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Research Article

Effects of Yeast Strain and Juice Nitrogen Status on Glutathione Utilisation during Fermentation of Model Media

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Background and Aims. An OIV resolution provides guidelines on using glutathione as a prefermentation additive when the amount of yeast assimilable nitrogen (YAN) of a juice or must is adequate, to avoid the metabolism of glutathione by the yeast. The effect of YAN concentration on glutathione metabolism by yeast had not been determined. This study explored whether nitrogen management could be used to control glutathione consumption during fermentation. *Methods and Results*. An HPLC-UV method was developed to quantify reduced L-glutathione (GSH) and oxidised glutathione (GSSG) and used to monitor yeast GSH metabolism during alcoholic fermentation with two yeast strains (AWRI 1688 and AWRI 2861). The addition of GSH had no impact on the fermentation rate of the chemically defined medium, even in a limited YAN environment; however, a decrease in glutathione concentration occurred regardless of YAN concentration. The effect of GSH on volatile sulfur compound formation was yeast strain-dependent. *Conclusions*. Increasing the YAN status of a chemically defined medium led to a decrease in GSH consumption during fermentation, but the loss of GSH could not be prevented entirely, even with a low initial GSH concentration and high initial YAN. *Significance of the Study*. In the presence of higher concentrations of GSH during fermentation, there is a risk of forming undesirable fermentative sulfur compounds that are not mitigated through nitrogen supplementation. Thus, it seems unlikely that an argument could be made for the inclusion of GSH in relevant food standards codes as a wine additive especially if a lack of GSH metabolism was a criterion.

1. Introduction

Glutathione (GSH) is a naturally occurring antioxidant present in grapes, wines, and other living organisms such as yeast [1]. It plays an important role in winemaking, preserving wine colour and aroma by reacting sacrificially with quinones, and acting as an antioxidant [2, 3]. As a result, GSH has been investigated as a potential alternative to sulfur dioxide (SO_2) for the preservation of wine freshness and aroma intensity [4]. Glutathione may contribute to the sensory attributes of wine aroma through the liberation of hydrogen sulfide (H_2S) during fermentation [5, 6], a process that is also dependent on the nitrogen status of the juice [7]. Elevated residual GSH concentrations in finished wine have also been shown to contribute to H_2S accumulation postbottling [8, 9]. Especially in combination with copper, GSH creates an environment that favours the accumulation of volatile sulfur compounds (VSCs), such as H_2S and methanethiol (MeSH) [9]. Glutathione may also contribute to the sensory attributes of wine by reacting with unsaturated aldehydes to produce varietal thiol precursors, which could be cleaved during fermentation to liberate potent thiols that modulate the varietal character of wines such as Sauvignon Blanc [10].

Aside from naturally occurring GSH in grapes, other potential sources in wine include yeast-derived products, some of which are marketed as GSH-enriched inactive dried

yeast preparations. Compared to grape processing interventions that can preserve GSH [11], however, the contribution of these additives to juice or wine is minimal, increasing GSH concentrations between 1 and 3.5 mg/L when used at recommended addition rates [12–14]. The environmental nutrient status may influence the uptake of GSH by yeast although contradicting results for the scale of impact are found in the literature. Kritzinger et al. [15] found that increased YAN concentration did not result in changes in GSH concentration, which suggests that neither GSH synthesis nor the metabolism of GSH by the yeast was influenced by the nutrient status of the must. However, Park et al. [16] found that GSH production was directly correlated to the total nitrogen and YAN content of the grape juice in their experiment. The fundamental differences in experimental design between the two studies may explain the contradicting reports, with Kritzinger et al. [15] adjusting the YAN concentration of a single must, whereas Park et al. [16] used various grape musts that contained different natural YAN levels. In a complementary study, Wegmann-Herr et al. [6] evaluated the effects of added diammonium phosphate (DAP) and GSH on sulfur metabolism by yeast and found that GSH addition (40–50 mg/L) did not increase VSC when a sufficient nitrogen pool was provided such that it was not necessary for the yeast to metabolise the added GSH.

In 2015, the International Organisation of Vine and Wine (OIV) passed two resolutions relating to the addition of GSH during wine production (OIV-OENO 445-2015 and OIV-OENO 446-2015). These resolutions state

(1) OIV-OENO 445-2015

  (i) 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

  (ii) The dose used should not exceed 20 mg/L

(2) OIV-OENO 446-2015

  (i) The addition of glutathione on storage and/or packaging, including the bottling of wine, is recommended

  (ii) The dose used should not exceed 20 mg/L

  (iii) The glutathione must be in a reduced form and comply with the prescriptions of the International Oenological Codex

The objectives of these resolutions are to limit the intensity of oxidation phenomena in must or wine due to the ability of GSH to act as an antioxidant, thereby preventing browning and protecting aroma compounds in wines (and particularly those of the thiol family) from oxidation. Notably, oxidised and 'reduced' aroma faults are two of the most common wine off-aromas that result in decreased shelf life and are directly influenced by the amount of oxygen a wine is exposed to during bottle ageing [17]. Depending on a wine's composition and the concentration of antioxidants such as SO_2 and GSH, trace amounts of the short-chain unsaturated aldehydes (methional and phenylacetaldehyde, among others) may be produced, imparting aromas described as sherry, honey, bruised apple, and others associated with oxidation spoilage [18]. Increased oxygen exposure can also result in the loss of tropical aromas associated with varietal compounds, particularly certain polyfunctional thiols including 3-sulfanylhexan-1-ol (3-SH), 3-sulfanylhexyl acetate (3-SHA), and 4-methyl-4-sulfanylpentan-2-one (4-MSP) [19]. On the other hand, depending on wine composition, 'reductive' characters may develop during bottle ageing of wines that have been completely protected from oxygen during storage [9]. These aroma characters are mainly ascribed to the presence of H_2S and MeSH, which impart aromas of rotten egg, sewage, burnt rubber, and putrefaction [20]. As a result, the addition of GSH at different stages of winemaking may have profound implications on the formation of volatile compounds that contribute positively or negatively to wine sensory properties.

Considering that GSH may become a regulated additive used during vinification, it was important to establish an accessible method for the analysis of GSH in reduced and oxidised (GSSG) forms that can be standardised for research and commercial laboratories, and wineries. The conventional methods for quantitating GSH and GSSG in tissue, plasma, and liquid matrices from human, animal, or plant material include high-performance liquid chromatography (HPLC) coupled to ultraviolet (UV) [21, 22], fluorometric [23–25], electrochemical [26], and mass spectrometric [27–29] detectors. Enzymatic assays have also been utilised [30], but due to its enhanced sensitivity, HPLC with tandem mass spectrometry (HPLC-MS/MS) is preferred for the analysis of blood and tissue samples, where the requirements for GSH and GSSG quantification can be as low as a few ng/L [31]. The use of HPLC-MS/MS for the analysis of wine and juice samples has also been widely implemented [32, 33]. Methods relying upon MS detection have the added benefit of being able to identify unknowns and GSH reaction products (if used in full scan mode). However, HPLC with UV detection is more accessible and completely adequate for quantifying typical GSH concentrations in juice, must, or fermenting wine, and when adding GSH or GSH products to ferments or for storage/packaging according to OIV regulations. Given that GSH can oxidise to GSSG during sampling and handling, it is necessary to derivatise GSH before analysis to preserve the natural ratio of GSH to GSSG. A derivatisation step has been included in some methods [27, 31, 34], and several derivatisation agents have been used, including p-benzoquinone [27], *N*-ethylmaleimide (NEM) [34–36], and 4,4-dithiodipyridine (DTDP) [37, 38]. None of the methods described above simultaneously analysed GSH and GSSG using simple HPLC with UV detection, with initial derivatisation of GSH to prevent autoxidation to GSSG during the sample preparation process.

