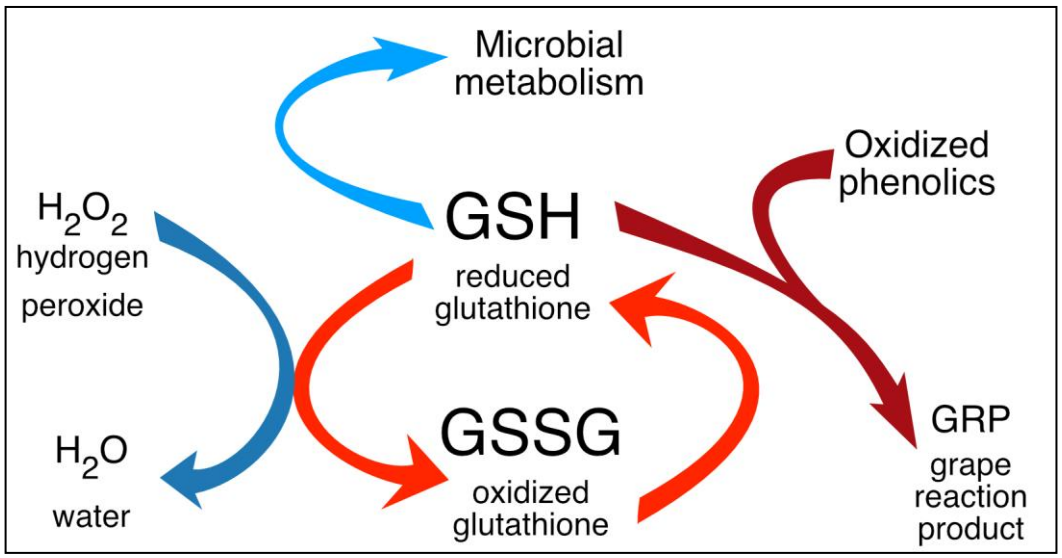
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Wine Australia

The Australian Wine Research Institute

Winemaking interventions to modulate glutathione status



FINAL REPORT TO WINE AUSTRALIA

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Table of Contents

1. Abstract 1

2. Executive summary 1

3. Background: 3

4. Project aims and performance targets 5

5. Methods 6

5.1. Conduct of small-scale fermentations for aromatic profiling 6

5.2. Conduct of pilot-scale winemaking experiments 6

5.2.1. Trial 1 – Effects of pre- and post-inoculation GSH additions in Riesling and Chardonnay winemaking 7

5.2.2. Trial 2 – Pre-inoculation GSH addition to Chardonnay juice 7

5.3. Analysis of wine chemical composition 8

5.3.1. Analysis of volatile fermentation products 8

5.3.2. Analysis of volatile sulfur compounds. 8

5.3.3. Analysis of organic acids 9

5.4. Sensory analysis 9

5.4.1. Trial 1 9

5.4.2. Trial 2 9

6. Results/Discussion 11

6.1. Development of an assay for reduced (GSH) and oxidised glutathione (GSSG) 11

6.2. How does nitrogen availability affect GSH consumption and H_2S production? 12

6.3. Is there an interaction between grape processing and GSH addition? 15

6.4. Vintage trial 1 – The impact of high GSH concentrations during fermentation on Chardonnay and Riesling wine 17

6.4.1. How does the concentration of GSH evolve over the course of fermentation when it is added during processing or following inoculation? 19

6.4.2. Effect of early GSH addition on the chemical composition of Chardonnay and Riesling wine . 24

6.4.3. Effects of early additions of GSH on sensory attributes of Chardonnay and Riesling wine. 25

6.5. Vintage trial 2 – The impact of small (20 mg/L) GSH additions prior to fermentation on Chardonnay wine 29

6.5.1. Effect of early GSH addition on the chemical composition of Chardonnay sparkling base and table wine 30

6.5.2. Effects of post-press additions of GSH on sensory attributes of Chardonnay wine. 31
6.6. Production of isotopically labelled GSH through synthesis 33
7. Outcome/Conclusion 37
7.1. Performance against planned outputs 37
7.1.1. Determine whether GSH addition to juice increases GSH concentration in wine and assess whether GSH concentration and its anti-oxidative efficacy are predictable 37
7.1.2. Examine winemaking factors (e.g. timing of addition, amino acids, oxygen, SO_2) that may influence the relationship between juice and wine GSH concentrations 37
7.1.3. Identify the extent to which GSH concentration relates to the concentration of GSH and cysteine conjugates of varietal thiols in juice and free thiols in wine, and determine if this can be manipulated to enhance wine typicity 38
7.1.4. Determine the effects of glutathione addition through sensory evaluation 38
7.1.5. Track the fate of added GSH using chemical and genetic tools 38
7.2. Could changing the methodology/technology have improved the outcome? 39
7.3. Practical implications 39
7.4. Benefits to industry 39
8. Recommendations 40
9. Appendix 1: Communication: 41
Communication of the outcomes 41
Articles written during project 41
10. Appendix 2: Intellectual Property 41
11. Appendix 3: References 42
12. Appendix 4: Staff 56
13. Appendix 5: Additional compositional and sensory data 47
Effect of GSH addition on the chemical composition of Chardonnay and Riesling wine. 47
Effect of GSH addition on the sensory attributes of Chardonnay and Riesling wine. 49
Effect of GSH addition on the chemical composition of Chardonnay sparkling base and Chardonnay table wine 52
14. Appendix 6: Budget reconciliation 55

1. Abstract

Glutathione (GSH) is a natural peptide and grape-derived antioxidant that has been proposed as an alternative to sulfur dioxide in wine. This project focused on the addition of pure GSH prior to or during fermentation. Its consumption during wine production was quantified and the impact of process variables such as grape juice nitrogen concentration and the degree of protection from oxygen on GSH concentration was assessed in both laboratory- and pilot-scale trials. It was demonstrated that low concentrations of GSH have little impact when added prior to fermentation and that higher addition rates caused undesirable sensory outcomes.

2. Executive summary

Sulfur dioxide (SO_2) is the antioxidant most commonly used to protect wine and preserve quality; however, more benign alternatives are continuously being sought. Reduced glutathione (GSH) is a naturally occurring antioxidant present in many organisms and as result it has been investigated for its potential to augment the use of SO_2 for the preservation of wine freshness and aroma intensity.

GSH is naturally present in both grapes and yeast and its concentration in grape juice is dependent on the conditions under which grape processing is conducted. Aside from grapes, other potential sources of GSH in wine include yeast-derived products, some of which are marketed as GSH-enriched inactive dried yeast preparations.

The work presented in this report will focus on the application of pure GSH as outlined in an International Organisation of Vine and Wine (OIV) resolution passed in 2015 that recommended GSH addition as an option during grape processing (OIV-OENO 445-2015). The addition of GSH to wine after alcoholic fermentation, as outlined in a second OIV resolution (OIV-OENO 446-2015), was not considered as part of this work and neither were sources of GSH from the grape berry or yeast-derived additives.

For this work to proceed, technical developments to methods for the quantification of GSH in grape were required. While conventional methods for quantitating reduced (GSH) and oxidised (GSSG) glutathione in human, animal and plant material existed, quantifying GSH to the low concentrations for which these methods were developed was unnecessary when determining the concentration of GSH in juice and fermenting wines to which GSH is added in the mg/L range. Therefore, a method was developed that enabled the determination of GSH and GSSG concentrations over a range from 2 to 300 mg/L in juice and fermenting wines using a simple, rapid and robust approach that required minimal sample preparation. This method was used in all subsequent fermentation-based monitoring of GSH.

A fundamental question arising from OIV-OENO 445-2015 was whether a level of juice nitrogen could be defined that prevented the metabolic consumption of GSH by yeast. To address this question, GSH was added to juices and defined media with increasing yeast assimilable nitrogen (YAN) concentrations, and the consumption of GSH was monitored throughout fermentation. Yeast strains that varied in their nitrogen demand were also evaluated. It was demonstrated that although increasing the YAN status of the juice did indeed decrease the consumption of GSH during fermentation, the loss of GSH could not be eliminated entirely. Even at a low initial GSH concentration and high initial YAN concentrations, some consumption of GSH was evident. This work raised some questions about whether the criteria for GSH usage as outlined in OIV-OENO 445-2015 could reasonably be met.

This project also sought to understand the impact of GSH addition during alternative processing regimes. Inert pressing of grapes has previously been shown to preserve both grape-derived GSH and much of the phenolic material that is commonly oxidised during more routine pressing of white grapes. Adding GSH to such juice could result in initial GSH concentrations that substantially exceed OIV guidelines. In such a scenario it was of interest to determine the likely consequences of this combination of processing and GSH addition steps. Inert pressing combined with GSH addition led to substantial formation of both 4-methyl-4-sulfanylpentan-2-one (4-MSP) and grape reaction product (GRP) in the finished wines. The concentrations of these were decreased in oxidatively produced wines when GSH was added. In the absence of GSH addition, little of either compound was detected. This demonstrated the capacity for GSH additions to interact with processing variables other than YAN to alter the wine composition.

Two pilot-scale experiments evaluated the impact of pre-ferment GSH addition in authentic winemaking circumstances. In one trial, the wines were bottled using a high and a low SO_2 regime to determine whether GSH could be used to supplement SO_2. Both high (250 mg/L) and OIV recommended (20 mg/L) additions of GSH, prior to or during fermentation, were trialled. High GSH addition trials were bottled with residual GSH concentrations of 50 mg/L indicating a potential consumption of 200 mg/L of GSH from all sources during wine production. This residual GSH concentration was insufficient to limit the loss of SO_2 during wine ageing, however, and did not limit the appearance of oxidative characteristics which were evident in the sensory evaluation of those wines. Overall, there were no compositional or sensorial differences between wines that had GSH added prior to or after inoculation. Wines with added GSH were consistently described as ‘cat pee’, ‘flinty’ or ‘baby sick’. As the wines aged the concentration of hydrogen sulfide (H_2S) increased dramatically. If low concentrations of GSH were added immediately post-pressing there were only subtle differences in the sensory characteristics of the wines, irrespective of the yeast strain used. Wines to which low concentrations of GSH had been added after pressing tended to be associated

with ‘apple’, ‘pear’ and ‘stone fruit’ sensory descriptors, but there was considerable overlap among all wines within the low GSH study, in which yeast strain choice appeared to have an equal impact.

The key benefit to industry of this work is the provision of evidence-based advice about GSH as an additive and the risks and benefits associated with its use. This work was undertaken as a collaborative project between the AWRI and the University of Adelaide.

3. Background

Although SO_2 is the antioxidant most commonly used to preserve wine quality, more benign alternatives are continuously being sought. Reduced glutathione (GSH) is a naturally occurring antioxidant present in organisms as diverse as bacteria, fungi, plants and animals, and its (bio)chemistry has long been the object of detailed scientific research (Kosower et al. 1978). As a result, it has also been investigated as a means to augment the use of SO_2 for the preservation of wine freshness and aroma intensity. GSH is a peptide that consists of three amino acids – glycine, cysteine and glutamate – with the reactive sulfur moiety on the side chain of cysteine key to its biological role and oenological interest. Within the cell, glutathione exists in two forms, predominantly as a reduced species (GSH) with the minority existing as oxidised dimer (GSSG). To some degree these two forms are in equilibrium and provide redox buffering to the intracellular environment (Sies 1999, Schafer et al. 2001). With the majority of living organisms being dependent on the presence of oxygen for survival, the formation of reactive oxygen species (ROS) is inevitable. These reduced forms of oxygen, predominantly peroxide, form as a direct result of respiration. The major role of GSH within the cell is the neutralisation and removal of ROS (i.e. it acts as an antioxidant). These same ROS are also relevant in the ageing of wine; hence the oenological interest in GSH.

Being a ubiquitous metabolite, GSH is present in the fundamental components of wine: it is in both grapes and yeast (Kritzinger, et al. 2013a). Its concentration in grape juice is dependent on grape processing conditions. During the early harvesting and processing phases of winemaking, oxygen reacts with oxidase enzymes and phenolic compounds of the berry (Day et al. 2015). This leads to the loss of GSH from juice or must, as it conjugates with quinones formed during oxidation, resulting in the formation of GRP (Singleton et al. 1985). High concentrations of SO_2 and/or protection from oxygen are required to prevent this type of reaction. Therefore, the primary factor influencing the concentration of GSH in must is exposure to oxygen during berry rupture (du Toit et al. 2007, Motta et al. 2014). Protection from air and oxidation, using an inert press for example, will generate juice with higher GSH and polyphenol concentrations. GSH in excess of 50 mg/L has been reported in juice following inert preparation (Pons et al. 2015), with some proportion of that being present at the conclusion of fermentation.

Aside from grapes, other potential sources of GSH include yeast-derived products, some of which are marketed as GSH-enriched inactive dried yeast (IDY) preparations. Compared to grape processing interventions that can preserve GSH, however, the GSH contribution of these additives to juice or wine is small, increasing GSH concentrations by between 1 and 3.5 mg/L when used at recommended addition rates (Kritzinger, et al. 2013b, Andújar-Ortiz et al. 2014, Rodriguez-Bencomo et al. 2014). Yeasts can also contribute GSH to ferments and finished wines through synthesis and export of the molecule during fermentation. Glutathione is the main sulfur compound in yeast and is produced and metabolised in ways that depend on the yeast's exposure to a variety of stresses (Penninckx 2000).

There are several resolutions relating to the use of GSH or GSH-containing products that have been ratified recently by the OIV. Most pertinent to the work being described in this report are the following:

1. OIV-OENO 445-2015
    - It is advised to add the glutathione while obtaining the musts or at the start of alcoholic fermentation, ensuring that, prior to and during alcoholic fermentation, the assimilable nitrogen level is sufficient to avoid the metabolism of glutathione by the yeast
    - the dose used should not exceed 20 mg/L

2. OIV-OENO 446-2015
    - The addition of glutathione on storage and/or packaging, including the bottling of wine, is recommended
    - the dose used should not exceed 20 mg/L
    - the glutathione must be in a reduced form and comply with the prescriptions of the International Oenological Codex

The objective of these resolutions has been to limit the intensity of oxidation phenomena in musts, thanks to the ability of GSH to trap quinones and decrease their oxidative activity and to protect aroma compounds present in wines (and particularly those of the thiol family) from oxidation, thanks to the antioxidant properties of the product.

The work undertaken as part of this project deals specifically with elements of proposal OIV-OENO 445-2015, namely addition of GSH while obtaining musts or at the start of alcoholic fermentation.

Oxidised and 'reduced' aroma faults are two of the most common wine aroma off-flavours that result in decreased shelf life, and these faults are directly influenced by the amount of oxygen a wine is exposed to during bottle ageing (Ugliano 2013). Depending on a wine's composition and the concentration of antioxidants such as SO_2 and GSH, trace amounts of short-chain unsaturated aldehydes such as methional and phenylacetaldehyde (among others) may be produced, thereby imparting aromas described as 'sherry', 'honey', and 'bruised apple', and other aromas associated with oxidation spoilage (Mayr et al. 2015). Increased oxygen exposure can also result in the loss of tropical and varietal aroma compounds, particularly certain polyfunctional thiols [3-sulfanylhexan-1-ol (3-SH), 3-sulfanylhexyl acetate (3-SHA), and 4-MSP] (Lopes et al. 2009). On the other hand, and

depending on wine composition, ‘reductive’ characters may develop during bottle ageing in wines that have been completely protected from oxygen during storage (Ugliano et al. 2011). The compounds responsible for these characters are mainly H_2S and methanethiol (MeSH), which impart aromas of ‘rotten egg’, ‘sewage’, ‘burnt rubber’, and ‘putrefaction’ (Siebert et al. 2010).

The chemistry of wine oxidation has been comprehensively described (Waterhouse et al. 2006). As with juice, phenolic compounds in wine are the primary targets of oxidation and the mediators of metal ion regeneration, fuelling the catalytic cascade of oxidation. The oxygen free radicals generated in this cascade have been shown to be capable of reacting with other susceptible elements of wine, such as potent polyfunctional thiols (Nikolantonaki et al. 2010). Careful management of total package oxygen (TPO) during bottling and attention to closure selection can only mitigate the effect for a limited time. As in the formation of GRP, GSH is capable of acting as a sacrificial nucleophile to preserve polyfunctional thiols during wine ageing (Nikolantonaki et al. 2014). For these reasons, GSH has been investigated as an alternative antioxidant with the potential to act synergistically with SO_2 such that the use of SO_2 could be decreased or eliminated altogether (Badea et al. 2015, Comuzzo et al. 2015).

The concentration of GSH can be manipulated through various winemaking practices, as well as direct or indirect addition. While GSH may contribute to production of quality wine, it has an ill-defined effect on expression of terroir and varietal character. Much of the work associated with GSH manipulation has targeted Sauvignon Blanc, aiming to preserve varietal thiols and colour in bottled wine. Thiols also contribute to the character of other varieties; however, the effect of GSH addition on the expression of varietal characters in wines other than Sauvignon Blanc is not well understood.

Addition of GSH at different stages of winemaking has potentially profound implications for the formation of compounds that contribute (positively or negatively) to wine sensory properties. It is not clear whether GSH applications as outlined by the OIV could be implemented solely for a beneficial impact on wine quality. Importantly, a scientific opinion by the European Food Safety Authority (EFSA) of GSH as food additive has not been made public to date and GSH has not been approved as an additive for winemaking by Food Standards Australia New Zealand.

4. Project aims and performance targets

This project aimed to extend understanding about the effects of GSH additions in juices and wines in varieties other than Sauvignon Blanc. Protective juice treatments, direct addition of pure GSH and microbial consumption of GSH were considered. Pilot-scale winemaking trials were used to assess the sensory and chemical impact of GSH treatments. This work elucidated whether GSH in combination with other factors can be used during grape processing to manipulate or preserve grape typicity, regional characters and wine quality.

Furthermore, this project aimed to determine the scope and limits of usefulness of GSH additions and whether or not there are off-target effects related to its use. Specifically, the objectives were to:

- Determine whether GSH addition to juice increases GSH concentration in wine and assess whether GSH concentration and its anti-oxidative efficacy are predictable
- Examine winemaking factors (e.g., timing of addition, amino acids, oxygen, SO_2) that may influence the relationship between juice and wine GSH concentrations
- Identify the extent to which GSH concentration relates to the concentration of GSH conjugates as well as cysteine conjugates of varietal thiols in juice and free thiols in wine, and determine if this can be manipulated to enhance wine typicity
- Determine the effects of GSH modulation through sensory evaluation
- Track the fate of added GSH using chemical and genetic tools.

In addressing these questions, the intention of the project was to develop foundational information that can inform the use of GSH in an environment where the regulated use of this additive may change and where practitioners may seek to augment or replace SO_2 as a protectant against oxidation.

5. Methods

5.1. Conduct of small-scale fermentations for aromatic profiling

Small-scale fermentations were conducted in 100 mL vessels with a sample port and directional-flow check valve to release CO_2. The vessels were stirred at 350 rpm and incubated at 18°C. All treatments were performed in triplicate. Grape juice was obtained from freshly pressed grapes and either used immediately or stored frozen at –20°C. Yeast growth was monitored by absorbance at 600 nm and fermentation progress was monitored by determining sugar concentration using an enzymatic assay as described by Schmidt et al. (2013).

