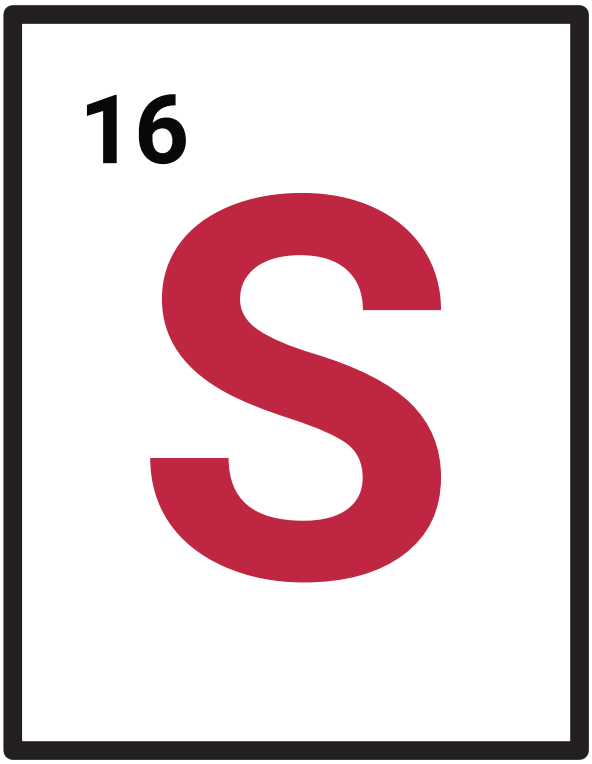


Figure S3. Overlaid selected ion chromatograms (*m/z* 160) showing (**A**) the corresponding peak intensity increase for both *cis*-**1** (same as **Figure 3**) and *trans*-**1** after spiking a commercial wine with increasing amounts of standard **1**, (**B**) an enlargement of chromatograms for *trans*-**1** compared with 3 times the signal-to-noise ratio (S/N) for this wine, and (**C**) overlaid chromatograms of four selected ions of the unspiked wine at the retention time of *trans*-**1**.

Chapter 7

Concluding Remarks & Future Perspectives



EIZE THE DAY

7.1. Conclusions

This thesis has focused on potent, odour-active polyfunctional thiols in wine and applied chemical synthesis, analytical chemistry (SIDA, chemical derivatisation, SPE, HPLC–MS/MS, HS–SPME GC–MS), winemaking trials by an automated robotic fermentation platform, and sensory evaluation to explore:

(1) chirality of polyfunctional thiols,

(2) relationship between thiol and precursor stereochemistry,

(3) impacts of various winemaking parameters on thiols and/or precursors,

(4) potential fate of polyfunctional thiols.

7.1.1. Chiral analysis of 3-SH and 3-SHA in wine by SIDA HPLC–MS/MS

Chiral analysis of polyfunctional thiols 3-SH and 3-SHA in wine has been achieved by a newly developed method. Simple chemical derivatisation of thiols in wine with DTDP and isolation of derivatives by SPE were applied to 3-SH and 3-SHA. Chiral separation of 3-SH and 3-SHA derivatives was evaluated with synthesised authentic derivatives on three polysaccharide chiral stationary phases. An Amylose-1 column afforded baseline resolution of the enantiomers of both 3-SH and 3-SHA derivatives, and was selected for further method development and validation. Deuterium labelled internal standards were utilised in the development of a stable isotope dilution assay, employing HPLC–MS/MS and detection in multiple reaction monitoring (MRM) mode. The method was optimised for major chromatographic parameters and validation encompassed calibration range, linearity, accuracy, precision, sensitivity, and matrix effect. The newly developed method was fast (analysis time: 30 min), accurate (recovery: 90% – 111%; *Z*-score: −1.8–1.9), precise (RSD_r < 8%), and sensitive (LOD: 0.1 ng/L

– 0.7 ng/L), and required a wine matrix (as opposed to a model wine solution) for quantitation purposes, especially for red and rosé wines (matrix effect: −20% – 61%). This new chiral method is by far the most sensitive method proposed for analysis of 3-SH and 3-SHA enantiomers in wine.