This study aimed to address a fundamental question regarding whether a level of juice nitrogen could be defined that prevented both the metabolism of GSH and the production of VSCs by yeast during alcoholic fermentation. To address this question, GSH was added to chemically defined

media (CDM) with increasing yeast assimilable nitrogen (YAN) concentrations, and fermentation was conducted with yeast strains that varied in their nitrogen uptake potential. A straightforward method involving NEM derivatisation and HPLC analysis with UV detection was developed to quantify GSH and GSSG simultaneously after GSH addition to juice, to monitor the changes in GSH concentration during fermentation, and to monitor for autoxidation of GSH to GSSG.

2. Materials and Methods

2.1. Chemicals. HPLC-grade acetonitrile (ACN) was purchased from Merck (Frenchs Forest, NSW, Australia). Ethanol (99.5%), formic acid (98%, FA), sodium hydroxide (97%), and propan-2-ol (99.5%) were purchased from Rowe Scientific Pty Ltd (Lonsdale, SA, Australia). Ethylene diaminetetraacetic acid (EDTA), L-glutathione reduced (GSH), L-glutathione disulfide (GSSG), and *N*-ethylmaleimide (NEM) were purchased from Sigma-Aldrich (Castle Hill, NSW, Australia). Water was purified using a Milli-Q water purification system (Millipore, North Ryde, NSW, Australia). Copper (II) sulfate pentahydrate (99%) was purchased from Ajax Chemicals (Sydney, NSW, Australia), and iron (II) pentahydrate (97%) was purchased from Acros Organics (Scoresby, VIC, Australia).

2.2. Assessing Method Parameters for Quantitation of GSH and GSSG by HPLC. Glutathione and GSSG were quantified after GSH was derivatised with NEM, similar to published protocols [31, 34] with modifications as described previously.

2.2.1. Model and Real Juice and Wine Samples. The CDM matrix was prepared as described by Schmidt et al. [39]. Chardonnay and Shiraz juice were obtained from grapes harvested in South Australia during the 2012 and 2016 vintages, respectively. Chardonnay and Shiraz wines from the 2016 vintage that were produced in South Australia were obtained from local wineries. Model wine consisted of 12% aqueous ethanol containing 10 g/L potassium hydrogen tartrate that was adjusted to pH 3.4 using 40% (w/v) tartaric acid solution.

2.2.2. Standard and Derivatisation Solutions. Stock solutions of GSH (5.5 g/L, 17.9 mM) and GSSG (5.5 g/L, 9.0 mM) were prepared with water containing EDTA (100 mg/L) and stored at −20°C. A stock solution of NEM (18.8 g/L, 150 mM) was prepared in ethanol and stored at −20°C. Two sets of working solutions were prepared for GSH (1.8 g/L, 6.0 mM and 0.4 g/L, 1.2 mM) and GSSG (1.8 g/L, 3.0 mM and 0.4 g/L, 0.6 mM). An eight-point calibration curve was prepared, in duplicate, for the expected concentration range of GSH (0–125 mg/L; 0–0.41 mM) and GSSG (0–125 mg/L; 0–0.20 mM), and a six-point calibration curve was prepared in duplicate for high range GSH concentrations (70–350 mg/L, 0.23–1.14 mM) to quantify GSH at the levels added to the ferments in this experiment. The matrix used to prepare the calibration levels was the same as that of the samples being analysed (i.e., calibration standards prepared in CDM for CDM and fermenting samples, and model wine for samples at the completion of fermentation). Calibration standards were prepared by adding an aliquot of CDM or model wine to 2 mL vial, followed by the addition of 50 μL of NEM solution (5 mM), and then an aliquot of GSH working solution specific to each calibration point was added. The range of the calibration curve was as follows: 0.00, 0.016, 0.033, 0.081, 0.16, 0.24, 0.33, and 0.41 mM. The sample was vortexed for 15 s and left for 5 minutes to derivatise at room temperature. A volume of GSSG working solution specific to each calibration point (0.00, 0.008, 0.016, 0.041, 0.082, 0.12, 0.16, and 0.20 mM) was then added, followed by 75 μL i-PrOH, with vortexing for 15 seconds. The total volume of the sample was 1.5 mL. Calibration samples prepared for the high range GSH curve (GSH: 0.00, 0.23, 0.33, 0.81, 0.98, 1.14 mM) were prepared in the same way and then diluted with 300 μL of respective model solution before analysis. A sample of derivatised GSH (1 mM) was prepared and analysed with each set of samples to monitor the GSH working solution for autoxidation to GSSG.

2.2.3. Sample Preparation. Model juice, fermentation, and wine samples were centrifuged for 5 min before 1450 μL of the supernatant was added to a 2 mL HPLC vial, followed by the addition of 50 μL of NEM solution (5 mM). The sample was vortexed for 20 seconds before the addition of 75 μL *i*-PrOH.

2.2.4. Analysis. High-performance liquid chromatography (HPLC) was performed with an Agilent 1100 instrument (Forest Hill, Victoria, Australia) equipped with a binary pump, degasser, autosampler, column oven, and an Agilent 1200 diode array detector (DAD). A Cosmosil HILIC column (250 mm × 2.0 mm ID, 5 μM, Nacalai Tesque, Inc. Kyoto, Japan) protected by a guard column packed with the same material. The solvents were (A) 0.1% FA in MilliQ water and (B) 0.1% FA in ACN. Two gradient profiles and flow rates were used based on the matrix. A sample volume of 8 μL was directly injected into the HPLC system. The column oven temperature was 35°C. For CDM and ferment matrices, a flow rate of 0.5 mL was used with the linear gradient for solvent B as follows: 0 min, 90%; 5 min, 90%; 10 min, 75%; 20 min, 20%; 28 min, 20%; 28.1 min, 90%, followed by 5 min of column equilibration with 90% B. For model wine, a flow rate of 0.7 mL/min was used with the linear gradient for solvent B as follows: 0 min, 90%; 5 min, 90%; 6 min, 75%; 15 min, 20%; 22 min, 20%; 22.1 min, 90%, followed by 5 min of column equilibration with 90% B. An injection volume of 4 μL was used for high-range standards and samples. The GSH derivative and GSSG were detected at 220 and 200 nm, respectively.

2.2.5. Linearity, Accuracy, Recovery, and Precision. Linearity was assessed by comparison of three calibration curves prepared on three separate occasions in CDM, juice, real wine, and model wine matrices. Intraday accuracy was

determined by evaluating the percentage recovery of three replicate samples spiked with GSH and GSSG at low, medium, and high concentrations (GSH: 0.0325, 0.163, and 0.325 mM; GSSG: 0.0163, 0.0816, and 0.163 mM) in each matrix. Interday accuracy was evaluated by analysing triplicate samples prepared on three separate occasions. Precision was expressed using relative standard deviation (RSD) (Table S1, Supporting Information). The limit of detection (LOD) and limit of quantitation (LOQ) were determined as $LOD = 3.3\sigma/S$ and $LOQ = 10\sigma/S$, where σ is the standard deviation of the response, and S is the slope of the calibration curve [40].

2.2.6. Stability during Storage. Calibration standards were prepared in model wine and Chardonnay juice on two separate occasions. The model wine samples were analysed immediately after derivatisation, then stored at −20°C for 10 days, and brought to room temperature (20°C) and reanalysed. Nonlinear regression analysis was used to compare the curve fit of the data sets to assess whether there were any significant differences between samples analysed immediately following preparation compared to samples that were stored and reanalysed after 10 days (Figure S3). Two sets of Chardonnay fermentation samples were simultaneously sampled from four ferments (prepared in triplicate, as described in the "Preparation of ferments" section below) that contained a different added level of GSH (0, 100, 250, 500 mg/L; 0, 0.163, 0.184, 1.627 mM) (Figure S4). One set of the four fermentation triplicate samples was derivatised along with calibration standards and analysed immediately. The second set was derivatised with its own calibration standards, and together, these were stored at −20°C, thawed, and analysed as a batch 10 days later (Figure S4).