5.2. Conduct of pilot-scale winemaking experiments

In parallel to small-scale laboratory fermentations, pilot-scale trials were performed across two vintages (Trial 1 and Trial 2) to evaluate the sensory impact of GSH addition on Chardonnay and Riesling wine. Wines produced in year 1 of the project were evaluated over two years to determine the effects of storage on wines with high initial concentrations of GSH. A standardised winemaking procedure, described in detail below, was used for all trials.

5.2.1. Trial 1 – Effects of pre- and post-inoculation GSH additions in Riesling and Chardonnay winemaking

Chardonnay and Riesling juice sourced from a commercial supplier was enzyme treated (Laffort Lafazym 600XL) and cold settled for 24 hours. Settled juice was racked to one of twelve 50 L stainless steel fermentation vessels and brought to 15°C. An inoculum of rehydrated active dried yeast (Laffort Zymaflore X5) was added at a rate of 25 g/hL. Measurements of total soluble solids (TSS) and temperature were recorded daily using a DMA35 hand-held electronic densitometer (Paar, Austria). Pre-inoculation addition of pure reduced GSH (Sigma-Aldrich G4251) occurred following racking of clarified must to the fermentation vessels. Post-fermentation addition of GSH occurred 24 hours after yeast inoculation. In both cases GSH was added at 250 mg/L. After completion of alcoholic fermentation, a standard addition of 40 mg/L SO_2 was made to the wines, which were settled off gross lees and racked to 18 L stainless steel kegs for storage until bottling. Prior to bottling, wines were adjusted to 0.6 or 0.8 mg/L of molecular SO_2. The entire trial consisted of 18 ferments bottled at two SO_2 concentrations to give 36 wines. A schematic of the trial is given in Figure 1.

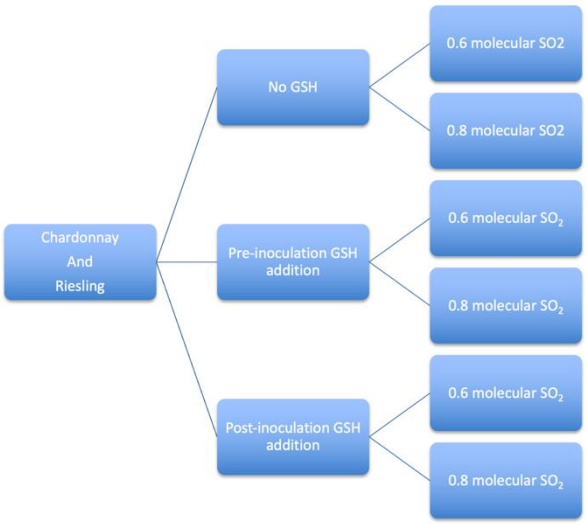


Figure 1. Schematic of experimental design for pilot-scale Trial 1.

5.2.2. Trial 2 – Pre-inoculation GSH addition to Chardonnay juice

Hand-picked Chardonnay grapes were sourced from the Adelaide Hills, cooled overnight at 4°C and pressed into a single tank. Post-press GSH concentrations were not detectable by HPLC. The juice was mixed and split into four vessels and 20 mg/L GSH was added to two of

them. Enzyme was added after GSH addition (Laffort Lafazym 600XL) and cold settled for 24 hours. Settled juice was racked into stainless steel fermentation vessels and brought to 15°C. Rehydrated active dried yeast (Laffort Zymaflore X5 or Laffort Zymaflore VL3) was added at a rate of 25 g/hL. Measurements of TSS and temperature were recorded daily using a DMA35 hand-held electronic densitometer (Paar, Austria). After completion of alcoholic fermentation, a standard addition of 40 mg/L SO_2 was made to the wines, which were prepared for bottling as described above. Prior to bottling, wines were adjusted to 0.8 mg/L of molecular SO_2. The entire trial consisted of 24 ferments. A schematic of the trial is given in Figure 2.

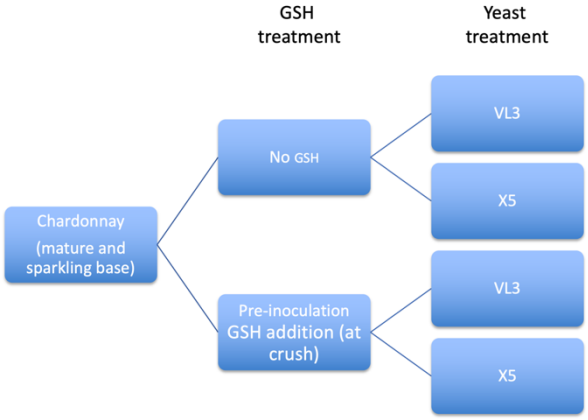


Figure 2. Schematic of experimental design for pilot-scale Trial 2.

5.3. Analysis of wine chemical composition

5.3.1. *Analysis of volatile fermentation products*.

Volatile fermentation products of the finished wines were determined using large volume, stable isotope dilution analysis headspace–GC-MS by Metabolomics Australia's node at the AWRI, as adapted from Siebert et al. (2005) and as described by Holt et al. (2013). Between 16 and 18 fermentation products were analysed with this method.

5.3.2. Analysis of volatile sulfur compounds.

Volatile sulfur compounds that contribute to sulfidic off-aromas often referred to as 'reduced' were analysed directly after primary alcoholic fermentation and then subsequently after bottling. They were determined by gas chromatography-sulfur chemiluminescence detection (GC-SCD) as described by Bekker et al. (2016). Polyfunctional thiols were measured using HPLC-MS/MS according to Capone et al. (2015) from samples taken directly into chilled glass vials and stored until analysis.

5.3.3. Analysis of organic acids

Concentrations of glycerol, acetate, ethanol and organic acids were determined as described by Schmidt et al (2011).

5.4. Sensory analysis

5.4.1. Trial 1

Wines were assessed in a preliminary one-hour session by a group of experienced judges. After independently assessing the wines blind with free choice comments and ratings for intensity of off-flavours, the wines were discussed. As a result of this session, it was determined that there was some within-treatment variation, but large between-treatment sensory differences. All samples were accepted for quantitative sensory descriptive analysis.

Samples were presented to panellists (one male, eight females, average age 53 years, SD = 6.8) in 30 mL aliquots in 3-digit-coded, covered, ISO standard wine glasses at 22 – 24°C, in isolated booths under daylight-type fluorescent lighting. Randomised presentation order was followed except in the practice sessions where there was a constant presentation order. All samples were expectorated. In practice booth sessions, the assessors were presented with four trays of four wines per tray. In the first formal booth sessions of two hours duration, the assessors were presented with four trays of four wines per tray. On the final day of assessment, the panel was presented with two trays of four wines per tray. The assessors were forced to have a 60-second rest between samples and a ten-minute rest between trays. During the ten-minute break assessors were requested to leave the booths. Formal evaluation was completed in five sessions.

The thirty-six wines from Trial 1, including each of the fermentation triplicates, were presented to assessors twice, in a modified Williams Latin Square incomplete random block design generated by Fizz sensory acquisition software (version 2.51, Biosystemes, Couternon, France). A different bottle was used for each of the two presentation replicates.

5.4.2. Trial 2

The projective mapping sensory method of Global Napping (GN) was used to holistically evaluate the treatments and replicate wines based on their perceived similarities and differences, with each wine type evaluated separately. Ultra-Flash Profiling (UFP) was also employed to provide indicative sensory descriptors of the sample set and groupings (Pagès 2005).

A panel of 12 assessors (11 females, 1 male) with an average age of 52 years (SD = 7.2) was convened to evaluate the Chardonnay wine. All panellists were part of the AWRI's trained

descriptive analysis panel and had extensive experience in wine quantitative sensory descriptive analysis (QSDA) and had completed GN/UFP tasks previously.

Prior to the sessions, a familiarisation exercise was conducted and the preformulated attribute list was discussed. The terms offered for the UFP step were primarily based on wine descriptive terminology introduced by Noble et al. (1987) and by paying particular attention to including common terms used in the expert bench tasting and descriptors used in previous Chardonnay sensory QSDA studies. The attribute list was initially discussed by the panellists, who were able to add any additional attributes if desired during the evaluations. The finalised list had 18 attributes (Table 1).

Table 1. Sensory attributes used in the ultra-flash profiling step following Napping

Aroma and flavour	**Palate and mouth-feel**
Citrus	Acidity
Tropical	Viscosity
Stone Fruit	Hotness
Apple/Pear	Bitterness
Confection	Astringency
Flint	Sweetness
Floral	
Grassy	
Sweaty	
Cheesy	
Drain	
Pungent	

For finalising the UFP data, two sensory analysts with wine expertise inspected and consolidated perceptually similar attributes upon discussion with judges, and taking into account known attribute correlations. Attributes describing the entire wine set were generally eliminated from the data, while attributes discriminating between wines were retained. Attributes with a low frequency of use, used only by one panellist and/or deemed not important to the sample set, were discussed by the analysts before being removed. This data processing helped to improve the clarity of the visualisation of the consensus maps produced, with the resulting maps highlighting only the attributes that discriminated the wines.

6. Results/Discussion

6.1. Development of an assay for reduced (GSH) and oxidised glutathione (GSSG)

The conventional methods for quantitating GSH and GSSG in human, animal and plant material consisting of tissue, plasma, and liquid matrices include high-performance liquid chromatography (HPLC) coupled to ultraviolet (UV), fluorometric (Neuschwander-Tetri et al. 1989, Toyo'oka et al. 1989) and mass spectrometric detectors (Mattivi et al. 2012). Enzymatic assays have also been used (Adams et al. 1993). The use of liquid chromatography–tandem mass spectrometry (LC-MS/MS), with its enhanced sensitivity, is preferred for the analysis of GSH and GSSG in blood and tissue samples, where the requirements for GSH and GSSG quantification can be as low as a few ng/L (New et al. 2008). The technique of LC-MS/MS has also been widely implemented in wine and juice samples (du Toit et al. 2007, Coetzee et al. 2013). Methods making use of mass spectrometry as a detector have the added benefit of increased sensitivity as well as the ability to identify unknowns and GSH reaction products. However, quantifying GSH to such low concentrations is not required when determining the concentration of GSH in juice and fermenting wines, nor when confirming the 20 mg/L of added GSH that is used as an antioxidant treatment according to OIV recommendations.

GSH can oxidise to GSSG during sampling and handling, which would negatively impact the accuracy of GSH quantification. Thus, it was necessary to derivatise GSH to prevent oxidation – such a derivatisation step has been included in some methods for this purpose (Giustarini et al. 2013).

In this study, a HPLC method with UV detection was developed for the quantification of GSH and GSSG in juice, fermenting wine and finished wines. The auto-oxidation of GSH to GSSG during the sample preparation, and subsequent overestimation of GSSG and underestimation of GSH, was avoided through the derivatisation of GSH with N-ethylmaleimide (NEM) to form the stable derivative, GSH-NEM (New et al. 2008). This method allowed the determination of GSH and GSSG concentrations in juice and fermenting wines in a simple, rapid, and robust way. An example of a calibration curve is shown in Figure 3, demonstrating that oxidised and reduced GSH can be quantified over a range from 1.8 to 50 mg/L. The assay is linear to concentrations of up to 300 mg/L, and was used to measure reduced and oxidised GSH in all subsequent work described in this report.

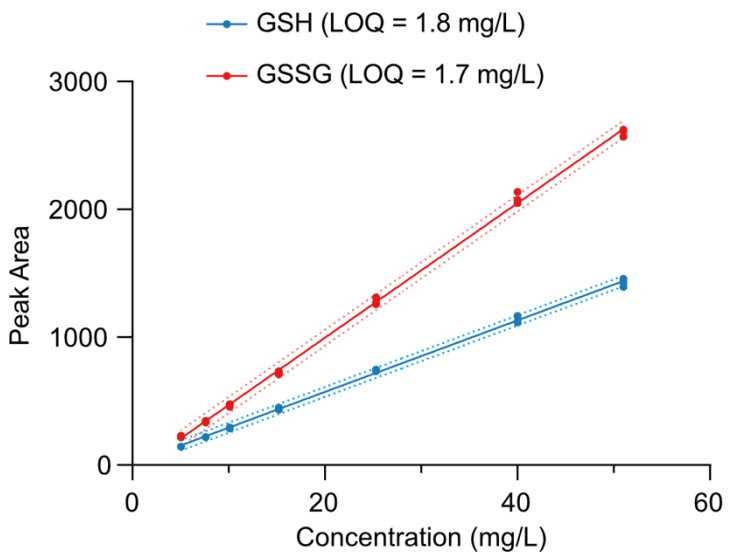


Figure 3. Relationship between reduced (GSH, blue) and oxidised (GSSG, red) glutathione concentration and HPLC peak area. Solid lines show the least squares regression model that was fitted to the data points.

6.2. How does nitrogen availability affect GSH consumption and H_2S production?

The OIV resolution on the use of GSH in wine production states that the juice should have an "assimilable nitrogen level sufficient to avoid the metabolism of glutathione by the yeast". A question then arises from this statement relating to the target concentration of assimilable nitrogen: how much nitrogen is sufficient to avoid the metabolism of GSH by yeast?

In this project this question was approached by quantifying the amount of GSH remaining after fermentation of defined media made with three different concentrations of YAN. Four different initial GSH concentrations were evaluated at each different YAN concentration, with the YAN being formulated from one-third ammonium and two-thirds amino acids. The yeast strain AWRI 1688 (an isolate of Zymaflore VL3) was used. This strain is known to contain oligopeptide transporters that permit the assimilation of complex nitrogen sources such as GSH (Marsit et al. 2015, 2016). A summary of one of these experiments is shown in Figure 4.

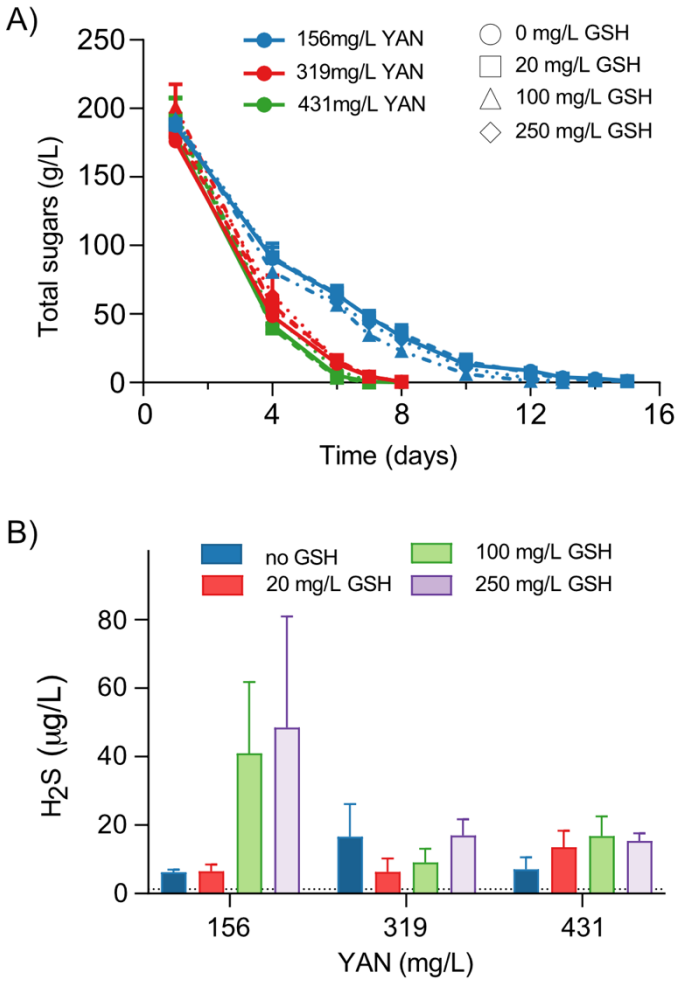


Figure 4. Effect of yeast assimilable nitrogen concentration on fermentation kinetics and hydrogen sulfide production by wine yeast strain VL3. A) Sugar utilisation during fermentation of defined medium at three yeast assimilable nitrogen concentrations (156, 319 and 431 mg/L) and four GSH concentrations (0, 20, 100 and 250 mg/L). B) Concentration of hydrogen sulfide (H_2S) after completion of alcoholic fermentation.

Figure 4A shows sugar consumption profiles at each combination of YAN and GSH. At 150 mgN/L, fermentations were slow, taking 15 days to reach a target sugar concentration of less than 1 g/L. Supplementing the YAN to either 320 or 430 mgN/L substantially decreased the duration of those ferments from 15 to 8 days. However, supplementing the medium with GSH did not decrease the fermentation time, suggesting that GSH was not being consumed as a supplementary nitrogen source in ferments where nitrogen was limiting (150 mgN/L).

The addition of GSH did give rise to higher final concentrations of H_2S at completion of fermentation (Figure 4B), but only in the low nitrogen fermentations to which either 100 or 250 mg/L GSH was added. Addition of 20 mg/L, as recommended in OIV-OENO 445-2015, did not result in a higher H_2S concentration on completion of alcoholic fermentation. Experiments with

only one yeast strain are shown here but other experiments with different yeast strains generated similar results. This suggests that a low concentration of GSH, when added to a fermentation, is not sufficient to stimulate H_2S formation, regardless of whether it is taken up by yeast.

The loss of GSH during these experiments was monitored by quantifying oxidised and reduced GSH upon completion of alcoholic fermentation. The decreases in total GSH concentration at all initial YAN concentrations mediated by two different yeast strains (VL3 and X5) are shown in Figure 5. These figures show that at all initial YAN concentrations and for all GSH concentrations, some loss of GSH occurs when GSH is added to the ferment. The consumption of GSH increased with increasing initial GSH concentration for both strains. In low YAN ferments containing an initial GSH concentration of 20 mg/L, a decrease of 13.1 (SD 1.2) and 9.6 (SD 0.3) mg/L of GSH was observed for strains VL3 and X5, respectively. In low YAN ferments containing an initial GSH concentration of 250 mg/L, a decrease in GSH concentration of 83 (SD 20) and 61 (SD 7) mg/L occurred. The magnitude of GSH loss in low-YAN conditions appeared to be independent of yeast strain. Furthermore, the consumption of GSH by the ferments decreased as initial YAN concentrations increased. Increasing the initial YAN concentration from 150 to 430 mg/L decreased the loss of GSH from an average across both strains of 72 (SD 16) mg/L to 30 (SD 5) mg/L.