This method has been successfully applied to the analysis of the enantiomers of 3-SH and 3-SHA in a set of commercial wines (n = 23) consisting of dry white, red, rosé, and botrytised wine. The results revealed that regardless of the variety, dry wines contained roughly equal amounts of (*S*)-3-SH and (*R*)-3-SH, whereas (*S*)-3-SHA was more abundant than (*R*)-3-SHA in most wines with an average ratio of 60:40. For botrytised wine, both 3-SH and 3-SHA were dominated by (*S*)-enantiomers (above 70%). The reported data were not only the most comprehensive data set published on chiral 3-SH and 3-SHA in wine, but this also led to more research questions around the biochemical drivers for such chirality patterns, particularly with respect to thiol precursor stereochemistry, which was subsequently explored.

7.1.2. Chiral polyfunctional thiols and their conjugated precursors upon winemaking with five *Vitis vinifera* Sauvignon blanc clones

The link between thiol and precursor stereochemistry has been comprehensively evaluated for the first time in a controlled laboratory winemaking trial using a fermentation robot. Sauvignon blanc grapes of five clones from a single vineyard were harvested and analysed for precursor diastereomers (Glut-3-SH, GlyCys-3-SH and Cys-3-SH) by a SIDA HPLC–MS/MS approach. After fermentation, 3-SH and 3-SHA enantiomers in the finished wines were assayed by the newly developed SIDA chiral HPLC–MS/MS method and compared against the concentrations of precursor diastereomers of Glut-3-SH and Cys-3-SH measured in grape juices. The correlation analysis demonstrated no obvious relationship (r = −0.35–0.06) between data sets of precursor diastereomers and thiol

enantiomers, strongly suggesting that other unknown factors may significantly influence the dynamic processes of degradation and/or formation of precursors and thiols. The impacts of grape clone (n = 5), yeast (n = 2), commercial nutrient (n = 1), and commercial enzyme (n = 1) on chirality of 3-SH and 3-SHA were also examined in this winemaking trial. Under identical fermentation condition, clonal variation of 3-SH and 3-SHA enantiomers was seemingly obvious, which indicated the fundamental influences of grape clones, regardless of yeast choices or the addition of winemaking additives. Two tested yeasts produced insignificantly different amounts and ratios of 3-SH and 3-SHA enantiomers in most cases, which suggested that there were minimal yeast influences on the chirality of 3-SH and 3-SHA. The addition of a commercial nutrient prior to yeast inoculation also showed limited impact on production of 3-SH and 3-SHA enantiomers, with no consistent nor obvious trends observed. In contrast, the use of a commercial enzyme significantly enhanced the production of 3-SH and 3-SHA enantiomers, apparently in a clone-dependent manner. Although the exact reasons of such enhancing effects were unclear, the results have demonstrated the potential of enzyme addition for managing thiol production.

7.1.3. Investigation of intraregional variation, grape amino acids, and pre-fermentation freezing on varietal thiols and their precursors for *Vitis vinifera* Sauvignon blanc.

A total of 21 Sauvignon blanc grape parcels from seven commercial vineyards (n = 7) in Adelaide Hills Geographical Indication of South Australia were harvested, processed to juice, and fermented at laboratory-scale under controlled conditions to assess sub-regional variation of thiols (3-SH, 3-SHA, and 4-MSP) and precursors (Glut-3-SH and Cys-3-SH). Thiol precursors in juices and thiols in finished wines were measured by SIDA HPLC–MS/MS methods, with the obtained data showing variation across sub-regions but with no obvious

correlation between precursors and thiols (r = −0.21−−0.38).

Grape amino acids were analysed by HPLC with fluorescence detection and compared with thiol precursors in juices and thiols in the wines. As with thiols and precursors, grape amino acids displayed sub-regional variation. More importantly, correlation analysis revealed, for the first time, moderate to strong correlations between certain amino acids and thiol precursors (e.g., $r \leq -0.73$ for glutamic acid, $r \geq 0.62$ for glycine) but much weaker correlations with free thiols. These novel results indicate the potential interactions between amino acids and thiol metabolism.