2.2.7. Effects of pH on Derivatised GSH and GSSG Quantitation. Model wine was adjusted to pH 3, 5, and 7 using formic acid or sodium hydroxide. A set of duplicate calibration standards of GSH (0, 0.5, 1.0, and 1.5 mM) and GSSG (0, 0.5, 1.0, and 1.5 mM) was prepared at each pH, and nonlinear regression analysis was performed to determine whether one curve could adequately fit all three datasets prepared at different pH levels. Additionally, four samples of derivatised GSH (1 mM) and GSSG (1 mM) were prepared at pH 3, 5, and 7 and analysed at four time points over 15 h to determine the stability of derivatised GSH and GSSG (Figure S5).

2.3. Preparation of Ferments. *Saccharomyces cerevisiae* AWRI 1688 and AWRI 2861 were obtained from the Australian Wine Research Institute (AWRI) culture collection. The CDM was prepared as described by Schmidt et al. [39] without the addition of sterols and fatty acids. The volume of nitrogen stock mix used was adjusted to obtain the desired low, medium, and high final YAN concentration in the media. YAN concentrations were determined by Affinity Labs (Adelaide, Australia) to be 155 mg/L (low), 320 mg/L (medium), and 430 mg/L (high) using the methods described in Dukes and Butzke [41] and Bergmeyer and Beutler [42]. L-GSH was dissolved in sterile MilliQ water and added prior to inoculation to the required concentration (0 mg/L, 20 mg/L, 100 mg/L, and 250 mg/L; 0, 0.0651, 0.325, 0.814 mM) for each YAN level and yeast strain. The sterile medium was inoculated with medium-acclimatised liquid culture at a starting rate of 0.02 absorbance units measured at 600 nm (Beckman Coulter DU530 UV Vis spectrophotometer, Beckman, NSW, Australia).

Small-scale fermentations (200 mL) were conducted in triplicate in 250 mL vessels with a sample port, a 25–2000 ppm H_2S detection tube (Kitagawa, Japan), and a 13.8 kPa directional-flow check valve to protect against overpressure. The vessels were stirred at 350 rpm and incubated at 18°C. Yeast growth was monitored by measuring absorbance at 600 nm, and fermentation progress was monitored using an enzymatic assay for reducing sugars as previously described [43] with adaptations [44] to allow the measurement in a 96-well microplate format [39]. Upon completion (residual sugar <1 g/L), ferments were cold-settled for 3 days at 4°C prior to compositional analysis.

2.4. Analysis of Volatile Sulfur Compounds. Volatile sulfur compounds were analysed directly after primary alcoholic fermentation and then after bottling, using gas chromatography with sulfur chemiluminescence detection (GC-SCD) as previously described [20].

2.5. Statistical Analyses. Significance tests (repeated measures ANOVA with Tukey mean comparison or Dunnet's mean comparison, nonlinear regression analysis, linearity, analysis of covariance, curve fit, derivatisation at different pH levels) were conducted using Prism statistics software (v6.04 GraphPad Software Inc., La Jolla, CA) at a significance level of $\alpha = 0.05$. Values are represented as means and standard deviation (SD). Where mean differences are presented, they are accompanied by 95% confidence intervals given in square brackets [lower CI, upper CI]. Where appropriate, multiplicity adjusted P-values are reported.

Duration of ferments as defined by the time (days) required for residual sugar concentration to reach 2 g/L was interpolated from a curve fit to the sugar concentration data. A least-squares approach was used to fit an asymmetric sigmoidal equation, weighted by $1/y^2$, and constraints: hillslope <0, top = 200, bottom = 0. Each ferment was fit independently, and the estimate of x (days) was calculated for $y = 2$. Two-way ANOVA of "time to completion" estimates was used to evaluate whether the duration of ferments with different concentrations of added GSH could be distinguished at different YAN concentrations. Each strain was evaluated independently. With limited interaction between GSH and YAN and limited contribution from GSH alone, Tukey's multiple comparison test was used to evaluate simple within-row effects with individual variances computed for each comparison.

Accuracy is presented as % recovery, calculated according to the following equation: accuracy (%) = $(\overline{y}/x) \times 100$ where $\overline{y}$ = mean measured value and x = expected value. Precision is given as the relative standard deviation which is calculated as follows: precision (%) = $(s/\overline{y}) \times 100$, where s = standard deviation of the measured values and $\overline{y}$ = mean measured value. The level of statistical evidence based on P-value is defined as follows: $P \geq 0.10$, "no evidence"; $P \leq 0.10$, "weak evidence"; $P \leq 0.05$, "evidence"; $P \leq 0.01$, "strong evidence"; $P \leq 0.001$, "very strong evidence."

3. Results and Discussion

3.1. Analysis of GSH and GSSG in Juices and Wines by HPLC. A robust HPLC method for quantification of GSH and GSSG (Figures 1(a) and 1(c)) was developed based on UV detection. Derivatisation of GSH using NEM to produce *N*-ethylsuccinimido-*S*-glutathione (Figure 1(b)) was used for analyte stability, thus avoiding GSH oxidation while awaiting analysis and preventing under-estimation of GSH and over-estimation of GSSG [31]. Enhanced stability was deemed useful to permit the storage of samples prior to analysis, increasing the accessibility of the method to wine analysis laboratories, analysis centres, and research laboratories.

Hydrophilic interaction chromatography (HILIC) was used for the separation of GSH and GSSG in the present study due to its strong retention of polar analytes [45]. The relationships between GSH and GSSG concentration with chromatogram peak area were evaluated in CDM, juice, and model and real wine.

Two GSH and GSSG concentration ranges were assessed, depending on the matrix being analysed. One calibration set was developed to cover the expected concentrations for GSH and GSSG in CDM and fermenting samples (5–125 mg/L) in accordance with previously reported ranges for juice and wine [13, 15, 24, 33, 46, 47]. A high-range calibration set covering the concentration range of 70–350 mg/L (0.228–1.139 mM) was also evaluated. The eight-point calibration functions were linear for both concentration ranges ($R^2 > 0.99$) in each matrix (Table S1). The LOD and LOQ values were similar for GSH and GSSG in the "expected" range calibration set for model wine and CDM, ranging from 2.80–3.22 mg/L (0.0046–0.0053 mM) and 6.36–7.32 mg/L (0.0207–0.0238 mM), respectively (Table S1).

Inter- and intraday accuracy and precision were determined for low (0.0325 mM), medium (0.163 mM), and high (0.325 mM) concentrations of GSH and GSSG (Table S1). Accuracy (99.1–103.1%) was equivalent for medium and high concentrations of GSH and GSSG in model wine and CDM. Measures of GSSG were less precise (0.7–5.0%) than GSH (0.4–1.6%) at these medium and high concentrations (Table S1). Measurement of low GSH and GSSG concentrations (0.0325 mM) in model wine and CDM was both less accurate than medium and high concentrations with GSH tending to be underestimated (accuracy 80.0–96.6%) and GSSG tending to be overestimated (accuracy 108.6–115.8%) (Table S1). The accuracy and precision of mid and high concentrations of GSH and GSSG determined using calibration curves prepared in Chardonnay and Shiraz wine were equivalent to GSH and GSSG concentrations determined using model solutions (Table S1). However, low concentrations of GSH were underestimated when calibration curves were prepared in Chardonnay and Shiraz juice, whereas low concentrations of GSSG were overestimated when lower concentrations were prepared either in Chardonnay and Shiraz juice or wine.