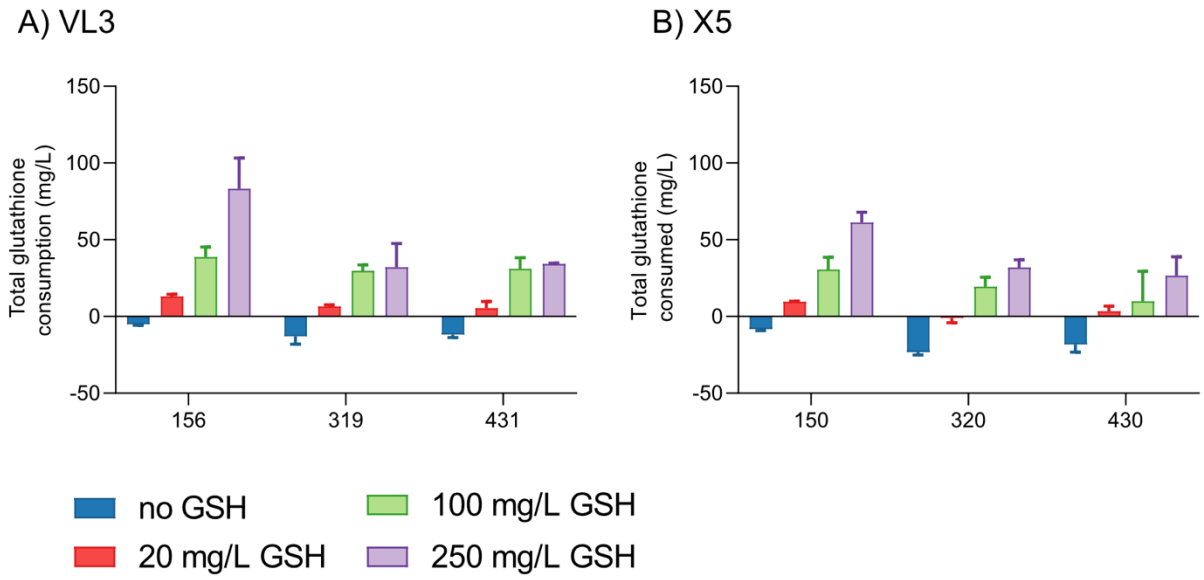


Figure 5. Decrease in GSH concentration mediated by A) VL3 and B) X5 during fermentations of model medium with different initial yeast assimilable nitrogen concentrations.

It is not possible to say from this experimental work whether this decrease in GSH concentration is entirely mediated by yeast metabolic activity and/or how much is lost through reaction with components of the medium. It is noteworthy, however, that these experiments were undertaken in defined medium with limited extraneous material that might interact with GSH, and that both oxidised and reduced GSH were quantified. Therefore, it is possible that there is not an optimal YAN concentration that will avoid the metabolism of GSH by yeast, as stipulated in the OIV resolution. It may only be the case that sufficiently low concentrations of GSH do not stimulate the production of yeast metabolites detrimental to wine quality.

In summary, this work found evidence of GSH consumption regardless of the initial concentration of assimilable nitrogen or GSH. Higher nitrogen concentration suppressed loss of GSH from the fermentation and also suppressed H_2S formation at higher GSH addition rates.

6.3. Is there an interaction between grape processing and GSH addition?

To better understand the impact of GSH addition during processing, the project explored the addition of GSH to grape juice that had been pressed either inertly or oxidatively. Inert pressing of grapes has previously been shown to preserve both grape-derived GSH and much of the phenolic material that is commonly oxidised during more routine pressing of white grapes (Motta et al. 2014, Day et al. 2019). Presses capable of inert pressing operations are in use in commercial wineries in Australia.

In the work described here, Sauvignon Blanc grapes were pressed using laboratory-scale (1kg) basket presses. Inert pressing was undertaken in an anaerobic hood and oxidative pressing was performed in air. The resulting juices were enzyme treated, settled and racked to clean bottles under nitrogen. Racking in the preparation of inertly pressed juice was performed in an anaerobic hood. Juices were inoculated with Zymaflore X5 and fermented at 17°C until the residual sugar concentration was less than 2g/L.

Inertly pressed juices were very slow to ferment and more variable in the time required to complete alcoholic fermentation than the other treatments, taking on average 30 days to finish. Inertly pressed juice to which GSH had been added finished within 20 days, as did both of the oxidatively pressed juices (Figure 6A). Overall, total GSH consumption was high, with 150 – 170 mg/L GSH lost during fermentation and settling in both inertly and oxidatively prepared juices. Of the remaining 70 mg/L in inertly pressed wine and 80 mg/L in oxidatively pressed wine, 44 and 46 mg/L respectively was in the reduced form (Figure 6B). The wine that had been pressed oxidatively had twice as much GSSG (36 mg/L) than the wine that had been processed inertly (16 mg/L) (Figure 6C).

Unlike the earlier work (Figure 4B), addition of GSH to these ferments did not result in significant differences in the concentration of H_2S in the resultant wines relative to wines that did not receive a GSH addition (Figure 6D) regardless of how the grapes were processed. Conversely, polyfunctional thiol concentrations varied dramatically in response to processing conditions and GSH addition. In particular, the concentration of 4-MSP, which was not detected in oxidatively pressed wines, was significantly higher (29 ng/L) in wines that had been prepared anaerobically. The concentration of 4-MSP was higher again in wines to which GSH had been added after pressing, with an additive increase of 45 ng/L in both inertly and oxidative processed wines. Significant treatment effects were not observed among the other main polyfunctional thiols 3-SH and 3-MSA. The compound 4-MSP is in part responsible for the characteristic 'box tree' aroma of Sauvignon Blanc (Darriet et al. 1995) and has an exceptionally low perception threshold (0.8 ng/L) (Tominaga et al. 2000).

While previous reports of the impacts of grape processing on the concentration of 3-SH and 3-SHA have been mixed, a consensus appears to have formed that the impact of pressing conditions are subtle at best (Allen et al. 2011, Herbst-Johnstone et al. 2013, Larcher et al. 2013). In addition, the use of antioxidants such as GSH was reported by Patel et al. (2010) to decrease 3-SH concentration. The observations in this project regarding 3-SH and 3-SHA are consistent with these previous observations. However, reports on the impact of oxidation control on 4-MSP are less prominent. Allen et al. (2011) reported no impacts of harvesting and processing conditions on 4-MSP concentrations in Sauvignon Blanc wine, which is in contrast to the observations in this study. It is possible that GSH stabilises oxidised phenolic compounds that might otherwise react with this potent aroma-active thiol.

The large losses in GSH recorded in these ferments (Figure 6B) may well be explained by the large increases in GRP observed in the wines with GSH added post-pressing (Figure 6F). Almost no GRP was observed in the absence of GSH addition. That GSH addition gives rise to GRP is expected due GSH being a substrate in the reaction that leads to GRP formation; however, it is surprising that higher concentrations of GRP were apparent in inertly pressed wines, since oxygen is also required in the reaction and typically suppression of quinone formation leads to the suppression of GRP formation (Singleton et al. 1985). It is possible that inert pressing contributed to the preservation of caftaric acid during the early processing stages, thus making it available for subsequent reaction with GSH and in this way contributing to elevated GRP concentrations. GRP has been shown to contribute to wine texture in ways that are dependent on the compositional context (Gawel et al. 2014). As a result, any further discussion as to the likely impact of the increased GRP concentrations observed in these wines is mere speculation. It should be noted that at 150 mg/L, the concentration of GRP in GSH-treated inertly pressed wine is considerably

higher than the concentrations that have previously been used in the sensory assessments of this compound (Vèrette et al. 1988).

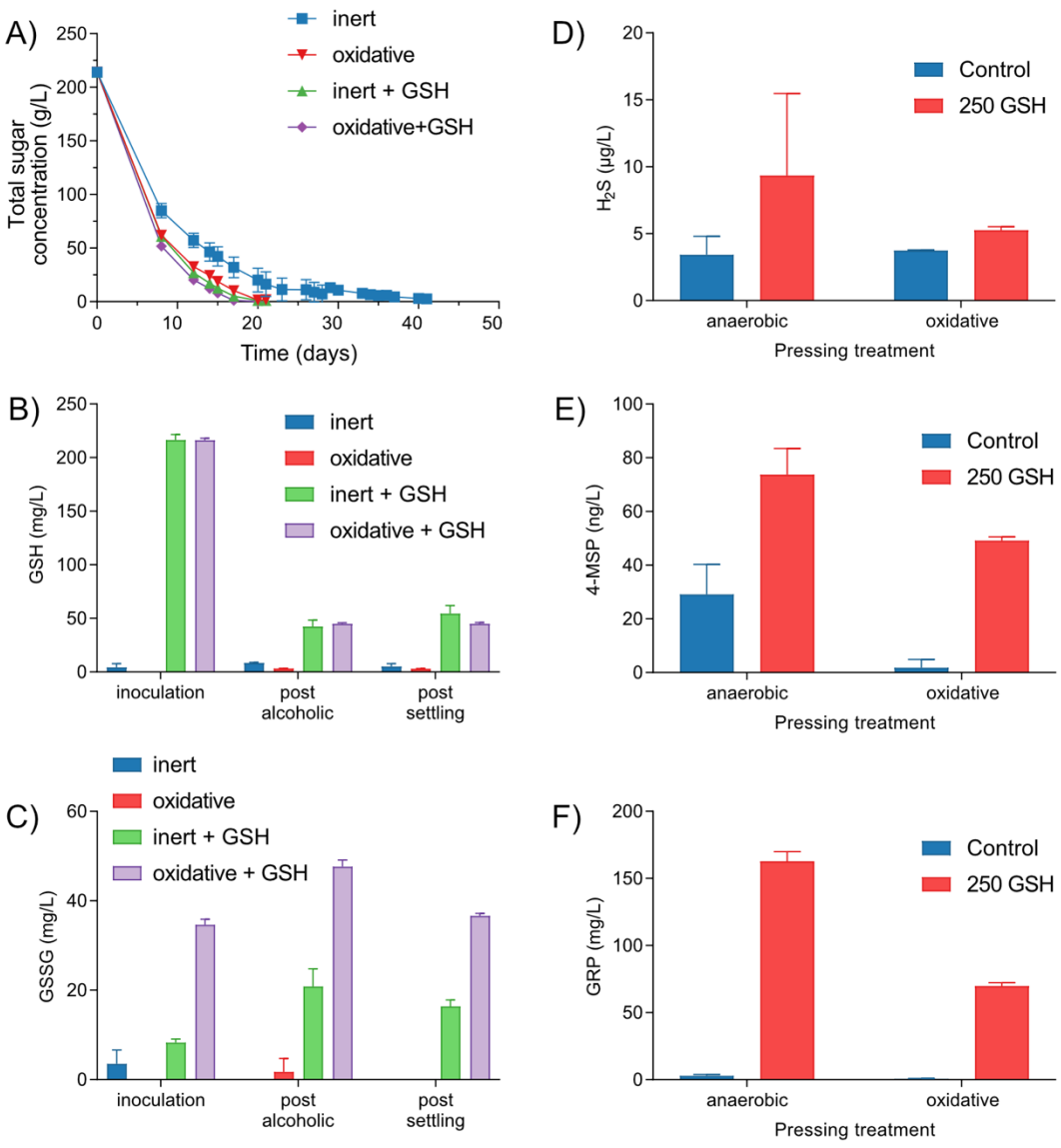


Figure 6. The effect of a 250 mg/L GSH addition to inertly or oxidatively pressed Chardonnay juice on A) sugar utilisation during fermentation, and post-fermentation concentrations of B) reduced glutathione (GSH), C) oxidised glutathione (GSSG), D) hydrogen sulfide (H_2S), E) 4-methyl-4-sulfanylpentan-2-one (4-MSP) and F) grape reaction product (GRP). Bar heights show means of three replicates with error bars showing standard deviation.

6.4. Vintage trial 1 – The impact of high GSH concentrations during fermentation on Chardonnay and Riesling wine

In the laboratory experiments presented in previous sections, and as reported by others, GSH consumption was shown to be dependent on both the amount of GSH used and on the initial nitrogen content of the must. A variety of compositional effects can be induced, most notably the

potential for increases in low molecular weight volatile sulfur compounds through GSH addition. However, it is difficult to gauge the true impact through laboratory-based trials alone. In the first of two pilot-scale trials, an experimental design was constructed to test the following hypotheses:

a. GSH addition during early stages of wine production will have significant compositional and sensory effects
b. GSH addition pre-fermentation will differ significantly from GSH addition during fermentation
c. Residual GSH addition at bottling will decrease the SO_2 requirement at bottling
d. The effect of GSH addition on different wine types will be different.

An overview of the design is presented in Figure 1. In this experiment, 250 mg/L of reduced GSH was added to Riesling and Chardonnay juice either 24 hours pre-inoculation or 24 hours post-inoculation. The basic analytical parameters of the juice are provided in Table 2. An amount of GSH was added in this experiment such that the concentration of GSH remaining at bottling would approximate a post-ferment GSH addition. The GSH addition rate chosen for this experiment was determined in preliminary laboratory trials. It is acknowledged that this addition (250 mg/L) is a great deal higher than concentrations recommended under resolution OIV-OENO 445-2015. Nevertheless, it is informative with regard to the experimental aims.

Table 2. Juice composition post-clarification

Analyte	*unit*	*Chardonnay*	*Riesling*
TSS	°Brix	21.2	19.8
pH		3.25	3.15
Titratable acidity (TA) pH 8.2	g/L	6.4	6.5
Malic acid	g/L	2.5	2.7
YAN	mg N/L	200	247
Free SO_2	mg/L	10	<5
Total SO_2	mg/L	41	17

6.4.1. How does the concentration of GSH evolve over the course of fermentation when it is added during processing or following inoculation?

The evolution of glutathione was monitored in two sets of fermentations, Chardonnay and Riesling, which were bottled at two SO_2 concentrations (Figure 7). Both juices were prepared using standard aerobic pressing methods. GSH was undetectable when not added to the fermentations, but it accumulated continuously to approximately 14 and 9 mg/L in the Chardonnay and Riesling wines, respectively, by the completion of alcoholic fermentation. The Chardonnay fermentations were sluggish, with remedial action being undertaken after 20 days, but eventually completing after 44 days. During cold settling of the finished wines, GSH concentrations continued to decrease moderately in Chardonnay and increased in Riesling, reaching respective concentrations of 8 and 12 mg/L before bottling. Cellaring of the wines for a further three months saw GSH concentrations decline in both wines, with the higher concentrations of SO_2 associated with accelerated decrease of GSH in the Chardonnay wine. Both the overall concentration of GSH observed in these wines and the SO_2-associated decrease in GSH are consistent with the observations of others (Panero et al. 2015, Pons et al. 2015).

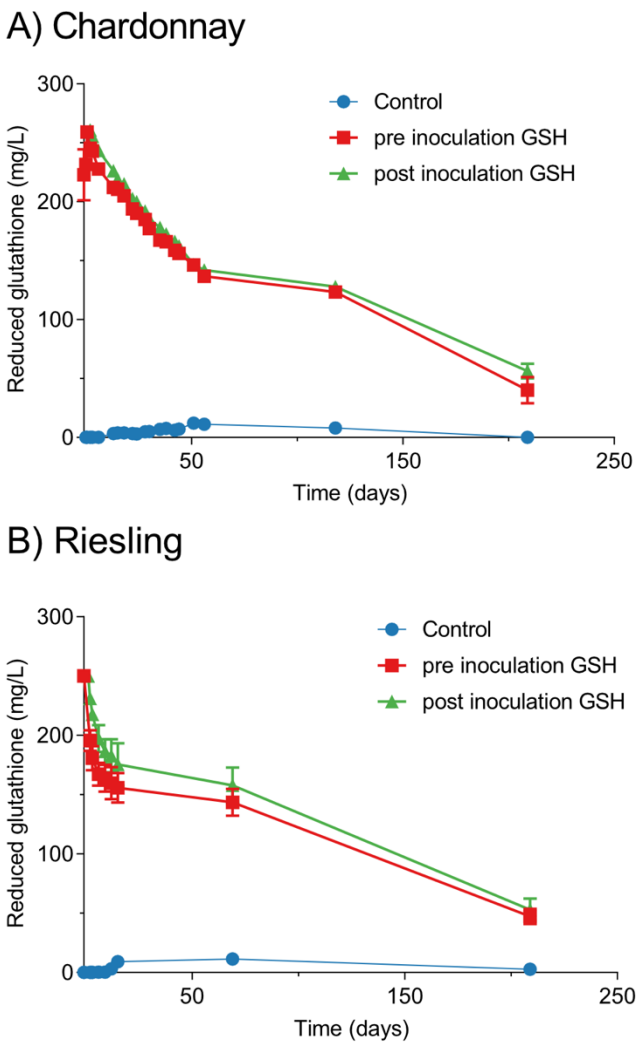


Figure 7. GSH evolution during fermentation and post-fermentation treatment in A) Chardonnay and B) Riesling following addition of 250 mg/L of reduced glutathione (GSH) to ferments pre-inoculation (red) or post-inoculation (green). Untreated control wines are shown in blue. Points represent the mean of three replicates, error bars show standard deviation.

When GSH was added either before or after inoculation, its concentration declined almost linearly with time until alcoholic fermentation ceased. The fermentation stage was the period of most rapid GSH loss over the entire production process, with a 100 mg/L decrease in GSH concentration observed in both the Chardonnay and Riesling ferments. During the subsequent cellaring period, which included settling of yeast lees, racking and cold stabilisation, a further 20–30 mg/L GSH was lost. GSH loss during filtration and bottling was again substantial, and by the time the wines were prepared for sensory evaluation, only 38 – 59 mg/L remained in the Chardonnay and 45 – 56 mg/L remained in the Riesling (Figure 8). This demonstrates a GSH consumption potential of 180–212 mg/L over the production life of a wine. Slightly more

GSH was lost from wines prepared under a low-SO_2 bottling regime. Although no statistical interactions were observed between the timing of GSH addition and SO_2 concentration at bottling, SO_2 concentration was responsible for 35% of the variation in GSH concentration in the Chardonnay and 24% of the variation in GSH concentration in the Riesling. In summary, higher SO_2 concentrations at bottling helped preserve GSH in bottle.

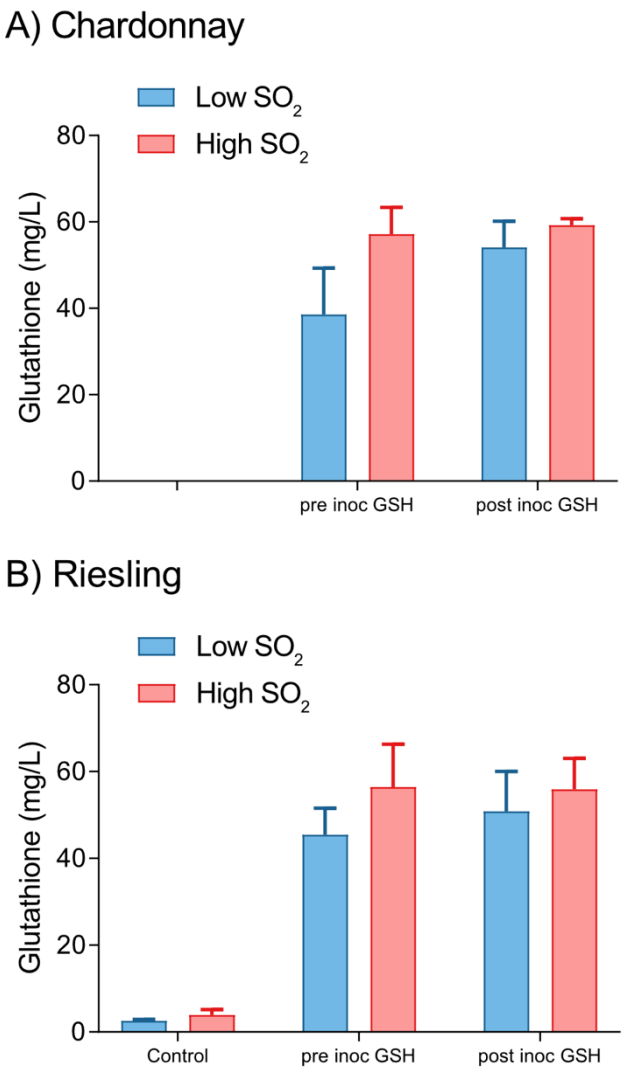


Figure 8. Concentrations of reduced glutathione (GSH) in bottled wine at the time of sensory evaluation in A) Chardonnay and B) Riesling. Bar heights represent the mean of three replicates, error bars show standard deviation. Where no bars are shown, no glutathione was detected in the sample.