In addition, pre-fermentation freezing treatment (1-month storage at −20 °C), applied both on fresh grapes and freshly obtained grape juices from the same grapes, was tested through an identical winemaking trial for its potential impact on thiols and precursors from an aroma enhancement perspective. After pre-fermentation freezing, the frozen grapes showed a significant increase in Glut-3-SH and Cys-3-SH concentrations, being 11–19 and 4–6 fold, respectively, compared to those in fresh grapes. In the wines made from frozen grapes, 3-SH, 3-SHA, and 4-MSP were also significantly increased by around 2–10, 3–7, and 2–8 times, in contrast to wines made from fresh grapes. Such results clearly demonstrated the possibility of significant thiol enhancement through a rather simple freezing treatment.

7.1.4. Identification and quantitative analysis of 2-methyl-4-propyl-1,3-oxathiane in wine

Curiously, after decades of research, knowledge related to the fate of 3-SH in wine is still rather limited. This prompted the search for 3-SH related compounds, leading to a new volatile sulfur compound, 2-methyl-4-propyl-1,3-oxathiane that is reminiscent of passion fruit aroma, being identified and quantitated in wine for the first time.

The presence of this oxathiane in wine was theorised based on the co-existence of 3-SH and acetaldehyde. 2-($^2H_3$)methyl-4-propyl-1,3-oxathiane was synthesised as deuterium labelled internal standard for development of a SIDA GC–MS approach. SPME fibres, GC capillary columns, and matrix effects were evaluated and the optimised method demonstrated good performances in linearity (R^2=0.9997) through the calibration range (0–500 ng/L), repeatability (RSD < 4%), recovery (94–106%), and sensitivity (LOD 2.6 ng/L). The identification of *cis*-2-methyl-4-propyl-1,3-oxathiane in wine was verified by the comparison of LRIs and the mass spectra with authentic 2-methyl-4-propyl-1,3-oxathiane, and with co-injection experiments that involved spiking increasing amounts of authentic 2-methyl-4-propyl-1,3-oxathiane to a commercial wine. Notably, no *trans*-isomer was identified in wines, which can be explained on the basis of formation pathways and relative distribution of the geometric isomers.

Quantitative analysis of *cis*-2-methyl-4-propyl-1,3-oxathiane in a selection of commercial wines revealed that concentrations ranged from undetectable to 460 ng/L and that Sauvignon blanc wines tended to contain greater amounts of the oxathiane than other varieties. The 3-SH concentrations in the same wines were measured by SIDA HPLC–MS/MS and compared against *cis*-2-methyl-4-propyl-1,3-oxathiane data, revealing a strong positive correlation (r = 0.72), which indicates the potential link between 3-SH and the oxathiane. The ODT of this newly identified compound was determined by an untrained panel to be 7.1 µg/L, using a mixture of 15%:85% *cis*-:*trans*-2-methyl-4-propyl-1,3-oxathiane in a neutral dry white wine . Although the threshold was well below the concentrations found in wines, *cis*-2-methyl-4-propyl-1,3-oxathiane could still play a significant role in wine aroma through its possible molecular interaction with the potent odour-active 3-SH.

7.2. Future perspectives

As some of the most potent odour-active odorants that have been identified in wine, certain polyfunctional thiols have been the focus of wine aroma chemists for almost three decades, and will remain an active topic while many important scientific questions around them continue to be resolved.

7.2.1. Thiol analysis

The analysis of polyfunctional thiols in wine has progressively evolved from complicated and laborious methods to more simplified analytical approaches, as systematically reviewed in **Chapter 2**. Already acknowledged for their fundamental role in thiol analysis throughout this thesis, modern sample preparation and analytical techniques will still be expected to make significant contributions for simpler extraction, more efficient separation, and highly sensitive analytical methods, either for targeted or non-targeted analysis.

In terms of specific thiol extraction, new possibilities could be found by developing new extraction protocols using novel extraction materials or new derivatisation reagents. For example, a SPE phase that features Ag^+ (instead of Hg) has been proposed for volatile thiol extraction from beer and hops [1]. This commercially available SPE cartridge contains a selective sorbent for thiols, which essentially leads to good extraction efficiency of thiols in their native forms. Another category of selective extraction material used for volatile analysis is molecularly imprinted polymers (MIP). The potential of molecularly imprinted solid-phase microextraction (MIP–SPME) for volatile analysis has been demonstrated in various samples [2]. Apart from SPME, MIP can also been used with SPE techniques, with some MIP–SPE cartridges having already been commercialised for special analytes [3, 4] and used for food analysis [5].