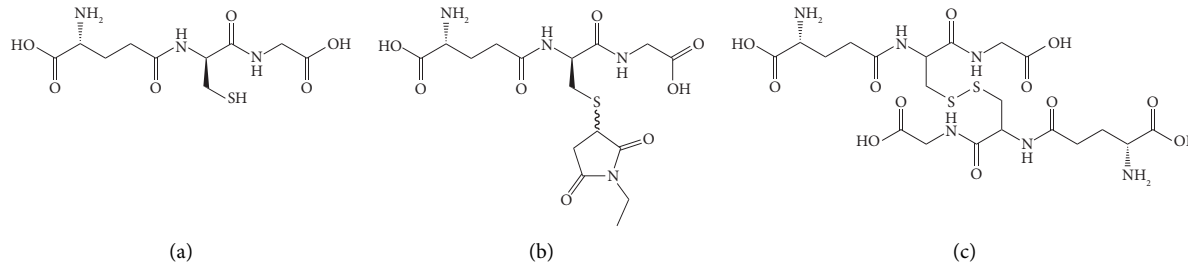
3.2. Stability of Derivatised GSH and GSSG. The stability of derivatised GSH and GSSG in samples stored at room temperature (20°C) was evaluated by preparing calibration standards in CDM, model wine, and Chardonnay juice and wine. There was no evidence ($\alpha = 0.05$) that calibration samples stored for 24 h (CDM, juice, and wine) or up to 5 days (model wine) produced different regression models (Figures S1 and S2). However, calibration standards prepared in juice and analysed immediately showed changes in peak area relative to stored samples (Figures S3c and S3d), with strong evidence that regression models for fresh and stored samples were different ($P < 0.0001$ for both). Nonetheless, there was no evidence that storing samples resulted in a different calculated concentration when freshly prepared samples (with GSH added at 100 and 250 mg/L) and calibration standards in CDM were compared to a replicate set that was stored at −20°C and analysed after 10 days (Figures S4a and S4b).

Overall, analysis of freshly prepared samples is advisable, but if that is not possible, then storing samples at −20°C for later analysis can be considered if GSH concentrations are less than 100 mg/L (0.325 mM). Furthermore, a set of calibration standards should be prepared and stored with the samples for subsequent analysis in the same batch.

The effect of matrix pH on the stability of the derivatised GSH and GSSG was also evaluated. There was no evidence that pH affected the quantification of GSH ($P = 0.857$) or GSSG ($P = 0.720$) when assessed within the timeframe of this experiment (Figures S5a and S5b).

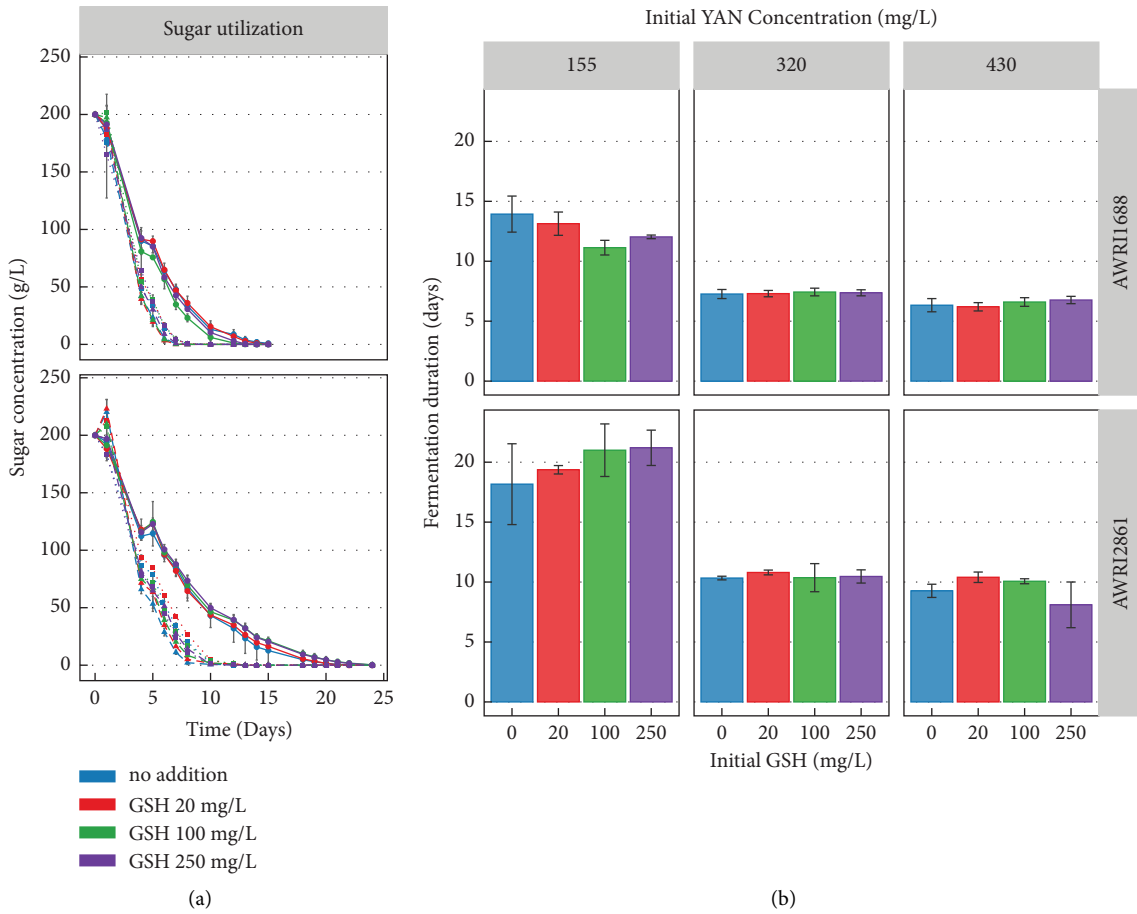
3.3. Role of Juice Nitrogen Status on GSH Consumption and VSC Liberation. According to the OIV resolution pertaining to the use of GSH in wine production, juice should have an "assimilable nitrogen level sufficient to avoid the metabolism of GSH by the yeast." In the absence of any other reports, it was unclear what a sufficient nitrogen concentration might be. As such, an evaluation of GSH consumption potential was undertaken by determining GSH and GSSG concentrations by HPLC upon fermentation of CDM prepared with three YAN concentrations (155 mg/L, 320 mg/L, and 430 mg/L) and four GSH concentrations (0, 20, 100, and 250 mg/L; 0, 0.0651, 0.325, and 0.814 mM) for each assimilable nitrogen level (total of 12 treatments).

The total amount of GSH remaining after fermentation was considered an indicator of the degree of GSH-related yeast metabolic activity. Two yeast strains were used in this work, with AWRI 1688 (an isolate of Zymaflore VL3) chosen because it contains an expanded set of oligopeptide



(a) (b) (c)

Figure 1: Chemical structures of (a) glutathione (GSH), (b) *N*-ethylsuccinimido-*S*-glutathione (derivatised GSH present as a pair of unresolved diastereomers), and (c) oxidised glutathione (GSSG).



(a) (b)

Figure 2: Mean values from defined medium fermentations ($n = 3$) using yeast strains AWRI 1688 and AWRI 2861 showing (a) sugar utilisation and (b) total fermentation time for three yeast assimilable nitrogen concentrations (155, 320, and 430 mg/L) and four GSH concentrations (0, 20, 100, and 250 mg/L). Error bars show the standard deviation of the replicates.

transporter genes (*FOT1* and *FOT2*) on a section of a horizontally transferred genomic island known as Region C. These transporters have been shown to permit the assimilation of complex nitrogen sources such as GSH [48]. The other yeast strain was AWRI 2861, selected as a comparison due to its genetic similarity to VL3 [49] but lack of *FOT1* or *FOT2*.