Although SO_2 can help preserve GSH during bottling, a more pertinent question is whether having GSH present during bottling will help preserve SO_2 and therefore support lower SO_2 use during production. In wines containing GSH, the decline in SO_2 concentrations in bottle is expected to be decreased. Wines in this study were bottled at two different SO_2 concentrations and SO_2 concentration was determined at bottling and at the time of the sensory evaluation, some three months later (Figure 9). Chardonnay wines bottled with low and high SO_2 (0.6 and 0.8 mg/L molecular SO_2) saw decreases in free SO_2 of 13 mg/L and 22 mg/L, respectively, during the time they were stored prior to sensory evaluation. Similar decreases were observed in the Riesling wines. None of the wines to which GSH was added during fermentation and which contained on average 50 mg/L of reduced GSH at the time of the sensory evaluation, differed from the control in the amount of free SO_2 present. This shows that while having higher concentrations of SO_2 at bottling helps to preserve the GSH that is present, the converse is not true. Having GSH present at bottling does not help to preserve SO_2.

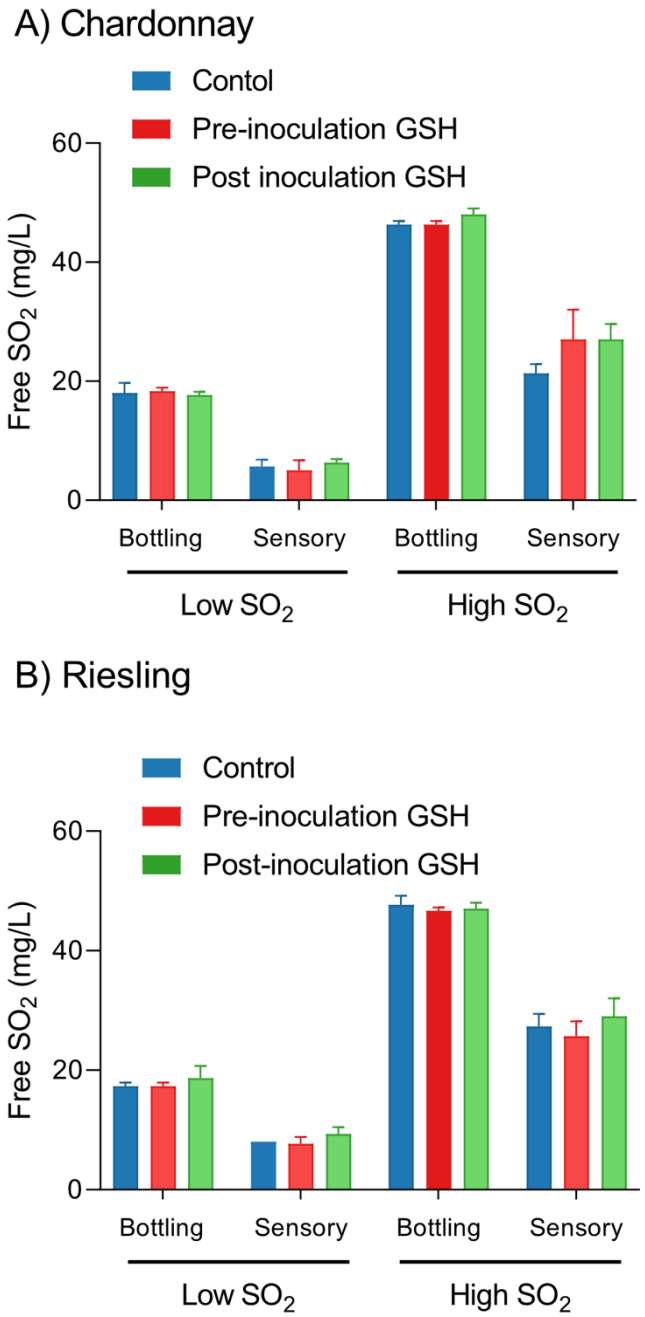


Figure 9. Concentrations of free SO_2 in bottled wine at the time of bottling and sensory evaluation in A) Chardonnay and B) Riesling. Bar heights represent the mean of three replicates, error bars show standard deviation.

The finding that GSH present at bottling does not interact with SO_2 is consistent with the work of Panero et al. (2015) who reported that post-ferment GSH addition did not help preserve the concentration of SO_2 over time, that SO_2 did not preserve GSH concentrations over time, and that GSH addition offered no protective effect against white wine browning. Earlier work by Ugliano et al. (2011) showed that post-ferment GSH addition could suppress the loss of

polyfunctional thiols such as 3-SH at the expense of higher concentrations of H_2S. For both of these compounds, there were complex interactions with wine copper concentrations and oxygen ingress through the closure. Thus, despite promising theoretical work indicating potential useful post-ferment applications for GSH, these two studies point toward limited practical outcomes and added risk with potential for in-bottle evolution of low molecular weight sulfur compounds that can negatively impact wine quality.

6.4.2. Effect of early GSH addition on the chemical composition of Chardonnay and Riesling wine

The chemical composition of the Chardonnay and Riesling wines was comprehensively assessed. The initial assessment was undertaken at the same time as the sensory evaluations conducted three months post-bottling. There was little variation in the chemical composition of the wines that could be attributed to treatment effects. Two-way ANOVA (Table 4, Appendix 5) considering GSH addition and SO_2 at bottling as main experimental variables did not reveal any significant interactions between these two treatments and their impact on the concentrations of wine analytes (volatile and non-volatile).

Considering GSH addition as single factor, only H_2S, MeSH and 3-SH differed significantly across the treatments. The concentration of these metabolites was generally higher in GSH treatment wines relative to wines in which GSH had not been added during fermentation. Considering SO_2 concentrations at bottling as a single factor, the concentration of MeSH and 3-SH were higher in wines bottled with higher concentrations of SO_2 whereas the concentration of CS_2 was lower.

Overall, the experimental variable with a dominant influence on wine composition was GSH addition; however, the impacts on wine composition were small, except for the effect of GSH on H_2S concentration (Figure 10). The concentration of H_2S increased four-fold in wines that had received GSH during production relative to untreated controls. These wines were reanalysed 18 and 22 months post-bottling. H_2S concentrations increased dramatically over the 15 months following the initial analysis, with concentrations in excess of 100 µg/L apparent in some of the wines. By comparison, the concentration of H_2S in wine that had received no GSH addition during production remained within acceptable limits, reaching a maximum of 4 µg/L over the duration of the maturation period.

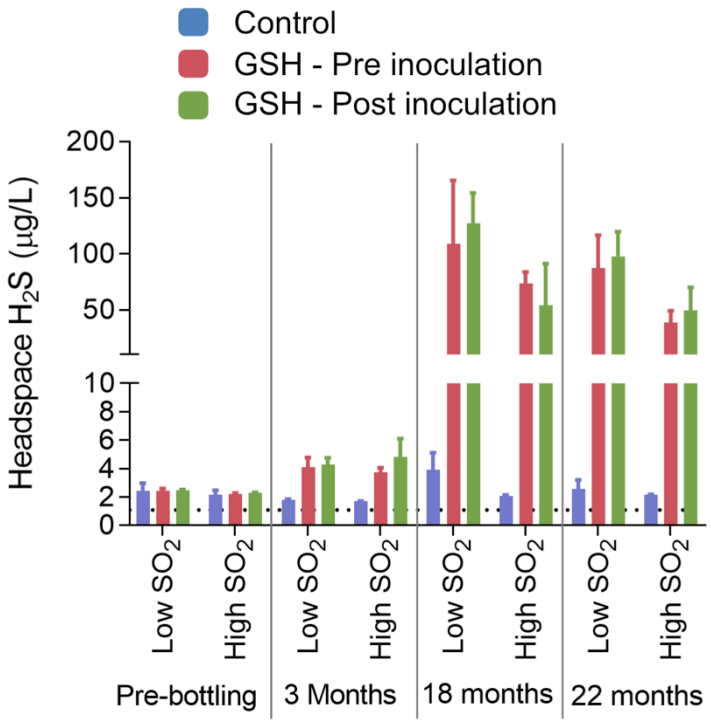


Figure 10. Effect of GSH addition prior to, or during, fermentation and SO_2 concentration at bottling on H_2S evolution in Chardonnay wine. The dotted line indicates the detection threshold for H_2S (1.1 µg/L).

6.4.3. Effects of early additions of GSH on sensory attributes of Chardonnay and Riesling wine.

Descriptive analysis of wine sensory attributes was undertaken for Chardonnay and Riesling wines in a single unified sensory study, such that major effects of variety, GSH treatment, and sulfur concentration at bottling and their potential interactions could be assessed.

GSH addition to Chardonnay ferments produced wines that were rated highly for 'box hedge/cat pee aroma', 'cheesy/baby vomit aroma', 'drain aroma' and 'flint aroma' regardless of SO_2 level. The control wines were rated highly in 'overall fruit aroma' and 'stone fruit aroma' (Figure 11). 'Stone fruit' flavour appeared to be suppressed in wines bottled with low molecular SO_2 concentrations.

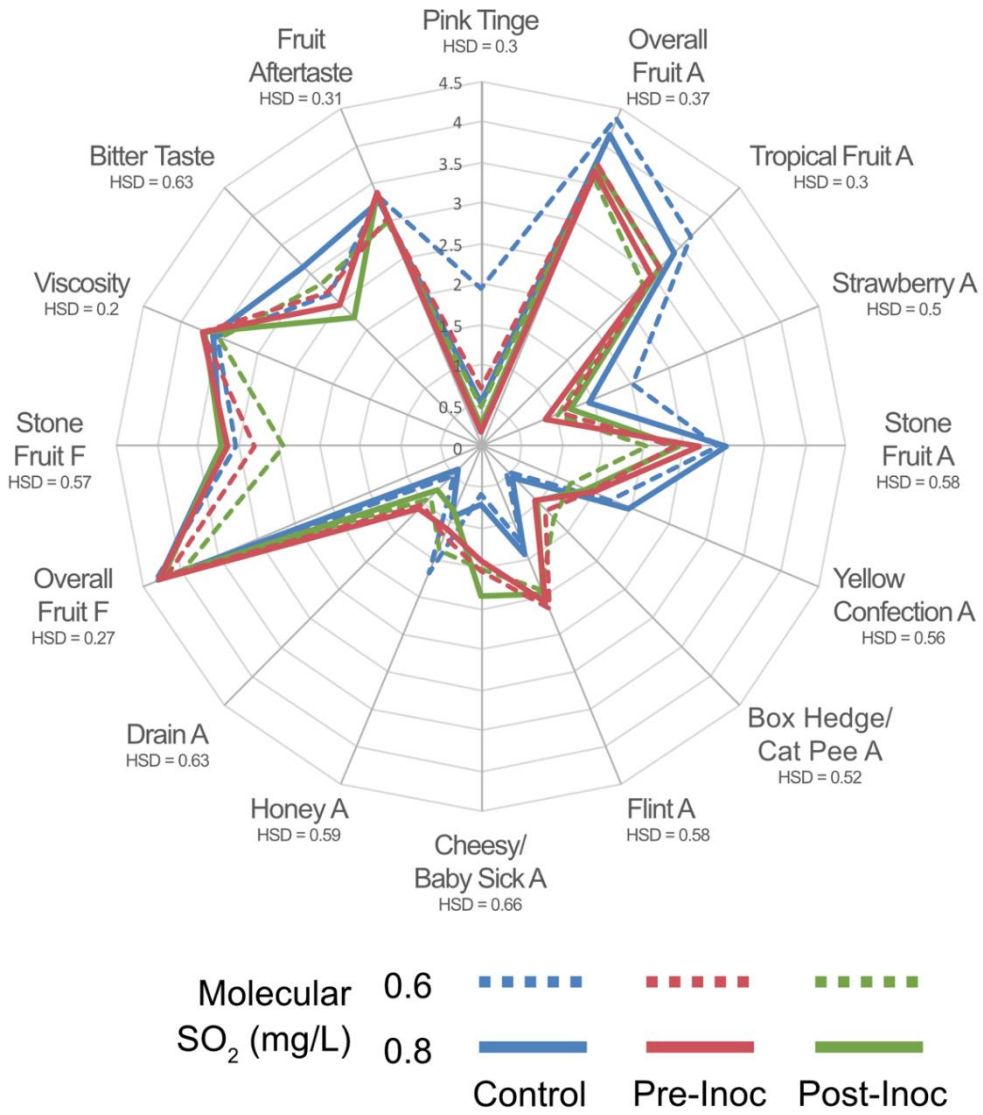


Figure 11. The effect of pre- and post-inoculation glutathione (GSH) addition on the sensory attributes of Chardonnay wine. Plot shows means of the of all attributes with $P < 0.1$. A = aroma, F = flavour.

Glutathione addition to Riesling ferments (Figure 12) produced wines that were generally rated highly in 'box hedge/cat pee aroma' and 'flint aroma'. Conversely, both of the low SO_2 treatments (i.e. control and pre-inoculation GSH addition wines) were rated highly for 'honey aroma'. Riesling wines bottled with low molecular SO_2 concentrations were rated more highly for yellow colour.

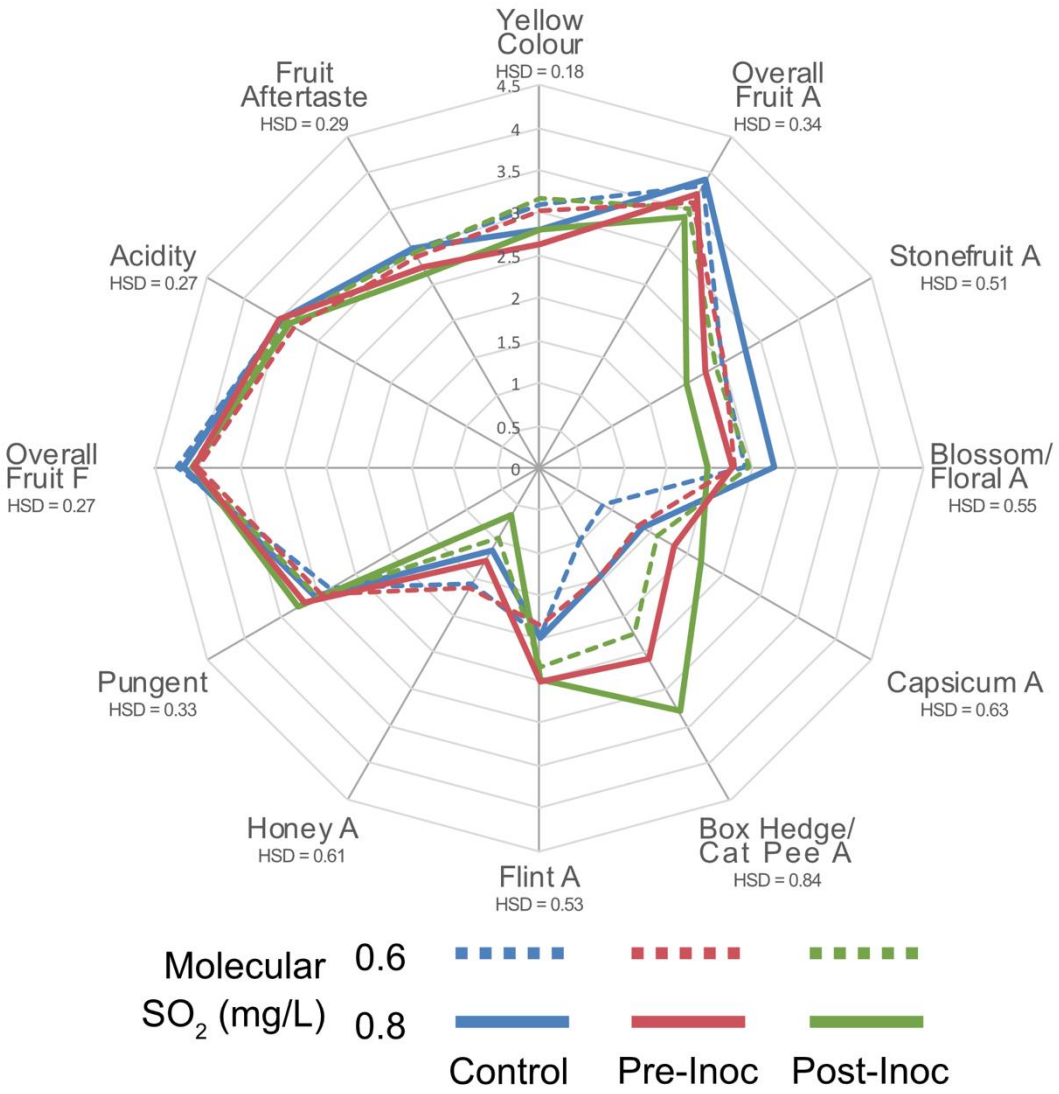


Figure 12. The effect of pre- and post-inoculation glutathione (GSH) addition on the sensory attributes of Riesling wine. Plot shows means of all sensory attributes with $P \leq 0.1$. A = aroma, F = flavour.

It is also worth noting that all wines showed some inter-fermentation replicate variation, which may have been caused by the compounded variation inherent with increasing variables within a treatment (i.e. the complex experimental design).

The experimental design and approach to sensory analysis permitted an investigation of GSH addition, SO_2 level and grape variety and their potential two- and three-way interactions using ANOVA. Overall, few interactions were evident between GSH and either SO_2 or grape variety. The attributes 'pink tinge' and 'yellow colour', both associated with oxidation, were influenced by interactions among all the main factors. There was no 'pink tinge' described in the Riesling wines; however, in the Chardonnay wines without GSH and bottled with 0.6 mg/L molecular SO_2 'pink tinge' was evident. This was suppressed in wines made with GSH or bottled with

0.8 mg/L molecular SO_2. The attribute 'honey aroma', also associated with oxidation, was higher in pre-inoculation GSH addition and 'no addition' control wines whereas post-inoculation GSH addition wines had a lower mean intensity of this attribute. These effects of GSH addition on oxidation-related sensory attributes suggest some effect of early GSH addition on post-fermentation oxidation phenomena.