With the development of MIP design, the application of MIP to thiol extraction, either in free thiol or derivative forms, would be optimistic. Other than using novel extraction materials, testing new chemical reagents for derivatisating thiols in wine is another direction for thiol extraction [6]. For example, newly proposed *N*-(4-(carbazole-9-yl)-phenyl)-*N*-maleimide [7] or 2-bromo-3'-methoxy acetophenone [8] could potentially be used as candidate reagents for thiol derivatisation.

For chromatographic separation of thiols in wine, future trends include separations by novel stationary phases and separation techniques, to achieve better separation efficiency and resolution than conventional chromatographic approaches for thiols (or thiol derivatives). As for the novel stationary phases of interest, a commercially available chiral polysaccharide-based LC column has not only been demonstrated for its resolution ability for the enantiomers of two of the most important polyfunctional thiols (3-SH and 3-SHA), but also displayed the possibility for the simultaneous separation of other important achiral polyfunctional thiols [9]. Another stationary phase that could potentially be useful for thiol analysis is the new generation of superficially porous silica particle columns (SPSPCs), which can typically reduce run times down to a few minutes with ultra-fast HPLC [10, 11]. If adopted for thiol analysis, this could greatly shorten run times of the LC-based chromatographic methods. Besides relying on new stationary phases, exploring new separation techniques can also be considered to improve separation efficiency, such as the use of ultraperformance convergence chromatography (UPC²) for thiol analysis in wine that has achieved speedy separation (7 min run time per sample) [12].

Regarding the trend for detection during thiol analysis, triple quadrupole (QqQ) and high-resolution MS (Q–TOF or Orbitrap) are expected to be more prevalent for both identification and quantitation [6]. Some major analytical advantages of using QqQ and high-resolution MS have been reviewed recently (see **Chapter 2**). Briefly, QqQ, Q–TOF, and Orbitrap MS offer unparalleled ability for screening new volatile thiols based on precursor ion scan mode with diagnostic fragmented ions for QqQ and high resolution measurements for the determination of molecular formulas with Q–TOF and Orbitrap instruments. QqQ in MRM mode (both GC [1] and LC [9]) will likely be applied more frequently for quantitating known volatile thiols.

Analytical chemistry development focusing on volatile thiols is essential and a prerequisite for thiol research. As already reviewed in **Chapter 2**, the knowledge of thiols in wine expands in parallel with the analytical methods developed for polyfunctional thiols. Through developing more sensitive, simpler, greener, more informative analytical methods to be used for thiol evaluation and exploration, crucial understanding of the biological and chemical pathways of these potent polyfunctional thiols has been achieved (such as the results presented in **Chapter 3–6**) and will still be expected to be expanded. Some of the aspects of biological and chemical pathways of polyfunctional thiols are suggested in the following sections.

7.2.2. Thiol chirality, precursors, and management

Chirality of 3-SH and 3-SHA in wine has been examined and discussed in **Chapter 2** and **Chapter 3**. Based on these results, 3-SH enantiomers are almost evenly distributed, in contrast to 3-SHA that is present with the (*S*)-form in excess, which leaves a number of aspects to be resolved regarding stereoselectivity and involvement of chemical and enzymatic reactions. The anticipated outcomes would contribute to a better understanding of chirality of thiols in wine (and thus their influence on sensory properties). For instance, acetylation of 3-SH to form 3-SHA is thought to be catalysed by *Saccharomyces cerevisiae* alcohol-*O*-acyltransferases. This enzymatic process should obviously proceed with a high level of enantioselectivity, so more research is needed to elucidate why this does not appear to be the case. In addition, the evolution of 3-SH and 3-SHA enantiomers throughout alcoholic fermentation could also be studied to understand how and when the resultant ratios arise.