As anticipated, YAN concentration was a primary mediator of the sugar consumption rate for both yeast strains (Figure 2). It is known that YAN concentration can range from 53–411 mgN/L (average of 175 mgN/L) and that YAN content of must correlates to maximum CO_2 production rate and thus provides information on fermentation health and duration [50]. Fermentations were slow at 155 mgN/L, taking an average of 12.5 days (SD 1.2) for AWRI 1688 and 19.9 (SD 1.4) days for AWRI 2861 to reach a target sugar concentration of less than 2 g/L. This was consistent with the common understanding that 155 mg/L YAN is the lower limit for reliable white wine fermentations [51]. There was strong evidence that YAN concentration was the major factor responsible for variations in fermentation time ($P < 0.0001$ for both strains) (Tables S2 and S3). The addition of

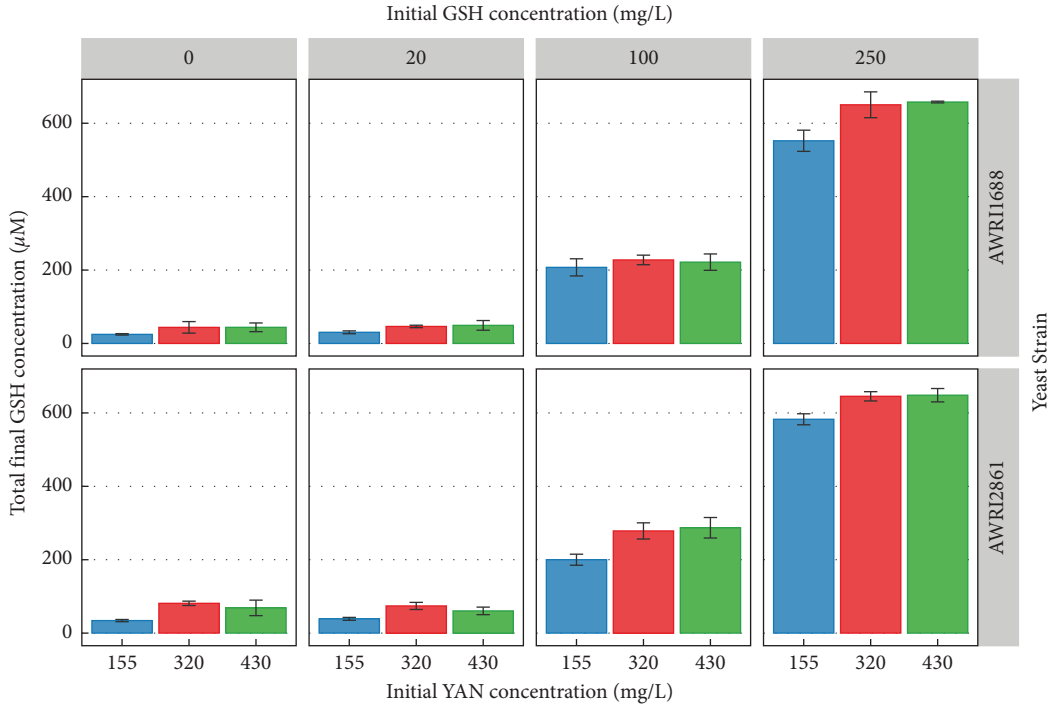


Figure 3: Mean concentration of GSH remaining (sum of GSH and GSSG) after completion of alcoholic fermentation ($n = 3$) following fermentation by AWRI 1688 or AWRI 2861 yeast strains. Fermentation was initiated in a medium containing three initial yeast assimilable nitrogen concentrations (155, 320, and 430 mg/L) and four GSH concentrations (0, 20, 100, and 250 mg/L (0, 0.0651, 0.325, 0.814 mM)). Error bars show the standard deviation of the replicates.

either 320 or 430 mgN/L decreased the respective fermentation durations by 6.6 days, 95% CI [5.4, 7.9] and 7.6 days, 95% CI [6.3, 8.8] for AWRI 1688 and by 7.8 days, 95% CI [9.9, 10.7] and 8.9 days, 95% CI [6.0, 11.8] for AWRI 2861 ($P < 0.0001$). There was no evidence for differences in fermentation time between the two higher YAN conditions.

In fermentations using yeast strain AWRI 2861, fermentation time appeared unaffected by GSH addition ($P = 0.321$), nor was there evidence for an interaction between GSH and YAN ($P = 0.152$); however, with AWRI 1688, there was weak evidence that supplementing the low YAN medium with GSH affected fermentation time ($P = 0.082$) and strong evidence for an interaction between GSH and YAN ($P = 0.002$) (Tables S2 and S3). The effects of GSH on fermentation time were only apparent at low YAN concentration (155 mg/L) and higher GSH addition rates [100 and 250 mg/L (0.325 and 0.814 mM)]. The magnitude of the effect was small, however, compared to the effects of YAN. Mean differences in ferment duration of 2.8 days, 95% CI [1.0, 4.6], and 1.9 days, 95% CI [0.1, 3.7] were observed between control ferments and those with 100 mg/L and 250 mg/L GSH addition, respectively. The altered fermentation time in response to GSH addition suggested that AWRI 1688 used GSH as a nitrogen source under nitrogen-limited conditions but only when GSH concentrations were high. The uptake of GSH by AWRI 1688 was consistent with both its genetic background, having an expanded set of oligopeptide transporters relative to AWRI 2861, and with the previously reported ability of FOT1-2 to mediate GSH uptake by yeast [48].

During these experiments, the change in total GSH concentration was monitored by quantifying and summing oxidised and reduced GSH upon completion of alcoholic fermentation. The total "end of ferment" GSH concentration for the two yeast strains is shown in Figure 3. End of ferment GSH concentrations were compared with initial GSH concentration using one-way ANOVA and Dunnett's test (Table S4). This data provided strong evidence that GSH concentration decreased during fermentation regardless of the initial YAN or GSH concentration, or yeast strain used for fermentation. In contrast, small increases in GSH concentration were observed in ferments without added GSH. There was strong evidence for both yeast strains that the magnitude of GSH loss was greater in ferments with higher initial GSH concentrations, especially at low initial YAN concentrations ($P < 0.0001$). In low YAN/low GSH ferments of AWRI 1688 (155 mg/L, 0.073 mM GSH), the mean decrease in total GSH concentration was 0.043 mM, 95% CI [0.022, 0.063], whereas, in low YAN/high GSH ferments (155 mg/L, 0.798 mM GSH), the mean decrease in total GSH concentration was 0.246 mM, 95% CI [0.181, 0.311]. Notably, loss of GSH during fermentation was inversely related to the initial YAN concentration. Again, in fermentations by AWRI 1688 with the highest initial GSH concentration (0.798 mM GSH), there was strong evidence ($P < 0.0001$) that an increase in YAN concentration from 155 mg/L to 430 mg/L was associated with a mean decrease in the postferment total GSH concentration of 0.140 mM, 95% CI [0.075, 0.206]. Nevertheless,

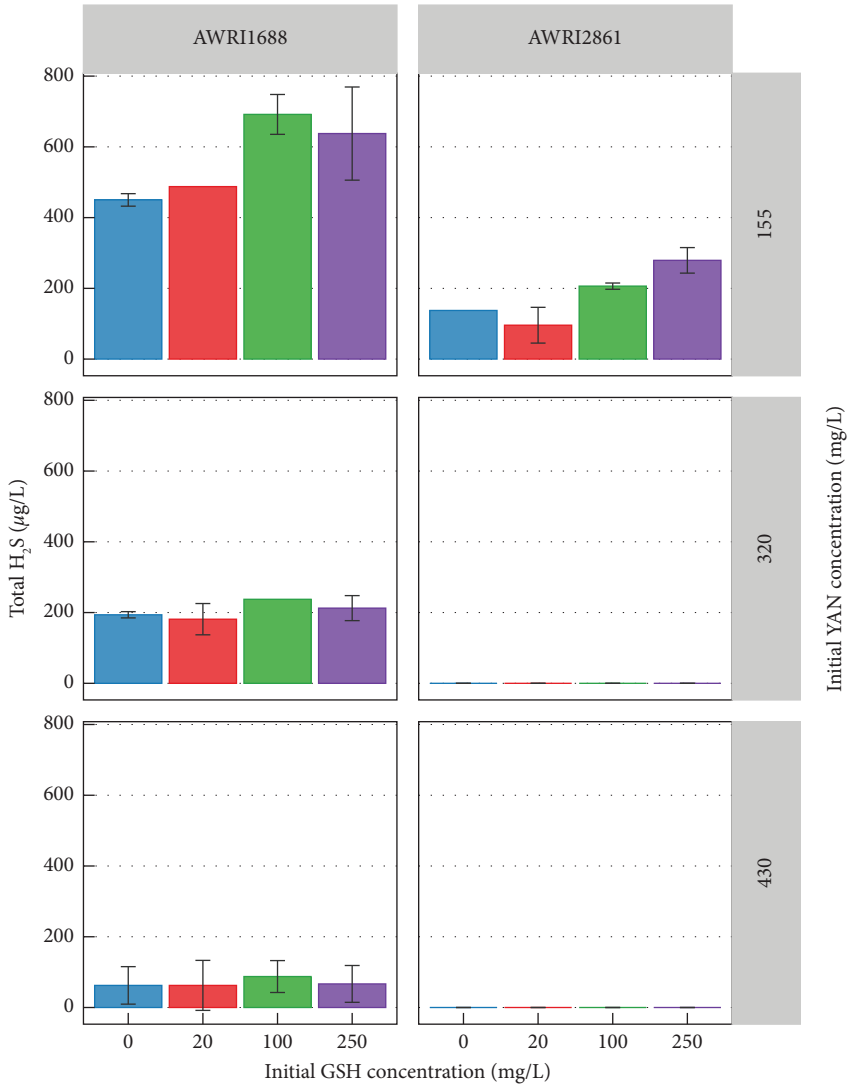


FIGURE 4: Total mean hydrogen sulfide (H_2S) production during fermentation ($n = 3$) with AWRI 1688 or AWRI 2861 yeast strain relative to initial nitrogen concentration. Fermentations were initiated in media containing three initial assimilable nitrogen concentrations (155, 320, and 430 mg/L) and four glutathione (GSH) concentrations (0, 20, 100, and 250 mg/L (0, 0.0651, 0.325, 0.814 M)). Error bars show the standard deviation of the replicates.