Twenty-six attributes (Table 7, Appendix 5) differed significantly ($P \leq 0.05$) as a function of variety (V) (2 appearance, 11 aroma, 6 flavour, 7 taste and mouth-feel attributes). The fact that variety was a dominant factor in the description of these wines is not unexpected. Somewhat fewer effects on sensory attributes could be discerned as a result of GSH addition: 16 attributes were significant for the main effect with 'overall fruit aroma', 'tropical aroma', 'strawberry aroma', 'bruised apple aroma', 'stone fruit aroma', 'yellow confectionary aroma', 'floral aroma', 'capsicum aroma', 'drain aroma', 'overall fruit flavours', 'tropical fruit flavour', 'stone fruit flavour', and 'capsicum flavour' all differing significantly ($P \leq 0.05$). The fruit-related aroma attributes 'overall fruit', 'tropical fruit', 'strawberry', 'apple/bruised apple', 'stone fruit', 'confection', and 'floral', as well as the fruit flavour attributes 'overall fruit', 'tropical fruit', 'stone fruit', and 'fruit aftertaste', were all rated significantly higher in the controls compared to the GSH treatments. Conversely, the GSH addition treatments were rated higher in 'dank/drain aroma', 'pungent', and 'capsicum aroma and flavour'. Four attributes differed significantly by SO_2 level (column S, Table 7) with no interactions being significant, meaning that there were consistent differences as a result of the SO_2 additions independent of the GSH or variety effects. The SO_2level effect was significant ($P < 0.05$) for the attributes 'honey aroma', 'pungent aroma', 'acidity' and 'astringency'.

In summary, residual GSH in the finished wine resulting from the high concentrations added during processing or immediately following the initiation of fermentation appeared to offer some limited protection against oxidation, as indicated by the suppression of pink tinge formation in Chardonnay or yellow colour formation in Riesling, in conditions where SO_2 was limiting (0.6 mg/L molecular at bottling); however, the action of 0.8 mg/L SO_2 was superior in the prevention of yellow colour development. In addition to ineffective protection of the wine against oxidation, the use of such high concentrations of GSH during processing had detrimental effects for Chardonnay in particular, with those wines being rated more highly in attributes that are typically perceived negatively.

6.5. Vintage trial 2 – The impact of small (20 mg/L) GSH additions prior to fermentation on Chardonnay wine

The first pilot-scale experiment (Trial 1) demonstrated that high concentrations of GSH added in the early stages of wine production had significant compositional and sensorial impacts, that pre-inoculation and post-inoculation additions did not differ significantly, that both Chardonnay and Riesling responded to GSH addition similarly, and that post-fermentation residual GSH did not moderate the requirement for SO_2 in bottle. The second of two pilot-scale trials sought to explore whether earlier GSH additions would be useful from the perspective of oxidation control. In principle, earlier GSH additions maximise the potential for reaction with grape berry components such as thiols. Glutathionylated compounds can be used by yeast to release flavour-active molecules, but yeast vary in their capacity to utilise these precursors. With these points in mind, an experimental design was constructed to test the following hypotheses:

a. Very early GSH addition will affect wine composition and sensory properties.
b. The impact of GSH will be dependent on the yeast strain used for alcoholic fermentation

An overview of the trial design is presented in Figure 2. In this experiment, 20 mg/L of reduced GSH as recommended in OIV-OENO 445-2015 was added immediately after pressing of Chardonnay grapes. No SO_2 was used during grape processing. Two yeast strains were used in the making of these wines, VL3 and X5. These two yeasts share an exceptionally high degree of genetic similarly (Borneman et al. 2016) but differ in their complement of oligopeptide transporters (Marsit et al. 2016). These transporters confer an ability to utilise a greater diversity of oligopeptides (such as GSH) as sources of nitrogen. Strain VL3 has two of the transporters (*Fot1* and *Fot2*) whereas X5 does not. VL3 has also previously been demonstrated to have the capacity to release volatile aroma compounds from their non-volatile precursor forms (Murat et al. 2001). The basic analytical parameters of the juice are provided in Table 3.

Table 3. Chardonnay juice composition post-clarification

Analyte	*unit*	*Chardonnay*
TSS	°Brix	21.26
pH		3.54
TA 8.2	g/L	5.6
Malic acid	g/L	2.78
YAN	mg N/L	366

6.5.1. Effect of early GSH addition on the chemical composition of Chardonnay sparkling base and table wine

The chemical composition of the Chardonnay wine in Trial 2 was comprehensively assessed. The assessment was undertaken at the same time as the sensory evaluations conducted approximately three months post-bottling. As was the case with the wines in Trial 1, there was little variation in the chemical composition that could be attributed to treatment effects. Two-way ANOVA (Table 8, Appendix 5) considering GSH addition and yeast strain as main experimental variables demonstrated few significant interactions between these two treatments and the concentration of wine analytes. Significant interactions were only evident for 3-SH, butanoic acid, octanoic acid and ethyl hexanoate ($P < 0.05$). Of those four compounds, only 3-SH showed significant variation with respect to GSH treatment (Figure 13A) and the remainder showing limited overall variation between the main variables. Yeast strain accounted for 48% of the variation observed in 3-SH concentration. Wines made with the strain X5 were generally higher in 3-SH concentration relative to wines made with VL3. GSH addition contributed a further 35% to the variation in 3-SH concentration, in particular to the wines made using yeast strain X5. There were no significant differences that could be attributed to GSH addition in wines made with VL3.

Increases in the concentrations of 3-SHA (Figure 13B), 2- and 3-methylbutyl acetate, hexyl acetate and DMS were associated with GSH addition prior to fermentation, but in all cases the increases were small and more than likely not oenologically relevant. Most impacted among these compounds was 3-SHA, where the concentration doubled in wines made with VL3 and GSH relative to VL3 alone.

A substantially larger number of compounds were influenced by the choice of yeast strain, with 1-butanol, 2-phenylethyl acetate and 3-SHA (Figure 13B) exhibiting the largest changes. Here again 3-SHA was the most affected, with strain choice alone leading to a doubling in concentration of this compound.

In summary, the small addition of GSH immediately post-pressing had a measurable impact on the composition of the finished wine. However, the magnitude of the observed compositional variation was small and typically overshadowed by the changes in wine composition associated with the yeast strain used to conduct the ferments.

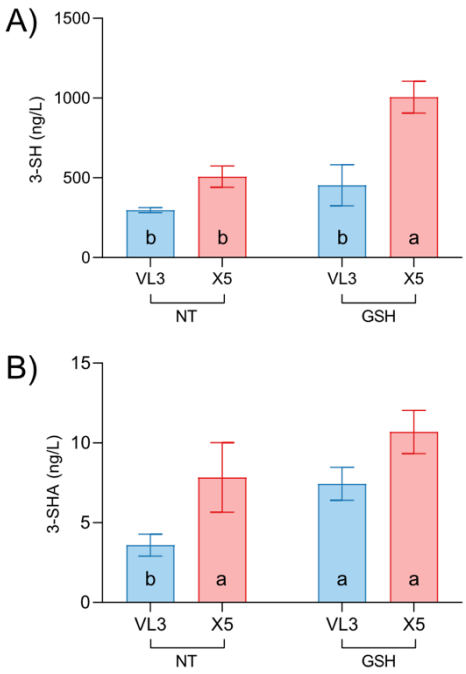


Figure 13. Effect of post-pressing glutathione (GSH) addition and yeast strain on the concentrations of A) 3-SH and B) 3-SHA in bottled wine relative to wines that received no GSH during production (NT). Bar heights show mean of three replicates with error bars showing standard deviation. Bars labelled with different letters are significantly different from each other at alpha = 0.05.

6.5.2. Effects of post-press additions of GSH on sensory attributes of Chardonnay wine.

The effect of post-pressing GSH addition and yeast strain on the sensory attributes of Chardonnay wine was evaluated using a rapid sensory analysis method known as Global Napping. This approach provides an indication of how similar samples are and together with ultra-flash profiling provides a description of the wines, but a quantitative assessment of those descriptors is not possible. Nevertheless, the method is a useful tool for sensory characterisation (Varela et al. 2012).

There were relatively small within-treatment differences for the Chardonnay wines arising from VL3 compared to the wide separation for the replicate X5 strain wines (Figure 14). Dimensions 1 and 2 explained 46.4% of the variance, with GSH appearing to drive separation along the first dimension and yeast strain separating wines in the second dimension. The VL3 wines with added GSH were described more often as ‘confection’, ‘stone fruit’, ‘apple/pear’ and high in ‘viscosity’, while the control VL3 wines were indicated more frequently as ‘grassy’, ‘astringent’, ‘bitter’ and ‘acidic’ (Figure 15). Two of the three replicates for the X5 wines with added GSH were also plotted to the right of Figure 15 with ‘viscosity’ related to these wines, and ‘astringency’ and ‘bitterness’ less so. A ‘sweaty’ character was related to two X5 wines (added GSH R3 and control R1).

In summary, this study indicated that for Chardonnay table wine, an addition of GSH had an effect of enhancing wine 'fruity' attributes and 'viscosity', and decreasing 'astringency' and 'bitterness'. Sample sets had relatively large within-treatment variation, limiting the ability to conclude treatment-related sensory effects. In other words, the treatment effects were small relative to the technical variation. Furthermore, the effect of yeast strain was nearly as large as that of GSH addition. The subtle effects described in this sensory analysis are consistent with the limited compositional changes described in section 6.5.1.

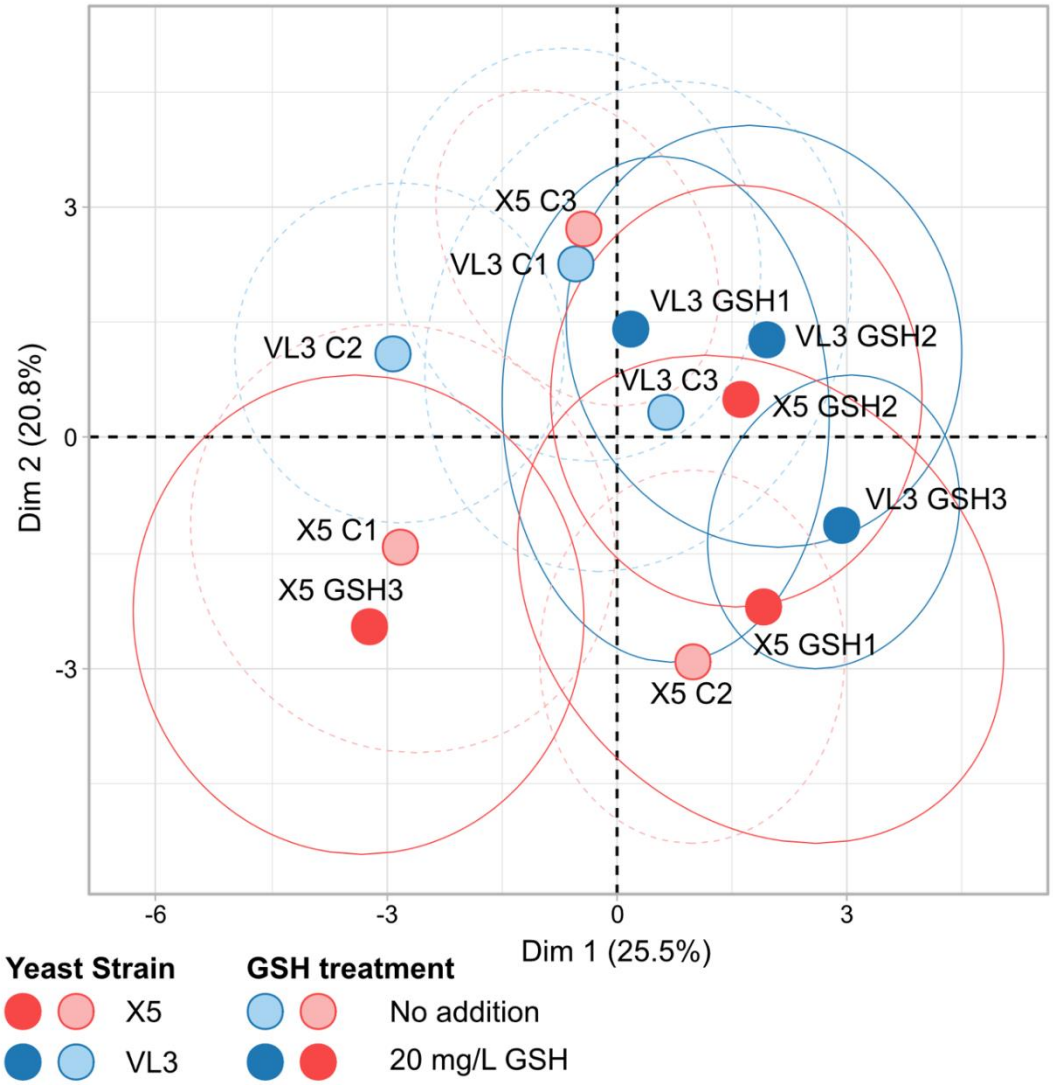


Figure 14. Consensus multiple factor analysis map for Chardonnay wines made in Trial 2 with 95% confidence ellipses. Dotted ellipses show 95% confidence boundaries for control wines and solid line ellipses show confidence boundaries for GSH-treated wines.

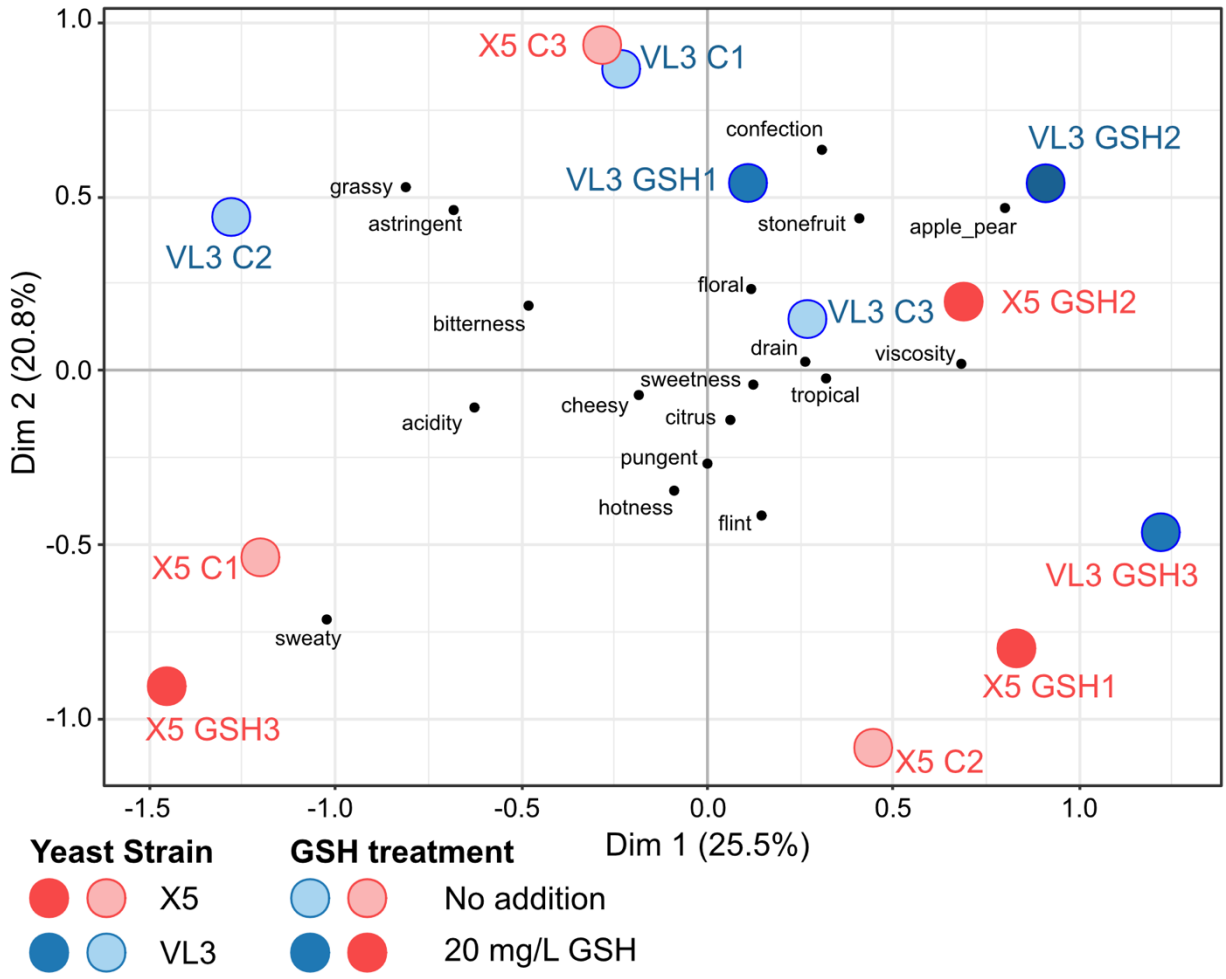


Figure 15. Consensus multiple factor analysis map for Chardonnay wines made in Trial 2 (as presented in Figure 14 but without confidence ellipses) with sensory descriptors overlaid.

6.6. Production of isotopically labelled GSH through synthesis

Glutathione addition prior to, or immediately after, the initiation of fermentation resulted in longer term development of volatile sulfur compounds, specifically hydrogen sulfide. The development of H_2S after a period of maturation appeared to be dependent on prior contact of GSH with yeast during fermentation. The underlying mechanism by which H_2S was produced, and whether GSH was in fact consumed by yeast or by reaction with the juice components remained an open question. One approach to resolve the fate of added GSH was to attempt to track the transfer of its sulfur moiety to its ultimate metabolic endpoint. Such an experiment is possible using isotopic tracers and mass spectrometry with isotopic mass resolution. This section describes endeavours by the project team to synthesise isotopically labelled GSH from elemental sulfur.

The initially planned and most expeditious route to introduce labelled sulfur into GSH required incorporation of sulfur into L-cysteine via benzyl mercaptan, using the benzyl group to protect the

cysteine thiol moiety from oxidation or nucleophilic side reactions based on the method described in Brock et al. (2014). The series of reactions was trialled with unlabelled sulfur (i.e. ^{32}S). To begin, elemental sulfur and potassium cyanide were reacted to form potassium thiocyanate, which was converted to benzyl thiocyanate and then to labelled benzyl mercaptan. On-reaction of benzyl mercaptan with methyl 2-((*t*-butoxycarbonyl)amino)-3-(tosyloxy)propanoate (tosylate) and sodium hydride (NaH) introduced the *S*-benzyl moiety into cysteine. *S*-Benzyl-L-cysteine was converted to *S*-benzyl-L-glutathione via established peptide coupling methods with protected amino acids (L-glutamatic acid and glycine). These steps proceeded smoothly and in reasonable yield.

With *S*-benzyl-L-glutathione in hand, issues were encountered when trying to remove the benzyl protecting group from the thiol of cysteine, which proved resistant to established methodology such as hydrogenation. Successful removal was ultimately achieved through dissolving metal reduction with sodium (Na) in liquid ammonia (NH_3). The extraction and purification (by flash chromatography on silica) of GSH from this reaction mixture required glutathione to be preferentially portioned into an organic solvent, and therefore the carboxylate (*O*-methyl ester, OMe) and amine (*t*-butyloxycarbonyl, BOC) protecting groups were left in place for the reduction step. Given the inability to monitor the reaction or control the amount of dissolved metal reducing agent, OMe was partially reduced to an alcohol in the presence of Na/NH_3 and protected GSH could not be resolved from the mixture containing this by-product. The unwanted ester reduction was prevented by replacing the OMe protecting group of the carboxylate with a *t*-butyl ester.