Currently, two major categories of thiol precursors – conjugates with amino acid /small peptides (Glut-3-SH, GluCys-3-SH, CysGly-3-SH, Cys-3-SH, Glut-4-MSP, GluCys-4-MSP, CysGly-4-MSP, Cys-4-MSP) and carbonyl precursor ((*E*)-2-hexenal) – have been identified. A recent study using a set of deuterated tracers to evaluate the conversion rates from conjugated precursors to 3-SH and 4-MSP has once again revealed that the proposed precursors can only partially explain the amounts of 3-SH and 4-MSP in wine, even when residual precursors (precursor availability) were taken into account when calculating the conversion yields [13]. This implies that there are other compounds potentially acting as precursors and that new pathways relating to thiol production remain to be discovered. As such, the use of alternative isotope labelled tracers of candidate precursors (^{13}C and ^{34}S) could be employed in future to tackle this question.

The identification of new precursors remains elusive and further research on some of the already-known precursors is still warranted. For instance, the recently identified γGluCys-3-SH is present as diastereomers in the ratio of 70:30 in grapes [14], but their accumulation as well as other precursors such as CysGly-3-SH during grape ripening, evolution during fermentation, relationship to 3-SH enantiomers, and impacts of various viticultural (cultivar, clone, viticultural management, etc.) and oenological practices are still unknown. Moreover, research could be directed to the investigation of the potential interactions and relation between grape metabolites and thiols/precursors. Addressed only in a few recent publications [15–17], including **Chapter 5** [18] in this thesis, it is evident that the metabolism of thiols and precursors is very likely linked to various grape metabolites and their biological pathways. Further research focusing on understanding the link between grape metabolites and thiols/precursors will create new knowledge, on which new approaches for thiol management could be proposed.

In recent years, some novel strategies for enhancing thiol production during winemaking have been suggested and tested, such as the use of grape tannins [19], addition of commercial winemaking additives, clone selection (**Chapter 3**), and pre-fermentation freezing of grape bunches (**Chapter 5**). Some promising results have been offered for thiol enhancement, but improvements can still be made with future research focusing on optimising the best conditions for specific treatments, such as pilot trials at larger scale with more varieties, and testing more commercial additives, alone or in combination. Additionally, novel viticultural or winemaking practices (e.g., the use of non-*Saccharomyces* yeast strains) could also be explored to offer other alternatives.

7.2.3. Fate of polyfunctional thiols

Other than the discovery of thiol precursors, research should also focus on better understanding the fate of polyfunctional thiols (which could assist in calculating a proper mass balance). With *cis*-2-methyl-4-propyl-1,3-oxathiane being identified (notably, this is also a chiral molecule, in **Chapter 6**) and suggested to be a new volatile sulfur compound that is closely associated with 3-SH, future research could be conducted to investigate the chemical, sensorial, and microbiological impacts on 2-methyl-4-propyl-1,3-oxathiane. Studies involving its occurrence, chirality, stability, biogenesis, and sensory contributions remain unknown and the endeavour to discover additional new volatiles to address the fate of polyfunctional thiols would be strongly encouraged.

7.3. References

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List of Abbreviations

2-MFT	2-methyl-3-furanthiol
3-SH	3-sulfanylhexan-1-ol
3-SHA	3-sulfanylhexyl acetate
4-MSP	4-methyl-4-suylfanylpentan-2-one
AAT	alcohol acetyl transferase
AC	affinity chromatography
ACMPC	amylose 3-chloro-5-methylphenylcarbamates
ADH	alcohol dehydrogenase
ADMPC	amylose 3,5-dimethylphenylcarbamates
ADY	active dry yeast
AED	atomic emission detector
AEDA	aroma extract dilution analysis
AENM	acridone-10-ethyl-*N*-maleimide
ANOVA	analysis of variance
BET	best-estimate threshold
BMT	benzenemethanethiol
BQB	ω-bromoacetonylquinolinium bromide
BtCl	1-chlorobenzotriazole
BtH	1H-benzotriazole
CI	chemical ionisation
CRM	consecutive reaction monitoring
CSP	chiral stationary phase
Cys-3-SH	3-*S*-cysteinylhexan-1-ol