even at the highest YAN and lowest initial GSH concentrations, measurable decreases in GSH concentration were observed (mean decrease of 0.024 mM, 95% CI [0.004, 0.044], $P = 0.025$), such that GSH metabolism by yeast could not be ruled out.

GSH metabolism by yeast may manifest as an increase in H_2S production during active fermentation. The yeast-specific production of H_2S is shown in Figure 4 with AWRI 1688 and AWRI 2861 differing in both their overall production of H_2S during fermentation and in their response to different nitrogen concentrations. There was evidence that high preferment concentrations of GSH (250 mg/L, 0.814 mM) increased the total production of H_2S in low YAN conditions relative to ferments where no GSH addition was made for strain AWRI 2861 ($P = 0.02$, Table S5). The imprecise nature of the tube-based H_2S measurement rendered it impossible to draw more fine-grained conclusions about the effect of GSH treatment on H_2S production. Nevertheless, this apparent trend was consistent with previous studies [52]. Both yeast strains responded to higher YAN concentrations with decreased H_2S production. Overall, AWRI 2861 produced less H_2S than AWRI 1688 at any YAN level. Indeed, H_2S production by AWRI 2861 was only detected in low YAN ferments. The lower overall production of H_2S by AWRI 2861 was consistent with its genetic background, carrying a heterozygous R301G mutation in *MET2*, a dominant allele contributing to the decrease in H_2S production in such strains [53].

There is strong evidence that high GSH addition rates (100 and 250 mg/L (0.325 and 0.814 mM)) increased post-ferment H_2S concentration in low nitrogen fermentations (155 mg/L) (Figure 5, Table S6), with mean H_2S concentration increases of 35 μg/L ($P = 0.013$) and 42 μg/L ($P = 0.002$) (AWRI 1688) and 32 μg/L ($P = 0.01$) (AWRI 2861 with 250 mg/L GSH). The effects of GSH addition were most evident in ferments conducted using strain AWRI 1688

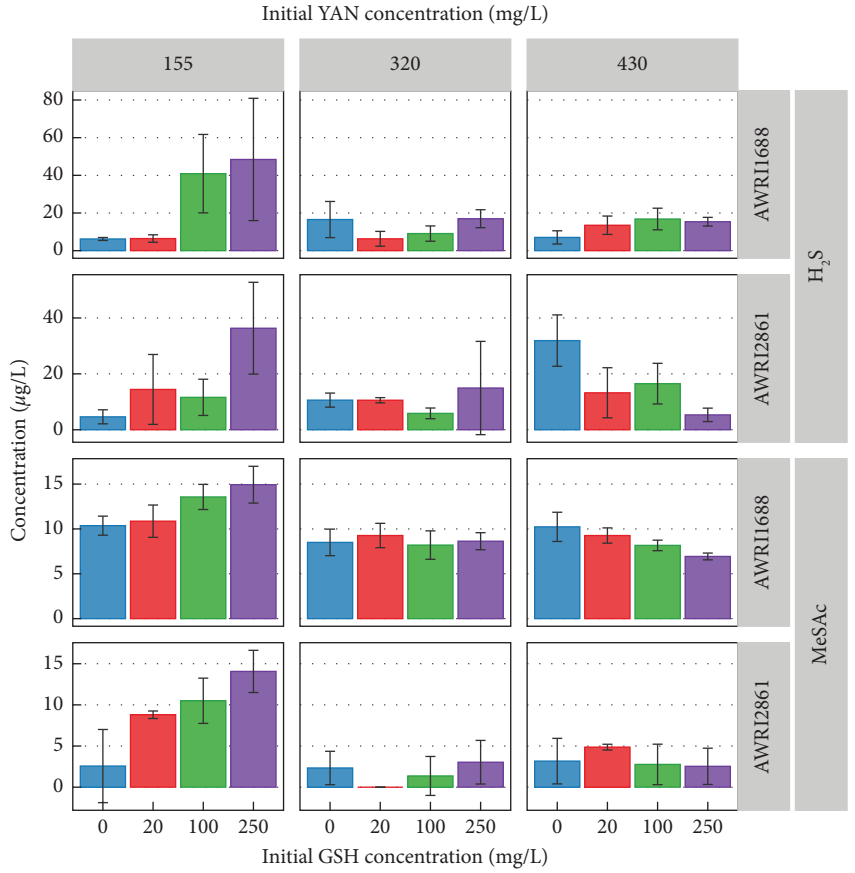


FIGURE 5: Mean concentration of hydrogen sulfide (H_2S) and methyl thioacetate (MeSAc) produced by yeast strains AWRI 1688 and AWRI 2861 during fermentation ($n = 3$) under different YAN and GSH conditions. Fermentations were initiated in media containing three initial assimilable nitrogen concentrations (155, 320, and 430 mg/L) and four GSH concentrations (0, 20, 100, and 250 mg/L (0, 0.0651, 0.325, 0.814 mM)). Error bars show the standard deviation of the replicates.

although similar trends were observed with strain AWRI 2861. There was no evidence that a 20 mg/L GSH addition (0.0651 mM), as recommended in OIV-OENO 445-2015, increased postfermentation H_2S concentrations. These data suggested that adding a low concentration of GSH to a fermentation would not be sufficient to stimulate H_2S formation, regardless of whether the added GSH is metabolised by yeast.

Postfermentation levels of MeSAc were also affected by YAN and GSH concentrations (Figure 5). Generally, higher concentrations of MeSAc were observed at the end of fermentations with the lowest YAN, and greater amounts of MeSAc were produced in response to increased GSH additions under this YAN condition. AWRI 1688 produced more MeSAc than AWRI 2861 at all YAN concentrations. Two-way ANOVA provided strong evidence ($P = 0.001$ and 0.002 for AWRI 1688 and AWRI 2861, respectively) for an interaction between GSH and YAN that contributed to the overall strain-specific variation in MeSAc concentration, with YAN concentration being the dominant factor for both AWRI 1688 ($P < 0.0001$) and AWRI 2861 ($P < 0.0001$) (Figure 5, Table S7). There was no evidence for GSH-related alterations in MeSAc concentration at higher initial YAN concentrations.

These experiments do not enable conclusions to be drawn about the causes of GSH concentration decrease. Both yeast metabolic activity and reaction with medium components could have contributed to the decrease in GSH concentration. However, it is noteworthy that these experiments were undertaken in a defined medium with limited extraneous material that could interact with GSH (e.g., phenolic compounds) and that both oxidised and reduced GSH were quantified. Therefore, it is plausible that contrary to the stipulation in the OIV recommendation, no YAN condition exists that can avoid the metabolism of GSH by yeast during fermentation, especially considering that yeast produce GSH through their own metabolic action, which may be accompanied by the formation of undesirable VSCs. Depending on yeast strain and YAN status of must or juice, the best-case scenario may be that sufficiently low concentrations of GSH do not stimulate the production of VSCs at concentrations that are detrimental to wine quality.