With this alternative protecting group, *S*-benzyl-glutathione was again reduced with Na/NH_3 to glutathione. Deprotection of the *t*-butyl group from the carboxylic acid in the presence of the free thiol moiety resulted in the liberated *t*-butyl reacting with the thiol group, however, forming *S*-*t*-butyl-glutathione. Various conditions were trialled for the deprotection step and in an attempt to prevent migration of the protecting group onto the sulfur, and considering only limited success with refluxing in HCl, changes to the synthetic pathway were required. Overcoming the issue involved removal of the *t*-butyl group prior to reduction of *S*-benzyl-glutathione in Na/NH_3. With the specified changes to the synthetic pathway, GSH was produced with 15% overall yield beginning with elemental sulfur (Figure 16).

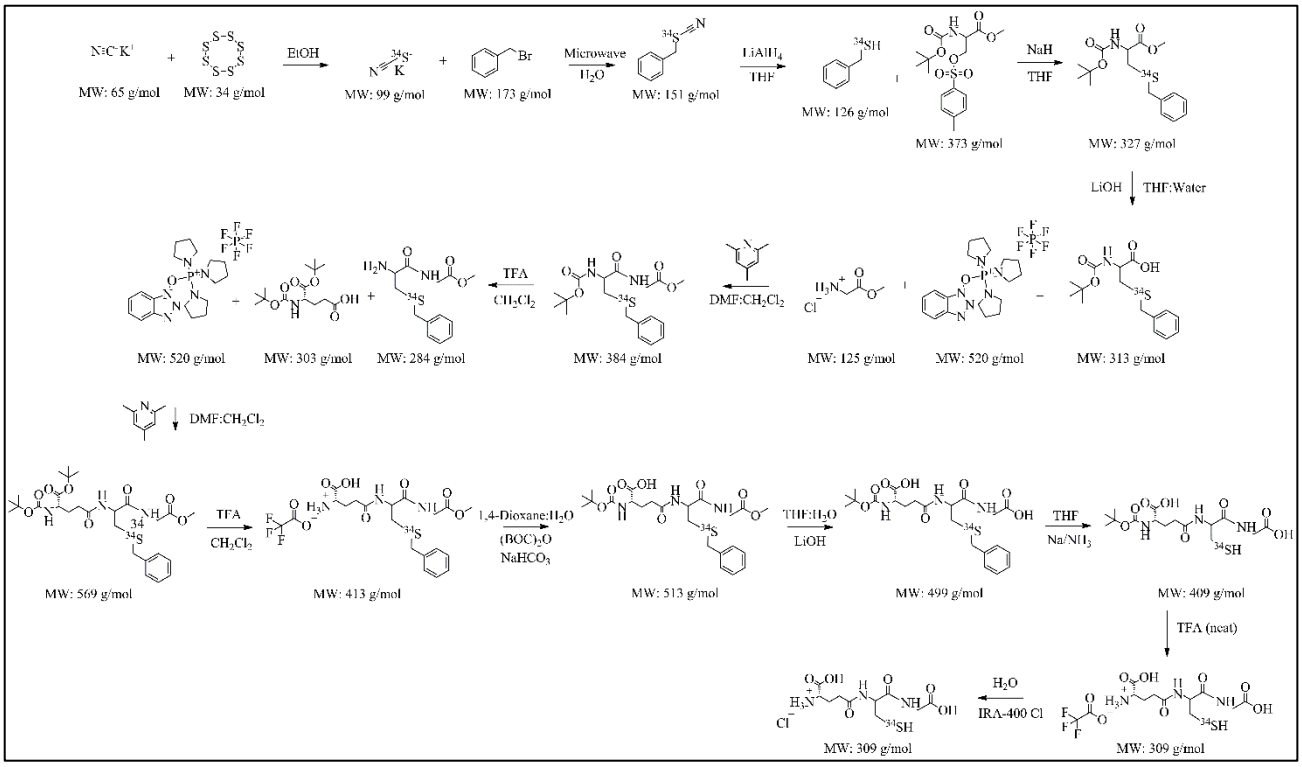


Figure 16. Initial scheme trialled for the preparation of isotopically labelled GSH

Yeast can only utilise amino acids in the L-configuration and once the synthesised glutathione was on hand it was analysed through polarimetry. This revealed the material to be a mixture of D,L- and L,L-diastereomers, and at that stage, 340 mg of ^{34}S GSH had been produced as a 7:3 mixture that was more abundant in the natural L-stereochemistry. The production of glutathione epimers was attributed to the racemisation of the cysteine stereocentre due to proton removal with sodium hydride during the formation of *S*-benzyl-cysteine. Despite numerous attempts and modifications to that reaction, epimerisation of the cysteine stereocentre could not be prevented.

Considering the issues encountered by using benzyl mercaptan, including the need to prevent epimerisation of the cysteine stereocentre, an alternate synthetic pathway was developed that did not require the coupling of benzyl mercaptan to the tosylate to produce *S*-benzyl-glutathione, thus eliminating the need for a dissolving metal reduction. Following the same initial steps, labelled elemental sulfur was reacted with potassium cyanide to form ^{34}S-potassium thiocyanate, which was directly reacted with the tosylate to produce methyl *N*-(*t*-butoxycarbonyl)-^{34}*S*-cyano-L-cysteinate. The cyano group was reduced with samarium(II) iodide to yield protected ^{34}S-cysteine (of which 470 mg was reserved for later use), which was oxidised to ^{34}S-cystine to protect the thiol group and prevent epimerisation during peptide coupling. ^{34}S-Cystine was coupled with glycine and glutamic acid using benzotriazol-1-yloxytripyrrolidinophosphonium hexafluorophosphate (PyBOP) as a coupling reagent to produce oxidised ^{34}S-glutathione (i.e., G^{34}S^{34}SG in 8% overall

yield beginning with elemental sulfur) as described in Gutierrez et al. (2015). Deprotection of oxidised ^{34}S-glutathione and reduction with tris(2-carboxyethyl) phosphine (TCEP) yields optically and chemically pure ^{34}S-L-glutathione after purification. The material was stored as the disulfide to prevent oxidation that would occur when storing the free thiol. A complete overview of the final synthetic pathway can be found in Figure 17. As with the previous scheme, this route provides access to labelled cysteine and dipeptide intermediates.

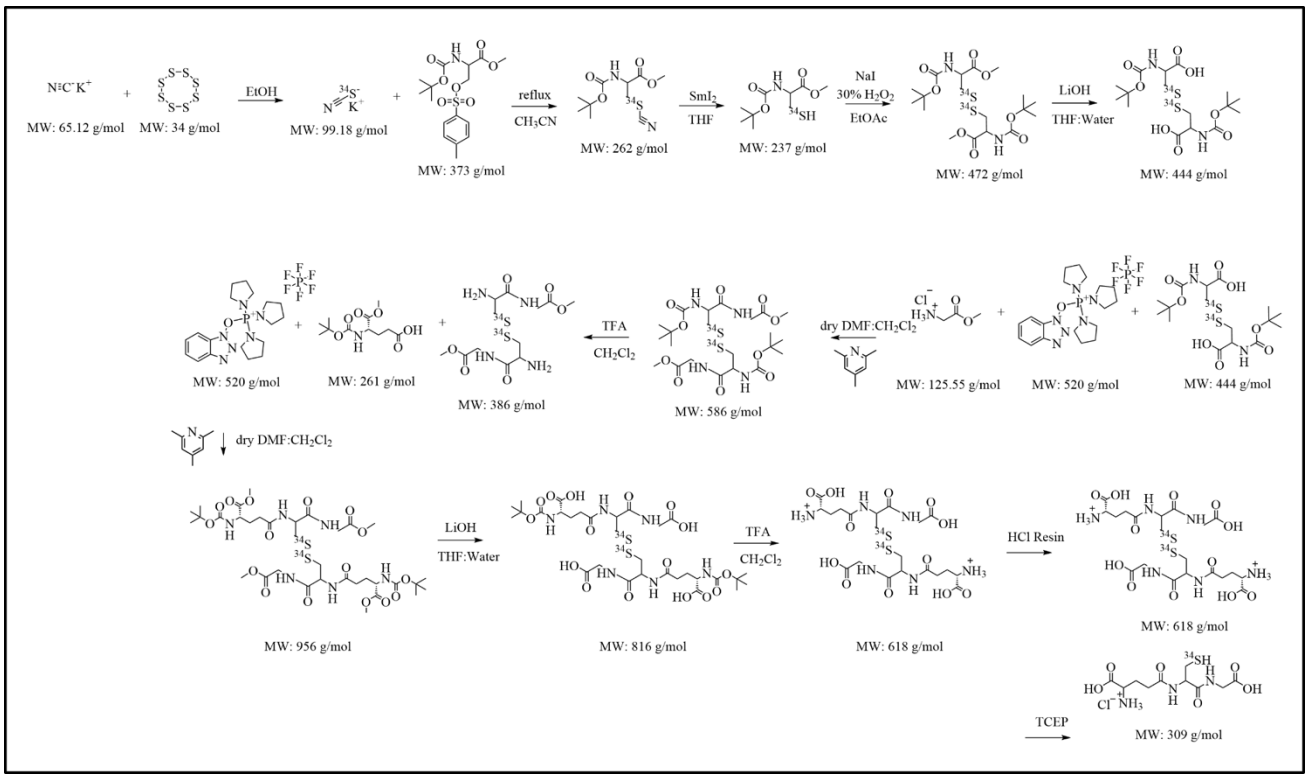


Figure 17. Final scheme for the synthesis of isotopically labelled GSH

7. Outcome/Conclusion

7.1. Performance against planned outputs

A summary of project performance against the specific objectives listed in Section 4 is given below. Overall, the project achieved the objectives set out in the original application, with the exception that attempts to produce isotopically labelled GSH for tracking the fate of sulfur through metabolism by yeast or reaction with juice constituents did not yield sufficient quantity of labelled ^{34}S-GSH by the project end date and ultimately was not used during the project.

7.1.1. Determine whether GSH addition to juice increases GSH concentration in wine and assess whether GSH concentration and its anti-oxidative efficacy are predictable

Through laboratory and pilot-scale work it was demonstrated that between 150 and 200 mg/L of GSH is required to be added to juice in order for it to be found in measurable quantities after completing the winemaking process. A consumption potential of 150 mg/L, either as a result of metabolic transformation by yeast or reaction with wine constituents, was consistently observed. Excess GSH, that is, the amount not consumed during the process of winemaking, did not contribute substantially to the antioxidative potential of the finished wines. Wines made with lower concentrations of SO_2 at bottling appeared oxidised at three months post-bottling, although inhibition of pinking was observed in Chardonnay wines, indicating some limited contribution to the antioxidant capacity of this particular wine.

7.1.2. Examine winemaking factors (e.g. timing of addition, amino acids, oxygen, SO_2) that may influence the relationship between juice and wine GSH concentrations

Glutathione concentrations in juice and wine were shown to be influenced by a variety of processing variables. It was demonstrated that a higher SO_2 concentration at bottling helped preserve GSH during wine ageing, but that the converse was not true, that is, GSH did not help preserve SO_2. Post-ferment GSH concentrations were not affected by the timing of GSH addition: both pre-inoculation and post-inoculation GSH additions resulted in similar GSH concentrations post-bottling. When GSH was added very early during grape processing (i.e. immediately after pressing), no GSH was detectable in the juice within an hour of addition, indicating very rapid reaction with juice components. It was also demonstrated that the concentration of reduced GSH post- alcoholic fermentation was unaffected by either inert or oxidative pressing and racking of juice when GSH was added after pressing.

The amount of GSH consumed during fermentation was proportional to the amount of GSH added. Increasing the nitrogen concentration suppressed GSH consumption during fermentation but not entirely. It is therefore difficult to envisage how the stipulation within the

OIV resolution that “the assimilable nitrogen level [of the juice] is sufficient to avoid the metabolism of glutathione by the yeast”, can be achieved in practice.

7.1.3. Identify the extent to which GSH concentration relates to the concentration of GSH and cysteine conjugates of varietal thiols in juice and free thiols in wine, and determine if this can be manipulated to enhance wine typicity

Changes in the concentration of varietal thiols in wine made with and without GSH addition were significant, but not of a magnitude sufficient to induce a shift in the varietal profile of the wine. The most marked effect was not in relation to varietal thiols but in the concentration of grape reaction product, the concentration of which increased substantially through GSH addition, particularly in wines that had been made from inertly prepared juice. Overall, the typicity of the wines made for this study, using OIV recommended dosages of GSH, did not vary beyond the bounds of what might be expected of a Chardonnay or Riesling.

7.1.4. Determine the effects of glutathione addition through sensory evaluation

Pre-fermentation GSH additions had little measurable effect on the organoleptic qualities of Chardonnay wine when added at low concentrations. At best, the effects of GSH addition could be described as subtle, showing enhancement of ‘apple’, ‘pear’ or ‘stone fruit’ attributes. Higher concentrations of GSH added either prior to, or following, initiation of fermentation demonstrated a high potential toward the development of ‘reductive’ characters, especially as the wines aged. This result demonstrates the risks inherent in the use of GSH in winemaking.

7.1.5. Track the fate of added GSH using chemical and genetic tools

Within this project, considerable effort was expended in an attempt to produce ^{34}S-labelled GSH. ^{34}S is a naturally occurring, stable, non-radioactive isotope of elemental sulfur and when incorporated into a compound will change its molecular weight sufficiently to be detectable by mass spectrometry. The production of ^{34}S-labelled GSH from elemental sulfur as a starting point, through synthesis of cysteine and then addition of the amino acids glutamate and glycine, was ultimately successful. However, successive technical hurdles culminated in significant delays in this aspect of the project with the result that ^{34}S-labelled GSH was not used within the project itself. Nevertheless, a set of materials was produced that has been made available to researchers in other Wine Australia-funded projects, and it is hoped that this will be used to resolve the fate of sulfur compounds during wine production and bottle ageing of wine.

Careful yeast strain selection, based on a detailed knowledge of wine yeast strain genetic composition, was used to probe the question of whether the consumption of GSH was increased in strains with a diversified genetic capacity for oligopeptide uptake. The strains X5

and VL3 were used for this purpose due to their varying complements of oligopeptide transporters, but otherwise indistinguishable genomic structure. This work showed that the amount of GSH lost during fermentation, across different initial GSH and YAN concentrations, was indistinguishable in ferments conducted by the different yeast strains. This suggests that strain choice would not be a promising mediating factor in the control of GSH consumption during fermentation.

7.2. Could changing the methodology/technology have improved the outcome?

Some aspects did not progress to the extent that was originally envisaged, at least in part due to technical limitations, particularly in the production of isotopically labelled GSH. Some of the choices made were guided by reagent cost and availability, and therefore could only be changed in hindsight. Two primary difficulties with synthesis of ^{34}S-labelled GSH related to the formation of epimers and the deprotection of sulfur or amine groups, with the latter case resulting in by-product formation where the protecting group reacted with the free-thiol group of GSH. Alternative synthetic reagents only became commercially available toward the end of the project, making modified approaches to the synthesis of ^{34}S-cysteine feasible, thus avoiding the issue of epimer formation. Alternative deprotection methodologies were also ultimately incorporated into the synthesis approach, which prevented the formation of GSH by-products.

7.3. Practical implications

The work outlined in this report has practical implications for Australian wine producers in the event that GSH gains regulatory approval as additive through FSANZ and in export markets. At the time of writing this report, no such approval has been granted and to the knowledge of the authors, no application for its use in Australia has been made. This research finds that there is little to be gained by the addition of GSH prior to or during fermentation, with limited and at best subtle effects on finished wines. Its use does not appear to facilitate a decrease in the concentration of SO_2 used during bottling and therefore one of the main potential drivers for its uptake as an additive in winemaking is repudiated. Considering the data presented here and elsewhere, it seems unlikely that an argument for GSH to be added to the relevant food standards codes as a wine additive will benefit wine producers or consumers.

7.4. Benefits to industry

The primary benefit to industry stemming from this work is a contribution to the body of knowledge surrounding the use and impact of a potential additive for winemaking. Should GSH become available as an additive this work creates a foundation that will inform how it should be used. If an application is made to FSANZ for the use of GSH in winemaking, this work may provide some scientific basis for appropriate applications and dosage rates.

8. Recommendations

The work undertaken in the course of this project was highly focused on the addition of pure GSH during the early stages of wine production. Clearly, high concentrations of GSH added during fermentation had a detrimental long-term impact on wine quality, with the evolution of high concentrations of volatile sulfur compounds after 12 months in bottle. While previous work in this area has shown that post-ferment GSH additions have led to small increases in the concentrations of the same volatile sulfur compounds (Ugliano et al. 2011, Bekker et al. 2018), it is unlikely that the pathways to the evolution of these compounds are the same for pre-fermentation and post-fermentation GSH additions. In the course of the work undertaken in this project, the GSH-induced evolution of 'reductive' characters was at least in part stimulated by contact with fermenting yeast, and later additions did not give rise to the same concentrations of H_2S in bottle. Certainly, in the work reported by Ugliano et al. (2011) and Bekker et al. (2018) the observed evolution of H_2S was far lower than the evolution of H_2S reported here.

The isotopically labelled GSH produced in this project provides a unique opportunity for future research investigating the various pathways to volatile sulfur compound evolution in bottle. It will enable a direct demonstration of whether there are multiple pathways to their evolution, and if there are, which are the most influential and amenable to remediation.

In addition to the potential future research directions outlined above, there are obvious potential policy implications that flow directly from the work presented. The use of GSH as a pure product in the process of winemaking comes with some obvious, inherent risks. GSH is known to participate in thermal reactions that can lead to the generation of sulfur-containing aroma compounds with 'cooked meat' aroma attributes (Lee et al. 2010) and commercial sources of GSH have been noted as having these same attributes. Hence, product quality issues with analytical grade GSH may exist that would need to be overcome to avoid the possibility of introducing a taint. There is also risk associated with overuse of GSH, either during production or bottling, potentially giving rise to product quality issues. In contrast to these risks there appears to be little to be gained through the application of pure GSH in winemaking and therefore a strong case would need to be made for it to be added to the food standards code pertinent to wine production.

9. Appendix 1: Communication:

Communication of the outcomes

Information has been extended to industry stakeholders through articles in trade journals, webinars and posters at the 17th Australian Wine Industry Technical Conference held in Adelaide in 2019.

Articles written during project

Schmidt, S.A., Bekker, M.Z., Sanders, R., Cuijvers, K.M., Puglisi, C., Jeffery, D.W. 2020. There's something about glutathione. *AWRI Technical Review* 246: 5–12.

Carne, N. 2016. If you could add glutathione, would you? Wine Australia publication retrieved 6 June 2021 from https://www.wineaustralia.com/news/articles/if-you-could-add-glutathione,-would-you

Posters

Cuijvers, K., Sanders, R., Kulcsar, A., Bekker, M.Z., Capone, D., Espinase-Nandorfy, D., David Jeffery, D.W., Schmidt, S. 2019. The effect of pre-ferment glutathione addition on Chardonnay and Riesling wine characteristics. T*he 17th Australian Wine Industry Technical Conference,* 21–24 July 2019, Adelaide SA.