List of Abbreviations

CysGly-3-SH	3-*S*-cysteinylglycinehexan-1-ol
DAP	diammonium phosphate
DBU	1,8-diazabicyclo[5.4.0]undec-7-ene
DI	decadal increase
DPIS	double precursor ion scan
DTDP	4,4'-dithiodipyridine
DVB/CAR/PDMS	divinylbenzene/carboxen/polydimethylsiloxane
ebselen	2-phenyl-1,2-benzisoselenazol-3(2H)-one
ECD	electron-capture detector
EDTA	ethylenediaminetetraacetic acid
EDTA 2Na	ethylenediaminetetraacetic acid disodium salt
EI	electron ionisation
ESI	electrospray ionisation
EtOH	ethanol
ETP	ethyl propiolate
FD	flavour dilution
FPD	flame photometric detector
FPD	flame ionisation detector
FT	2-furfurylthiol
FTMS	Fourier transform mass spectrometry
GABA	γ-aminobutyric acid
GC	gas chromatography
GC–O	gas chromatography with olfactometry

List of Abbreviations

GC×GC	two-dimensional GC
GGT	glutamyltranspeptidase
GI	Geographical Indication
Glu	glutamic acid
GluCys-3-SH	*S*-3-(hexan-1-ol)-γ-glutamyl-cysteine
Glut-3-SH	S-3-(hexan-1-ol)-glutathione
Glut-3-SH	3-*S*-glutathionylhexan-1-ol
Glut-3-SH-al	*S*-3-(hexanal)-glutathione
Glut-3-SH-SO_3	*S*-3-(1-hydroxyhexane-1-sulfonate)-glutathione
Glut-4-MSP	4-*S*-glutathionyl-4-methylpentan-2-one
Gly	glycine
GP–MSE	gas purge microsyringe extraction
GSH	glutathione
HorRat	Horwitz ratio
HPL	hydroperoxide lyase
HPLC	high-performance liquid chromatography
HR–LSIMS	high resolution liquid secondary ion mass spectrometry
HS–SPME	Headspace solid-phase microextraction
IS	internal standard
ITMS	Ion trap mass spectrometry
LOD	limit of detection
LOQ	limit of quantitation
LOX	lipoxygenase

List of Abbreviations

LRI	linear retention index
MeCN	acetonitrile
MeOH	methanol
MIP	molecularly imprinted polymers
MRM	multiple reaction monitoring
MS	mass spectrometry
MS/MS	tandem mass spectrometry
MW	model wine
NCR	nitrogen catabolite repression
NMR	nuclear magnetic resonance
O	olfactometry
OAV	odour activity value
ODT	odour detection threshold
OPA	*o*-phthaldialdehyde
P+T	purge and trap
PA	polyacrylate
PC	principal component
PCA	principal component analysis
PDMS	polydimethylsiloxane
PFBBr	2,3,4,5,6-pentafluorobenzyl bromide
PFF	pre-fermentation freezing
PFPB	pulsed flame photometric detector
*p*HMB	*p*-hydroxymercuribenzoate

List of Abbreviations

PIPD	1-(4-(1*H*-phenanthro[9,10-d]imidazol-2-yl)phenyl)-1*H*-pyrrole-5-dione
Pro	Proline
PTI	purge and trap injector
Q	single quadrupole
QC	quality control
QqQ	triple quadrupole
RI	retention index
RP	reversed-phase
RSD	relative standard deviation
SAFE	solvent-assisted flavour evaporation
SBSE	stir bar sorptive extraction
SCD	synthetic complete dextrose
SCD	sulfur chemiluminescence detector
SD	standard deviation
SIDA	stable isotope dilution assay
SIM	selected ion monitoring
SPE	solid phase extraction
SPSPC	superficially porous silica particle column
SRM	selected reaction monitoring
TA	titratable acidity
TDU	thermal desorption unit
TOF	time-of-flight
TSS	total soluble solids

List of Abbreviations

UHPLC	ultra high-performance liquid chromatography
UPC^2	ultraperformance convergence chromatography
VSC	volatile sulfur compound
YAN	yeast assimilable nitrogen
YPD	yeast extract-peptone-dextrose
YPS	yeast peptone sucrose

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