4. Conclusion

A fundamental hypothesis was tested in this work regarding whether a level of juice nitrogen could be defined that prevented the metabolic consumption of GSH by yeast. To

address this gap and provide evidence or otherwise for the adoption of GSH addition during winemaking, GSH was added to defined media with increasing YAN concentrations, and the change in GSH concentration was monitored. Facilitating this, a robust HPLC method with UV detection was developed to quantify GSH and GSSG in juice, ferments, and finished wines, using GSH derivatisation to preserve the levels of each analyte in existence at the time of sampling.

It was demonstrated that increasing the YAN status of the media minimised the decrease in GSH concentration during fermentation, but the loss of GSH could not be entirely eliminated. Decreases in GSH concentration were still evident with a low initial GSH concentration and high initial YAN concentration, with both of the yeast strains trialled. However, higher YAN concentrations were found to suppress H_2S formation when higher concentrations of GSH were applied. This work raises doubt about the possibility of implementing a level of juice nitrogen that is sufficient to prevent the metabolism by yeast of GSH added prior to fermentation.

Data Availability

All relevant data are within the manuscript or have been deposited with Dryad available at doi.org/10.5061/dryad.3ffbg79nh.

Conflicts of Interest

The authors declare that they have no conflicts of interest.

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Supplementary Materials

Figure S1. Nonlinear regression analysis for calibration curves to determine interday stability of derivatised GSH is shown (a) chemically defined media, (b) Chardonnay juice, (c) model wine analysed three times every 12 hours, (d) model wine analysed 5 days apart, and (e) Chardonnay wine analysed three times every 12 hours. R2 and *P*-value describing the comparison of fit for the reanalysed calibration curves are shown for each dataset in each plot. The dotted lines represent the 95% confidence bands that indicate how well the data define the best-fit curve. Figure S2. The results of the nonlinear regression analysis for calibration curves for GSSG to determine interday stability of GSSG are shown for (a) chemically defined media analysed three times every 12 hours, (b) Chardonnay juice analysed three times every 12 hours, (c) model wine analysed three times every 12 hours, (d) model wine analysed two times 5 days apart, and (e) Chardonnay wine analysed three times every 12 hours. The R2 and *P*-value describing the comparison of fit for the reanalysed calibration curves are shown for each dataset in each plot. The dotted lines represent the 95% confidence bands that indicate how well the data define the best-fit curve. Figure S3. Evaluating the stability of derivatised GSH (a) and GSSG (b) prepared in model wine and derivatised GSH (c) and GSSG (d) prepared in Chardonnay juice. The samples were analysed immediately after preparation and again after 10 days of storage at −20°C. The R2 and *P*-value describing the comparison of fit for the reanalysed calibration curves are shown for each dataset in each plot. The dotted lines represent the 95% confidence bands which indicate how well the data define the best-fit curve. Figure S4. The stability of derivatised GSH (a) and GSSG (b) measurements was evaluated in ferments prepared in chemically treated with three levels of GSH of 100, 250, and 500 mg/L (0.33, 0.81, 1.63 mM), and a control sample without added GSH. One set of samples was taken and derivatised with NEM and immediately analysed (blue bars). The second set of samples was also derivatised with NEM and then stored at −20°C for nine days before analysis (red bars). One-way ANOVA was used to determine whether the freshly prepared and analysed samples were significantly different from samples there were prepared, stored at −20°C, and analysed nine days later. Statements ascribing the level of statistical evidence in this report are as follows: $P \geq 0.10$ "no evidence" (ns); $P \leq 0.10$ "weak evidence" (*); $P \leq 0.05$ "evidence" (**), $P \leq 0.01$ "strong evidence" (***), and $P \leq 0.001$ "very strong evidence" (****). Figure S5. Evaluating the effect of pH on derivatised GSH (a, c) and on GSSG (b, d) quantification at pH 3, 5, and 7. Points are the means of duplicate samples with error bars showing standard deviation. The bar graphs in (c) and (d) represent four repeat analyses of the derivatised GSH (1mM) (c) and GSSG (1 mM) (d) samples over 15 hours to evaluate their stability at each pH level. The R^2 and *P*-value describing the comparison of fit for the reanalysed calibration curves are shown for each dataset in each plot. The dotted lines represent the 95% confidence bands that indicate how well the data define the best-fit curve. Table S1. Figures of merit for GS-NEM and GSSG for chemically defined media, juice, wine, and model wine. Table S2. Two-way ANOVA analysis of the effect of initial YAN and GSH on ferment duration by yeast strain AWRI1688. Post hoc test employed Tukey's multiple comparison test evaluating simple effects between GSH treatments (within-row effects) at each YAN concentration (within YAN). Contrast specific *P*-values are adjusted for multiple comparisons. Table S3. Two-way ANOVA analysis of the effect of initial YAN and GSH on ferment duration by yeast strain AWRI2681. Post hoc test employed Tukey's multiple comparison test evaluating simple effects between GSH treatments (within-row effects) at each YAN concentration (within YAN). Contrast specific *P*-values are adjusted for multiple comparisons. Table S4. One-way ANOVA comparison of initial with final GSH concentrations. ANOVA analyses were grouped by strain and initial

GSH concentration. Post hoc test employed Dunnett's test to compare the final concentration of GSH with the initial concentration. Six tests were conducted to evaluate GSH loss at different nitrogen concentrations. Table S5. One-way ANOVA comparison of total strain-specific H_2S concentrations produced in low YAN conditions (155 mgN/L) with different initial GSH concentrations. ANOVA analyses were grouped by strain and initial GSH concentration. Post hoc test employed Tukey's multiple comparison test with a single pooled variance. Contrast specific *P*-values are adjusted for multiple comparisons. Two one-way ANOVA analyses were conducted to evaluate H_2S production by each of the two yeast strains. Table S6. Two-way ANOVA analysis of the effect of initial YAN and GSH on end of ferment H_2S concentrations by yeast strains AWRI1688 and AWRI2861. ANOVA analyses were conducted separately for each strain. Post hoc test employed Dunnett's multiple comparison test with individual variances computed for each comparison. Contrast specific *P*-values are adjusted for multiple comparisons. Table S7. Two-way ANOVA analysis of the effect of initial YAN and GSH on end of ferment MeSAc concentrations by yeast strains AWRI1688 and AWRI2861. ANOVA analyses were conducted separately for each strain. Post hoc test employed Dunnett's multiple comparison test with individual variances computed for each comparison. Contrast specific *P*-values are adjusted for multiple comparisons. (*Supplementary Materials*)

References

[1] L. O. Santos, P. G. P. Silva, W. J. F. Lemos Junior, V. S. de Oliveira, and A. Anschau, "Glutathione production by *Saccharomyces cerevisiae*: current state and perspectives," *Applied Microbiology and Biotechnology*, vol. 106, no. 5-6, pp. 1879–1894, 2022.

[2] E. C. Kritzinger, F. F. Bauer, and W. J. du Toit, "Role of glutathione in winemaking: a review," *Journal of Agricultural and Food Chemistry*, vol. 61, no. 2, pp. 269–277, 2013.

[3] R. L. Binati, I. Larini, E. Salvetti, and S. Torriani, "Glutathione production by non-Saccharomyces yeasts and its impact on winemaking: a review," *Food Research International*, vol. 156, Article ID 111333, 2022.

[4] A. Pons, V. Lavigne, P. Darriet, and D. Dubourdieu, "Glutathione preservation during winemaking with Vitis Vinifera white varieties: example of Sauvignon blanc grapes," *American Journal of Enology and Viticulture*, vol. 66, no. 2, pp. 187–194, 2015.

[5] C. P. Hallinan, D. J. Saul, and V. Jiranek, "Differential utilisation of sulfur compounds for H_2S liberation by nitrogen starved yeasts," *Australian Journal of Grape and Wine Research*, vol. 5, no. 3, pp. 82–90, 1999.

[6] P. Wegmann-Herr, S. Ullrich, and D. Durner, "Approaches to limit S-off-flavors during white wine fermentation with specific emphasis on yeast nitrogen nutrition," *BIO Web of Conferences*, vol. 15, Article ID 02029, 2019.