Cuijvers, K., Sanders, R., Kulcsar, A., Bekker, M.Z., Capone, D., David Jeffery, D.W., Schmidt, S. 2019. Yeast strain dependent effects of glutathione addition on wine chemistry. T*he 17th Australian Wine Industry Technical Conference,* 21–24 July 2019, Adelaide SA.

10. Appendix 2: Intellectual Property

Nothing to report.

11. Appendix 3: References

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12. Appendix 4: Staff

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13. Appendix 5: Additional compositional and sensory data

Effect of GSH addition on the chemical composition of Chardonnay and Riesling wine.

Table 4. The effect of GSH addition immediately post-grape crush (PreInoc) or following yeast inoculation (PostInoc) on concentrations of volatile compounds in Chardonnay table wine. Control wines received no GSH. Wines were bottle at two SO2 concentrations (0.6 mg/L and 0.8 mg/L molecular). Summary table for two-way ANOVA with GSH addition SO2 concentration at bottling defined as the main variables. The table shows the mean analyte concentration with standard deviation in brackets and the calculated F statistic and P value for each of the main effects and the interaction.

analyte	units	Control 0.6	Control 0.8	PostInoc 0.6	PostInoc 0.8	PreInoc 0.6	PreInoc 0.8	F(2,12) GSH	Pval GSH	F(1,12) SO_2	Pval SO_2	F(2,12) Int	Pval Int
2-Methylbutanol	mg/L	21.6 (0.6)	22.1 (0.6)	21.8 (0.3)	21.5 (0.9)	21.7 (1.3)	21.5 (1.1)	0.14	0.87	0	0.98	0.34	0.7
3-Methylbutanol	mg/L	84 (3.6)	85.7 (1.7)	84.3 (1.3)	84 (2.6)	84 (2.8)	83.8 (5.1)	0.16	0.85	0.09	0.77	0.2	0.8
1-Butanol	µg/L	610.8 (9.8)	615.3 (17.5)	592.8 (38.2)	578.8 (63.3)	611.5 (26.1)	531.8 (44.9)	1.86	0.2	2.78	0.12	2.06	0.2
1-Hexanol	µg/L	970.8 (12.9)	930.1 (16.6)	948 (19.7)	925.9 (23.7)	933.5 (9.5)	936 (17.9)	1.45	0.27	6.05	0.03	2.35	0.1
2-Phenylethanol	mg/L	13.8 (1.2)	14.4 (0.4)	14 (0.6)	14.6 (1.2)	14.6 (1)	13.8 (1.3)	0.07	0.94	0.06	0.81	1.01	0.4
2-Methylpropanoic acid	mg/L	1.9 (0.3)	2.2 (0.1)	2.2 (0.1)	2.3 (0.1)	2.2 (0)	2 (0.4)	1.02	0.39	0.62	0.44	1.91	0.2
2-Methylbutanoic acid	µg/L	466.9 (14.4)	487.7 (3.6)	510.9 (2.5)	483.6 (6.8)	515.6 (4.4)	476.4 (32.5)	3.32	0.07	4.66	0.05	6.73	0
3-Methylbutanoic acid	µg/L	701.4 (86.7)	755.8 (16.3)	779 (4.3)	745.3 (17.1)	762.6 (6.9)	707 (89.7)	0.71	0.51	0.23	0.64	1.88	0.2
Acetic acid	µg/L	nd	nd	nd	nd	nd	nd	na	na	na	na	na	na
Propanoic acid	mg/L	1.5 (0.09)	1.3 (0.03)	1.3 (0.02)	1.4 (0.02)	1.3 (0.03)	1.3 (0.02)	6.46	0.01	12.33	<0.001	13.61	<0.001
Butanoic acid	mg/L	1.1 (0.15)	1.1 (0.01)	1.2 (0.1)	1.1 (0.04)	1.2 (0.08)	1.1 (0.08)	0.07	0.94	0.75	0.4	0.04	1
Hexanoic acid	mg/L	5.5 (0.56)	5.8 (0.07)	5.6 (0.34)	5.8 (0.02)	5.5 (0.59)	5.8 (0.1)	0.01	0.99	3.26	0.1	0.06	0.9
Octanoic acid	mg/L	7.7 (1.07)	7.6 (0.07)	7.5 (0.41)	7.8 (0.11)	7.6 (0.56)	7.7 (1)	0.01	0.99	0.07	0.79	0.1	0.9
Decanoic acid	mg/L	1.6 (0.26)	1.6 (0.1)	1.6 (0.09)	1.6 (0.11)	1.6 (0.11)	1.6 (0.34)	0.05	0.95	0.02	0.88	0.14	0.9
2-Methylpropyl acetate	µg/L	nd	nd	nd	nd	nd	nd	na	na	na	na	na	na
2-Methylbutyl acetate	µg/L	67.9 (4.4)	70.7 (0.6)	69.1 (0.9)	69.6 (2.4)	66.7 (1)	67.8 (3.9)	1.22	0.33	1.4	0.26	0.31	0.7
3-Methylbutyl acetate	mg/L	1.1 (0.13)	1.1 (0.01)	1.1 (0)	1.1 (0.04)	1.1 (0.04)	1.1 (0.15)	0.1	0.91	0.12	0.74	0.07	0.9
2-Phenylethyl acetate	µg/L	161.3 (19.3)	156.8 (0.6)	160.6 (1.1)	168.9 (12.8)	165.2 (11.8)	169.5 (21.4)	0.57	0.58	0.17	0.69	0.34	0.7
Hexyl acetate	µg/L	122.8 (11.6)	119.6 (1.2)	121.4 (4.1)	118.7 (1.1)	116.2 (0.6)	130.1 (10.2)	0.35	0.71	0.73	0.41	3.33	0.1
Ethyl acetate	mg/L	37.3 (0)	37.4 (0.6)	37 (1.2)	36.4 (0.6)	36.4 (0.5)	37.7 (0.3)	1.63	0.24	0.72	0.41	3.38	0.1
Ethyl propanoate	µg/L	59.6 (1)	59.8 (0.9)	60.9 (1.7)	57.4 (2.3)	59.7 (1.2)	56.5 (3.6)	0.98	0.4	5.14	0.04	1.5	0.3
Ethyl butanoate	µg/L	286.3 (9.9)	289.3 (4.9)	283.7 (2.8)	283.7 (4.8)	282.4 (4.4)	280 (11.5)	1.31	0.3	0	0.96	0.22	0.8
Ethyl hexanoate	µg/L	798.6 (5.6)	817.5 (2.8)	774.5 (13.6)	786.8 (10.6)	812.9 (12.8)	782.7 (37.4)	3.64	0.06	0	0.97	3.37	0.1
Ethyl octanoate	mg/L	1.1 (0.1)	1.1 (0)	1.1 (0)	1.1 (0)	1.1 (0)	1.1 (0.1)	0.41	0.67	0.13	0.73	0.19	0.8
Ethyl decanoate	µg/L	452 (90.3)	440.2 (14.7)	427.2 (51.3)	425.3 (65.4)	437.3 (51.3)	470.1 (63.2)	0.33	0.73	0.05	0.83	0.22	0.8
Ethyl 2-methylpropanoate	µg/L	71.2 (18)	81.2 (2.7)	81.8 (1.1)	77.1 (0.5)	77.3 (1.4)	69 (18.8)	0.52	0.61	0.04	0.84	1.25	0.3
Ethyl 2-methylbutanoate	µg/L	4.8 (0.6)	5.3 (0)	5.2 (0.1)	5.1 (0)	5 (0.2)	4.7 (0.2)	2.72	0.11	0.07	0.79	4.23	0

Ethyl 3-methylbutanoate	µg/L	9.2 (1.1)	10.1 (0.2)	10.2 (0.2)	9.7 (0.2)	9.5 (0.3)	9.6 (1)	0.75	0.49	0.45	0.52	1.77	0.2
H_2S	µg/L	1.8 (0.1)	1.7 (0)	4.3 (0.5)	4.8 (1.3)	4.1 (0.7)	3.7 (0.4)	28.88	<0.001	0	0.97	0.75	0.5
MeSH	µg/L	1.8 (0.3)	2.6 (0.1)	3 (0.2)	3.3 (0.3)	2.6 (0.3)	3.3 (0.1)	30.39	<0.001	29.26	<0.001	2.2	0.2
DMS	µg/L	6.4 (1.6)	6.3 (2.1)	7.8 (0.2)	7.8 (0.4)	7.9 (0.4)	8.6 (0.3)	4.91	0.03	0.11	0.74	0.22	0.8
DES	µg/L	0.3 (0)	0.3 (0.1)	0.3 (0)	0.2 (0.2)	0.3 (0)	0.3 (0)	1.3	0.31	0.4	0.54	1.3	0.3
CS_2	µg/L	0.6 (0.2)	0.4 (0.2)	1.1 (0.1)	0.7 (0.3)	1.2 (0.1)	0.8 (0.1)	14.02	<0.001	20.9	<0.001	1.49	0.3
3-SH	ng/L	0.2 (0.1)	0.4 (0)	0.6 (0.1)	0.7 (0.1)	0.5 (0.1)	0.6 (0)	35.11	<0.001	9	0.01	1.33	0.3
3-SHA	ng/L	nd	nd	nd	nd	nd	nd	na	na	na	na	na	na
4-MSP	ng/L	nd	nd	nd	nd	nd	nd	na	na	na	na	na	na
Benzenemethanethiol	ng/L	nd	nd	nd	nd	nd	nd	na	na	na	na	na	na
EtSH	µg/L	nd	nd	nd	nd	nd	nd	na	na	na	na	na	na
Alanine	mg/L	43.8 (2.9)	42 (2.9)	37.8 (3)	37.5 (2.3)	38.1 (1.1)	40.3 (3.8)	5.57	0.02	0	0.99	0.79	0.5
Arginine	mg/L	28.7 (5)	25.7 (0.4)	24.2 (2.2)	21.8 (1.6)	23.1 (4.1)	26 (3.9)	2.53	0.12	0.29	0.6	1.42	0.3
Asparagine	mg/L	10.6 (2.4)	10.7 (0.5)	9.8 (1)	9.8 (0.9)	10.2 (0.9)	9.4 (1.2)	0.92	0.43	0.2	0.66	0.2	0.8
Aspartic acid	mg/L	14.8 (2.8)	15.9 (0.3)	15.1 (1.5)	15.4 (0.8)	15.4 (0.4)	13.9 (2)	0.32	0.73	0	0.96	1.02	0.4
Cysteine	mg/L	4.3 (1.1)	6 (0.3)	7.7 (0.2)	8.1 (0.2)	6.2 (1.1)	5.4 (0.6)	25.21	<0.001	1.67	0.22	4.55	0
Glutamic acid	mg/L	35.5 (7.7)	37.2 (1.5)	35.8 (2.6)	35.6 (2)	36.4 (2.2)	34.7 (5.1)	0.06	0.94	0	0.97	0.24	0.8
Glutamine	mg/L	3.6 (0.6)	3.2 (0.2)	2.9 (0.2)	2.6 (0.4)	3.6 (0.8)	3.3 (0.7)	4.09	0.04	1.75	0.21	0.03	1
Glycine	mg/L	11.5 (2.4)	13.5 (0.7)	12.3 (1)	11.9 (0.8)	12.3 (0.2)	11.3 (1)	0.44	0.65	0.13	0.72	2.55	0.1
Histidine	µg/L	nd	nd	nd	nd	nd	nd	na	na	na	na	na	na
Isoleucine	mg/L	6.1 (1.9)	6.9 (0.6)	6.7 (0.6)	6.3 (0.1)	6.3 (0.4)	5.7 (2.4)	0.27	0.77	0.01	0.91	0.53	0.6
Leucine	mg/L	20.5 (6.6)	23.7 (1.6)	22.9 (1)	22.8 (0.8)	22.7 (1.3)	19.9 (5.7)	0.27	0.77	0	0.95	0.99	0.4
Lysine	mg/L	21.3 (2.6)	21.9 (0.5)	20.9 (1.2)	21 (1.4)	22 (0.2)	20.1 (2.2)	0.33	0.73	0.3	0.59	1.05	0.4
Methionine	mg/L	4.7 (1.1)	5.4 (0.1)	5.5 (0.3)	5.1 (0.1)	5.2 (0.2)	4.3 (1.1)	0.97	0.41	0.33	0.58	2.35	0.1
Methylpropanol	mg/L	10.6 (0.6)	10.7 (0.4)	10.6 (0.3)	10.6 (0.2)	10.7 (0.6)	10.6 (0.5)	0	1	0.01	0.93	0.13	0.9
Phenylalanine	mg/L	16.2 (3)	17.2 (0.6)	17.1 (1.5)	16 (0.5)	16.8 (1.4)	15.5 (3.1)	0.12	0.88	0.27	0.62	0.61	0.6
Proline	mg/L	818.5 (27.4)	814.4 (21.3)	764.7 (39.2)	786.9 (23.1)	802.5 (42.4)	801.3 (30.7)	2.55	0.12	0.14	0.71	0.31	0.7
Serine	mg/L	10 (2.4)	12.2 (0.2)	10.3 (0.6)	10.2 (0.1)	11.2 (0.7)	9.8 (1.9)	0.7	0.51	0.13	0.72	2.81	0.1
Threonine	mg/L	8.9 (2.3)	9.9 (0.1)	9.2 (0.9)	9.1 (0.7)	9.1 (0.2)	8.6 (1.7)	0.29	0.75	0.06	0.81	0.61	0.6
Tryptophan	mg/L	2.9 (0.8)	3 (0.1)	3.2 (0.2)	2.8 (0.1)	3 (0.2)	2.3 (0.7)	1.13	0.36	2.35	0.15	1.57	0.3
Tyrosine	mg/L	10.3 (1.7)	10.3 (0.1)	10.1 (0.8)	9.9 (0.6)	10.2 (0.4)	10.7 (2)	0.21	0.82	0.03	0.86	0.12	0.9
Valine	mg/L	8.7 (2.9)	9.6 (0.6)	9.3 (0.3)	9.2 (0.1)	9.7 (0.3)	8.4 (2.7)	0.02	0.98	0.05	0.82	0.75	0.5

Effect of GSH addition on the sensory attributes of Chardonnay and Riesling wine.

Table 5. Means of the significant (P≤0.05) and close to significant (P≤0.10) sensory attributes, Tukey's HSD and Mean Square Error values from the Chardonnay data and ANOVA.

Attribute	CTRL High SO_2	CTRL Low SO_2	POST-INOC High SO_2	POST-INOC Low SO_2	PRE-INOC High SO_2	PRE-INOC Low SO_2	HSD	MSE
Pink Tinge	0.55	1.96	0.26	0.49	0.18	0.73	0.30	0.357
Overall Fruit Aroma	4.17	4.37	3.74	3.60	3.66	3.77	0.37	0.561
Tropical Fruit Aroma	3.37	3.65	3.13	2.82	2.98	3.12	0.50	1.018
Strawberry Aroma	1.43	2.02	1.19	1.02	0.85	1.07	0.58	1.346
Stone Fruit Aroma	3.03	2.93	2.42	2.06	2.69	2.39	0.55	1.217
Yellow Confection Aroma	1.98	1.73	1.38	1.19	1.49	1.45	0.56	1.286
Box Hedge/Cat Pee Aroma	0.54	0.42	0.96	1.26	0.93	1.12	0.52	1.100
Flint Aroma	1.43	1.31	1.98	1.92	2.07	2.17	0.58	1.338
Cheesy/Baby Sick Aroma	0.72	0.60	1.85	1.51	1.42	1.53	0.66	1.780
Honey Aroma	0.95	1.68	0.87	1.38	1.10	1.20	0.59	1.421
Drain Aroma	0.41	0.49	0.75	0.89	1.08	1.02	0.63	1.611
Overall Fruit Flavour	4.31	4.37	4.24	3.97	4.31	4.18	0.27	0.285
Stone Fruit Flavour	3.20	3.03	3.20	2.45	3.13	2.79	0.57	1.330
Viscosity	3.59	3.54	3.70	3.49	3.71	3.71	0.20	0.167
Bitter Taste	3.12	2.64	2.23	2.81	2.46	2.69	0.63	1.621
Fruit Aftertaste	3.27	3.32	3.38	2.99	3.38	3.05	0.31	0.389

HSD = Tukey's honest significant differences (*P*≤0.10) value. MSE = Mean Square Error.

Table 6. Means of the significant (P≤0.05) and close to significant (P≤0.10) sensory attributes, Tukey's HSD and Mean Square Error values from the Riesling data and ANOVA.

Attribute	CTRL High SO_2	CTRL Low SO_2	POST-INOC High SO_2	POST-INOC Low SO_2	PRE-INOC High SO_2	PRE-INOC Low SO_2	HSD	MSE
Yellow Colour	2.80	3.10	2.79	3.17	2.62	3.01	0.18	0.135
Overall Fruit Aroma	3.90	3.84	3.41	3.52	3.71	3.59	0.34	0.475
Stone Fruit Aroma	2.79	2.46	2.00	2.39	2.26	2.49	0.51	1.033
Blossom Floral Aroma	2.75	2.40	1.97	2.45	2.27	2.29	0.55	1.214
Capsicum Aroma	1.39	0.86	2.19	1.61	1.82	1.33	0.63	1.588
Box Hedge/Cat Pee Aroma	1.45	0.97	3.30	2.24	2.59	1.46	0.84	2.846
Flint Aroma	1.99	1.97	2.49	2.34	2.52	1.85	0.53	1.132
Honey Aroma	1.11	1.58	0.65	0.95	1.26	1.62	0.61	1.522
Pungent	3.03	2.83	3.27	3.02	3.17	2.95	0.33	0.444
Overall Fruit Flavour	4.17	4.26	4.05	4.09	4.05	4.00	0.27	0.290
Acidity	3.50	3.48	3.39	3.44	3.51	3.31	0.27	0.294
Fruit Aftertaste	2.97	2.95	2.64	2.92	2.74	2.87	0.29	0.337

HSD = Tukey's honest significant differences (*P*≤0.10) value. MSE = Mean Square Error.

Table 7. Probability values and degrees of freedom (df) from analysis of variance for the combined Chardonnay and Riesling sensory descriptive data.

Attribute	V	G	S	V*G	V*S	G*S	V*G*S	MSE
Yellow Colour	0.000***	0.021*	0.000***	0.001***	0.000***	0.168	0.773	0.1615
Pink Tinge	0.000***	0.000***	0.000***	0.000***	0.000***	0.000***	0.000***	0.2426
Overall Fruit Aroma	0.000***	0.000***	0.786	0.076ǂ	0.495	0.826	0.161	0.586
Tropical Fruit Aroma	0.258	0.000***	0.829	0.110	0.464	0.426	0.130	1.0045
Strawberry Aroma	0.037*	0.000***	0.072ǂ	0.056ǂ	0.596	0.195	0.183	1.3037
Apple Bruised Apple Aroma	0.000***	0.044*	0.084	0.250	0.243	0.414	0.326	1.2208
Stone Fruit Aroma	0.032*	0.000***	0.377	0.357	0.045*	0.533	0.052ǂ	1.233
Citrus Aroma	0.079ǂ	0.778	0.340	0.704	0.146	0.364	0.253	0.776
Yellow Confection Aroma	0.142	0.002**	0.971	0.412	0.119	0.449	0.225	1.6568
Blossom Floral Aroma	0.472	0.001***	0.849	0.918	0.480	0.128	0.160	1.483
Capsicum Aroma	0.000***	0.000***	0.003**	0.182	0.005**	0.762	0.631	1.3375
Box Hedge/Cat Pee Aroma	0.000***	0.000***	0.001***	0.004**	0.000***	0.834	0.142	2.199
Dry Grass Aroma	0.007**	0.852	0.519	0.307	0.176	0.814	0.676	1.3498
Flint Aroma	0.000***	0.000***	0.086ǂ	0.040*	0.159	0.585	0.124	1.31
Cheesy/Baby Sick Aroma	0.000***	0.000***	0.969	0.003**	0.267	0.596	0.381	1.7189
Honey Aroma	0.985	0.005**	0.000***	0.047*	0.722	0.329	0.487	1.6384
Dank Drain Aroma	0.000***	0.000***	0.770	0.084ǂ	0.421	0.385	0.821	1.9068
Pungent	0.018*	0.060ǂ	0.008**	0.134	0.077ǂ	0.607	0.422	0.4124
Overall Fruit Flavour	0.006**	0.002**	0.308	0.288	0.133	0.191	0.444	0.342
Tropical Fruit Flavour	0.001***	0.010*	0.845	0.857	0.446	0.157	0.666	0.8878
Stone Fruit Flavour	0.000***	0.036*	0.149	0.711	0.001***	0.496	0.291	1.337
Citrus Flavour	0.000***	0.999	0.974	0.417	0.879	0.682	0.518	0.728
Flint Flavour	0.000***	0.793	0.203	0.253	0.911	0.227	0.853	0.832
Capsicum Flavour	0.000***	0.001***	0.359	0.361	0.395	0.155	0.111	1.1377
Sweetness Taste	0.000***	0.450	0.941	0.727	0.901	0.700	0.078ǂ	0.901
Viscosity	0.000***	0.393	0.027*	0.027*	0.645	0.719	0.018*	0.1744
Acidic Taste	0.000***	0.295	0.004**	0.005**	0.709	0.171	0.722	0.438
Hotness	0.010**	0.853	0.988	0.591	0.867	0.740	0.806	0.594
Astringency	0.000***	0.192	0.008**	0.785	0.346	0.312	0.836	0.612
Bitter Taste	0.000***	0.803	0.498	0.034*	0.672	0.008**	0.502	1.688
Fruit Aftertaste	0.000***	0.063ǂ	0.363	0.805	0.001***	0.684	0.012*	0.4525
df	1	2	1	2	1	2	2	625

†Significance levels are as follows: * $P \leq 0.05$; ** $P \leq 0.01$; *** $P \leq 0.001$; ǂ $P \leq 0.10$. df = degrees of freedom, Judge effect was significant for all attributes $P \leq 0.01$. G: Glutathione addition; S: SO_2level; V: Variety; MSE: Mean Square Error.

Effect of GSH addition on the chemical composition of Chardonnay sparkling base and Chardonnay table wine

Table 8. The effect of GSH addition immediately post-grape pressing on concentrations of volatile compounds in Chardonnay table wine. Summary table for two-way ANOVA with yeast strain and GSH addition defined as the main variables. The table shows the mean analyte concentration with standard deviation in brackets and the calculated F statistic and P value for each of the main effects and the interaction.

analyte	units	Cont VL3	Cont X5	GSH VL3	GSH X5	F(1,8) Yeast	Pval Yeast	F(1,8) GSH	Pval GSH	F(1,8) INT	Pval INT
pH		3.3 (0)	3.3 (0.01)	3.3 (0.01)	3.3 (0.01)	39.2	<0.001	28.8	<0.001	0	1
TA	g/L	6.5 (0)	6.6 (0.23)	6.4 (0.06)	6.4 (0)	1.47	0.26	7.12	0.03	0.53	0.49
VA	g/L	0.4 (0.01)	0.5 (0.03)	0.4 (0.02)	0.4 (0.01)	14.4	0.01	0.9	0.37	0.9	0.37
Residual_sugar	g/L	0.8 (0.1)	0.8 (0.2)	0.9 (0.2)	0.9 (0.1)	0.25	0.63	4	0.08	0.25	0.63
Free_SO_2	mg/L	30 (4.6)	28 (4.5)	36 (10.2)	28 (2)	2	0.19	0.72	0.42	0.89	0.37
Total_SO_2	mg/L	80 (5.6)	86 (4.6)	88 (12.9)	83 (2.5)	0.01	0.91	0.25	0.63	1.62	0.24
Malic_acid	g/L	2.17 (0.02)	2.11 (0.04)	2.21 (0.01)	2.16 (0.01)	17.02	<0.001	8.27	0.02	0.14	0.72
Ethanol	g/L	88.6 (0.5)	88.3 (0.5)	87.5 (2.9)	88 (0.7)	0.02	0.9	0.62	0.45	0.19	0.68
2-Methylpropanol	mg/L	15.9 (1.3)	17 (1.9)	16 (0.9)	15.2 (1)	0.04	0.85	1.1	0.32	1.45	0.26
2-Methylbutanol	µg/L	2000 (0)	2000 (0)	2000 (0)	2000 (0)	1	0.35	1	0.35	1	0.35
3-Methylbutanol	mg/L	123.6 (12.4)	120 (14.3)	101.6 (8)	122.3 (22.1)	0.96	0.36	1.27	0.29	1.94	0.2
1-Butanol	µg/L	900 (0)	1139 (40)	900 (0)	1081 (33)	194.06	<0.001	3.65	0.09	3.65	0.09
1-Hexanol	µg/L	1088 (84)	1086 (45)	1034 (26)	1293 (240)	2.92	0.13	1.05	0.34	3.03	0.12
2-Phenylethanol	mg/L	16 (0.1)	13.8 (1.7)	15.1 (0.3)	13.1 (1.5)	9.61	0.01	1.42	0.27	0.01	0.93
Glycerol	g/L	5.8 (0.1)	6.7 (0.1)	5.7 (0.3)	6.3 (0.2)	44.49	<0.001	6.15	0.04	2.44	0.16
2-Methylpropanoic acid	µg/L	1214 (110)	1083 (123)	1124 (80)	968 (38)	7.01	0.03	3.6	0.09	0.05	0.83
2-Methylbutanoic acid	µg/L	272 (15)	298 (37)	258 (14)	258 (9)	1.08	0.33	4.68	0.06	1.08	0.33
3-Methylbutanoic acid	µg/L	397 (22)	451 (66)	401 (25)	402 (13)	1.6	0.24	1.1	0.33	1.45	0.26
Acetic acid	mg/L	201.4 (11.9)	228.2 (35.5)	184.6 (2.4)	199.7 (11.8)	3.41	0.1	3.99	0.08	0.27	0.62
Propanoic acid	µg/L	1386 (527)	1081 (46)	937 (174)	1105 (35)	0.18	0.68	1.73	0.22	2.16	0.18
Butanoic acid	µg/L	1781 (200)	1343 (22)	1544 (118)	1457 (62)	14.2	0.01	0.78	0.4	6.39	0.04
Hexanoic acid	mg/L	7.1 (0.4)	5.4 (1.2)	6.2 (0.4)	6 (0.2)	6.84	0.03	0.15	0.71	3.96	0.08
Octanoic acid	mg/L	10.3 (0.4)	8.1 (1.3)	9.4 (0.4)	9.3 (0.4)	6.99	0.03	0.14	0.72	5.68	0.04
Decanoic acid	µg/L	3003 (257)	2524 (394)	2581 (176)	2811 (127)	0.69	0.43	0.2	0.66	5.62	0.05
Succinic acid	g/L	3.8 (0)	3.7 (0.1)	3.9 (0)	3.7 (0)	15.59	<0.001	0.89	0.37	0	0.96
2-Methylpropyl acetate	µg/L	25 (5)	23 (3)	28 (1)	26 (3)	1.09	0.33	2.03	0.19	0.01	0.93
2-Methylbutyl acetate	µg/L	47 (6)	44 (7)	53 (2)	54 (3)	0.09	0.77	8.7	0.02	0.82	0.39
3-Methylbutyl acetate	µg/L	828 (107)	785 (50)	918 (34)	963 (32)	0	0.98	13.21	0.01	1.42	0.27
2-Phenylethyl acetate	µg/L	156 (7)	107 (4)	158 (3)	118 (4)	294.82	<0.001	6.02	0.04	2.82	0.13
Hexyl acetate	µg/L	75 (11)	72 (6)	86 (6)	86 (6)	0.13	0.73	9.25	0.02	0.13	0.73
Ethyl acetate	mg/L	37.8 (2.5)	44.5 (4.9)	38 (0.7)	43.5 (1.3)	13.64	0.01	0.05	0.83	0.13	0.72
Ethyl propanoate	µg/L	148 (8)	200 (19)	146 (6)	187 (9)	51.37	<0.001	1.44	0.26	0.63	0.45
Ethyl butanoate	µg/L	422 (32)	353 (19)	416 (21)	385 (17)	14.57	0.01	0.92	0.36	2.08	0.19
Ethyl hexanoate	µg/L	1189 (58)	981 (125)	1155 (45)	1169 (51)	4.77	0.06	3	0.12	6.29	0.04

Ethyl octanoate	µg/L	1621 (66)	1403 (170)	1626 (18)	1612 (49)	4.51	0.07	3.79	0.09	3.49	0.1
Ethyl decanoate	mg/L	16.1 (2.4)	12.4 (1.3)	12.5 (3)	13.2 (6.2)	0.48	0.51	0.41	0.54	1.09	0.33
Ethyl 2-methylpropanoate	µg/L	130 (4)	126 (24)	132 (9)	108 (7)	3.31	0.11	1.12	0.32	1.72	0.23
Ethyl 2-methylbutanoate	µg/L	14 (1)	15 (1.7)	13.7 (1.2)	13.3 (0.6)	0.24	0.64	2.12	0.18	0.94	0.36
Ethyl 3-methylbutanoate	µg/L	21.3 (0.6)	23.3 (3.2)	21.3 (1.2)	20.7 (1.2)	0.4	0.54	1.6	0.24	1.6	0.24
H_2S	µg/L	2.6 (0.8)	2.5 (0.3)	2.6 (0.8)	2.6 (0.3)	0.05	0.83	0.04	0.84	0.01	0.94
MeSH	µg/L	1.7 (0.2)	2.1 (0.2)	2 (0.3)	2.2 (0.3)	3.05	0.12	2.03	0.19	0.12	0.74
DMS	µg/L	8.9 (0.42)	10.5 (0.69)	10.3 (1.33)	12.2 (1.22)	9.92	0.01	7.64	0.02	0.1	0.76
3-SH	ng/L	297 (16)	507 (67)	453 (129)	1006 (100)	55.47	<0.001	40.89	<0.001	11.23	0.01
3-SHA	ng/L	3.6 (0.7)	7.8 (2.2)	7.4 (1)	10.7 (1.4)	20.71	<0.001	16.52	<0.001	0.36	0.57
Benzenemethanethiol	ng/L	3 (1)	3 (0)	3 (1)	4 (0)	3.29	0.11	0.17	0.69	0.28	0.61

Table 9. The effect of GSH addition immediately post-grape crush on concentrations of volatile compounds in Chardonnay sparkling base wine. Summary table for two-way ANOVA with yeast strain and GSH addition defined as the main variables. The table shows the mean analyte concentration with standard deviation in brackets and the calculated F statistic and P value for each of the main effects and the interaction.

analyte	units	NT VL3	NT X5	GSH VL3	GSH X5	F(1,8) Yeast	Pval Yeast	F(1,8) GSH	Pval GSH	F(1,8) INT	Pval INT
pH		3 (0.01)	3 (0.01)	3 (0.01)	3 (0.01)	9.14	0.02	0.57	0.47	0.57	0.47
TA	g/L	8.4 (0)	8.6 (0.17)	8.4 (0.06)	8.6 (0.1)	9.31	0.02	0.08	0.79	0.08	0.79
VA	g/L	0.2 (0.01)	0.2 (0.03)	0.2 (0.01)	0.3 (0.01)	22.73	<0.001	7.81	0.02	1.32	0.28
Residual_sugar	g/L	0.1 (0)	0.1 (0)	0.1 (0)	0.1 (0)	1	0.35	1	0.35	1.00	0.35
Free_SO_2	mg/L	14 (0)	10 (3.6)	11 (1.5)	4 (3.2)	14.14	0.01	8.12	0.02	1.05	0.34
Total_SO_2	mg/L	61 (1.5)	65 (3.6)	60 (1.7)	67 (3.5)	11.25	0.01	0.04	0.84	1.10	0.33
Malic_acid	g/L	3.47 (0.05)	3.4 (0.08)	3.45 (0.02)	3.37 (0.03)	7.36	0.03	0.82	0.39	0.09	0.77
Ethanol	g/L	76.3 (0.7)	75.8 (1)	75.9 (1)	75 (0.5)	1.96	0.2	1.57	0.25	0.17	0.69
2-Methylpropanol	mg/L	19.1 (2.1)	16.8 (0.6)	17.3 (1.8)	18.2 (3)	0.32	0.59	0.02	0.89	1.82	0.21
2-Methylbutanol	µg/L	2000 (0)	2000 (0)	2000 (0)	2000 (0)	1	0.35	1	0.35	1.00	0.35
3-Methylbutanol	mg/L	112.1 (6.5)	129.2 (28.3)	99.6 (5.4)	113.9 (7.8)	3.14	0.11	2.46	0.16	0.03	0.88
1-Butanol	µg/L	900 (0)	900 (0)	900 (0)	900 (0)	1	0.35	1	0.35	1.00	0.35
1-Hexanol	µg/L	1712 (799)	2338 (465)	2006 (130)	2116 (155)	1.81	0.22	0.02	0.9	0.89	0.37
2-phenylethanol	mg/L	14.5 (0.3)	14.2 (2)	15.1 (1.5)	13.8 (1.4)	0.89	0.37	0.01	0.91	0.37	0.56
Glycerol	g/L	6 (0.1)	7 (0.3)	5.5 (0.1)	7 (0.3)	116.78	<0.001	6.28	0.04	3.55	0.1
2-Methylpropanoic acid	µg/L	1174 (78)	1097 (38)	1162 (10)	1082 (82)	5.15	0.05	0.15	0.71	0.00	0.96
2-Methylbutanoic acid	µg/L	263 (6)	282 (28)	258 (10)	291 (9)	8.32	0.02	0.05	0.83	0.55	0.48
3-Methylbutanoic acid	µg/L	420 (7)	432 (23)	416 (24)	439 (36)	1.55	0.25	0.01	0.94	0.15	0.71
Acetic acid	mg/L	181.9 (10.4)	228.1 (30.2)	200.2 (18.9)	261.8 (6.6)	24.53	<0.001	5.73	0.04	0.50	0.5
Propanoic acid	µg/L	917 (66)	1395 (280)	948 (133)	1356 (92)	21.56	<0.001	0	0.97	0.13	0.73
Butanoic acid	µg/L	1201 (112)	1263 (55)	1370 (317)	1122 (72)	0.85	0.38	0.02	0.89	2.38	0.16
Hexanoic acid	mg/L	5.4 (0.4)	4.5 (0.5)	5.6 (0.7)	4.1 (0.3)	18.92	<0.001	0.06	0.81	1.32	0.28
Octanoic acid	mg/L	8.9 (0.6)	7 (0.7)	9 (1.1)	6.5 (0.2)	28.4	<0.001	0.16	0.7	0.68	0.43
Decanoic acid	µg/L	2482 (184)	2315 (138)	2679 (370)	2159 (50)	7.36	0.03	0.03	0.88	1.95	0.2
Succinic acid	g/L	4.7 (0)	4.6 (0)	4.7 (0)	4.6 (0.1)	8.11	0.02	0	0.95	0.01	0.93
2-Methylpropyl acetate	µg/L	11 (1)	11 (1)	11 (2)	13 (2)	1.63	0.24	0.83	0.39	1.63	0.24
2-Methylbutyl acetate	µg/L	18 (2)	19 (3)	16 (3)	19 (2)	1.58	0.24	0.04	0.84	0.40	0.55
3-Methylbutyl acetate	µg/L	290 (54)	271 (23)	292 (30)	241 (33)	2.72	0.14	0.44	0.53	0.59	0.46
2-Phenylethyl acetate	µg/L	58 (3)	42 (2)	60 (4)	39 (1)	147.56	<0.001	0.2	0.67	2.39	0.16
Hexyl acetate	µg/L	53 (12)	47 (6)	58 (7)	40 (7)	6.93	0.03	0.02	0.89	1.46	0.26
Ethyl acetate	mg/L	28.2 (1.1)	34.4 (4.6)	31.3 (2.8)	40 (0.4)	22.1	<0.001	7.49	0.03	0.63	0.45
Ethyl propanoate	µg/L	138 (4)	203 (9)	144 (12)	218 (20)	94.74	<0.001	2.14	0.18	0.39	0.55
Eethyl butanoate	µg/L	247 (17)	241 (12)	264 (21)	230 (8)	4.94	0.06	0.14	0.72	2.62	0.14
Ethyl hexanoate	µg/L	805 (47)	701 (44)	865 (76)	656 (4)	29.42	<0.001	0.06	0.81	3.27	0.11
Ethyl octanoate	µg/L	1211 (64)	1046 (88)	1301 (99)	983 (7)	32.23	<0.001	0.1	0.76	3.21	0.11
Ethyl decanoate	mg/L	6.4 (5.1)	9.3 (1.7)	7.5 (5.9)	8.7 (1.1)	0.81	0.39	0.01	0.92	0.12	0.73
Ethyl 2-methylpropanoate	µg/L	146 (6)	134 (14)	156 (12)	144 (17)	2.65	0.14	1.73	0.22	0.00	0.98
Ethyl 2-methylbutanoate	µg/L	15.3 (0.6)	18.3 (2.1)	17 (1.7)	19.7 (2.1)	8.03	0.02	2.25	0.17	0.03	0.87
Ethyl 3-methylbutanoate	µg/L	27.3 (1.2)	29 (2.6)	29 (2.6)	32 (3.6)	2.31	0.17	2.31	0.17	0.19	0.68

H_2S	µg/L	1.9 (0.1)	1.8 (1.7)	1.7 (0.1)	2 (0.5)	0.05	0.82	0	0.98	0.08	0.79
MeSH	µg/L	0 (0)	1 (0.9)	0.9 (0.8)	1.4 (0.1)	4.71	0.06	3.49	0.1	0.63	0.45
DMS	µg/L	4.6 (0.4)	4.6 (0.55)	4.9 (0.51)	4.9 (0.34)	0	0.95	1.24	0.3	0.00	0.97
3-SH	ng/L	185 (45)	279 (86)	111 (10)	171 (67)	4.99	0.06	7.06	0.03	0.25	0.63
3-SHA	ng/L	0.3 (0.6)	2.1 (0.2)	1.2 (0.4)	2.6 (1)	19.35	<0.001	3.77	0.09	0.38	0.55
Benzenemethanethiol	ng/L	4 (0)	4 (1)	3 (0)	3 (1)	0.02	0.9	1.34	0.28	0.48	0.51

14. Appendix 6: Budget reconciliation

Budget details will be forwarded separately.