[7] M. Ugliano, B. Fedrizzi, T. Siebert et al., "Effect of nitrogen supplementation and Saccharomyces species on hydrogen sulfide and other volatile sulfur compounds in shiraz fermentation and wine," *Journal of Agricultural and Food Chemistry*, vol. 57, no. 11, pp. 4948–4955, 2009.

[8] M. Z. Bekker, E. N. Wilkes, and P. A. Smith, "Evaluation of putative precursors of key "reductive" compounds in wines post-bottling," *Food Chemistry*, vol. 245, pp. 676–686, 2018.

[9] M. Ugliano, M. Kwiatkowski, S. Vidal et al., "Evolution of 3-mercaptohexanol, hydrogen sulfide, and methyl mercaptan during bottle storage of Sauvignon blanc wines. Effect of glutathione, copper, oxygen exposure, and closure-derived oxygen," *Journal of Agricultural and Food Chemistry*, vol. 59, no. 6, pp. 2564–2572, 2011.

[10] D. L. Capone, M. A. Sefton, and D. W. Jeffery, "Application of a modified method for 3-mercaptohexan-1-ol determination to investigate the relationship between free thiol and related conjugates in grape juice and wine," *Journal of Agricultural and Food Chemistry*, vol. 59, no. 9, pp. 4649–4658, 2011.

[11] S. Motta, M. Guaita, M. Petrozziello, L. Panero, and A. Bosso, "Effect of reductive pressing on the concentration of reduced glutathione and phenols in the musts of four Italian cultivars," *American Journal of Enology and Viticulture*, vol. 65, no. 4, pp. 471–478, 2014.

[12] I. Andujar-Ortiz, C. Chaya, P. J. Martin-Alvarez, M. V. Moreno-Arribas, and M. A. Pozo-Bayon, "Impact of using new commercial glutathione enriched inactive dry yeast oenological preparations on the aroma and sensory properties of wines," *International Journal of Food Properties*, vol. 17, no. 5, pp. 987–1001, 2014.

[13] E. C. Kritzinger, M. A. Stander, and W. J. Du Toit, "Assessment of glutathione levels in model solution and grape ferments supplemented with glutathione-enriched inactive dry yeast preparations using a novel UPLC-MS/MS method," *Food Additives and Contaminants: Part A*, vol. 30, no. 1, pp. 80–92, 2013.

[14] J. J. Rodriguez-Bencomo, R. Schneider, J. P. Lepoutre, and P. Rigou, "Improved method to quantitatively determine powerful odorant volatile thiols in wine by headspace solid-phase microextraction after derivatization," *Journal of Chromatography A*, vol. 1216, no. 30, pp. 5640–5646, 2009.

[15] E. C. Kritzinger, F. F. Bauer, and W. J. Du Toit, "Influence of yeast strain, extended lees contact and nitrogen supplementation on glutathione concentration in wine," *Australian Journal of Grape and Wine Research*, vol. 19, no. 2, pp. 161–170, 2013.

[16] S. K. Park, R. B. Boulton, and A. C. Noble, "Formation of hydrogen sulfide and glutathione during fermentation of white grape musts," *American Journal of Enology and Viticulture*, vol. 51, no. 2, pp. 91–97, 2000.

[17] M. Ugliano, "Oxygen contribution to wine aroma evolution during bottle aging," *Journal of Agricultural and Food Chemistry*, vol. 61, no. 26, pp. 6125–6136, 2013.

[18] C. M. Mayr, D. L. Capone, K. H. Pardon, C. A. Black, D. Pomeroy, and I. L. Francis, "Quantitative analysis by GC-MS/MS of 18 aroma compounds related to oxidative off-flavor in wines," *Journal of Agricultural and Food Chemistry*, vol. 63, no. 13, pp. 3394–3401, 2015.

[19] P. Lopes, M. A. Silva, A. Pons et al., "Impact of oxygen dissolved at bottling and transmitted through closures on the composition and sensory properties of a Sauvignon Blanc wine during bottle storage," *Journal of Agricultural and Food Chemistry*, vol. 57, no. 21, pp. 10261–10270, 2009.

[20] T. E. Siebert, M. R. Solomon, A. P. Pollnitz, and D. W. Jeffery, "Selective determination of volatile sulfur compounds in wine by gas chromatography with sulfur chemiluminescence detection," *Journal of Agricultural and Food Chemistry*, vol. 58, no. 17, pp. 9454–9462, 2010.

[21] V. Cheynier, J. M. Souquet, and M. Moutounet, "Glutathione content and glutathione to hydroxycinnamic acid ratio in vitis-vinifera grapes and musts," *American Journal of Enology and Viticulture*, vol. 40, pp. 320–324, 1989.

[22] O. Yilmaz, S. Keser, M. Tuzcu et al., "A practical HPLC method to measure reduced (GSH) and oxidized (GSSG) glutathione concentrations in animal tissues," *Journal of Animal and Veterinary Advances*, vol. 8, pp. 343–347, 2009.

[23] B. A. Neuschwander-Tetri and F. J. Roll, "Glutathione measurement by high-performance liquid chromatography separation and fluorometric detection of the glutathione-orthophthalaldehyde adduct," *Analytical Biochemistry*, vol. 179, no. 2, pp. 236–241, 1989.

[24] M. L. Steele, L. Ooi, and G. Munch, "Development of a high-performance liquid chromatography method for the simultaneous quantitation of glutathione and related thiols," *Analytical Biochemistry*, vol. 429, no. 1, pp. 45–52, 2012.

[25] T. Toyooka, T. Suzuki, Y. Saito, S. Uzu, and K. Imai, "Fluorogenic reagent for thiols: 4-(*N, N*-dimethylaminosulphonyl)-7-fluoro-2, 1, 3-benzoxadiazole," *Analyst*, vol. 114, no. 4, pp. 413–419, 1989.

[26] W. Jin, W. Li, and Q. Xu, "Quantitative determination of glutathione in single human erythrocytes by capillary zone electrophoresis with electrochemical detection," *Electrophoresis*, vol. 21, no. 4, pp. 774–779, 2000.

[27] A. Tirelli, D. Fracassetti, and I. De Noni, "Determination of reduced cysteine in oenological cell wall fractions of *Saccharomyces cerevisiae*," *Journal of Agricultural and Food Chemistry*, vol. 58, no. 8, pp. 4565–4570, 2010.

[28] F. Mattivi, B. Fedrizzi, A. Zenato et al., "Development of reliable analytical tools for evaluating the influence of reductive winemaking on the quality of Lugana wines," *Analytica Chimica Acta*, vol. 732, pp. 194–202, 2012.

[29] A. Vallverdu-Queralt, A. Verbaere, E. Meudec, V. Cheynier, and N. Sommerer, "Straightforward method to quantify GSH, GSSG, GRP, and hydroxycinnamic acids in wines by UPLC-MRM-MS," *Journal of Agricultural and Food Chemistry*, vol. 63, no. 1, pp. 142–149, 2015.

[30] D. O. Adams and C. Liyanage, "Glutathione increases in grape berries at the onset of ripening," *American Journal of Enology and Viticulture*, vol. 44, pp. 333–338, 1993.

[31] L.-S. New and E. C. Y. Chan, "Evaluation of BEH C18, BEH HILIC, and HSS T3 (C18) column chemistries for the UPLC-MS-MS analysis of glutathione, glutathione disulfide, and ophthalmic acid in mouse liver and human plasma," *Journal of Chromatographic Science*, vol. 46, no. 3, pp. 209–214, 2008.

[32] C. Coetzee, K. Lisjak, L. Nicolau, P. Kilmartin, and W. J. du Toit, "Oxygen and sulfur dioxide additions to Sauvignon blanc must: effect on must and wine composition," *Flavour and Fragrance Journal*, vol. 28, no. 3, pp. 155–167, 2013.

[33] W. J. Du Toit, K. Lisjak, M. Stander, and D. Prevoo, "Using LC-MSMS to assess glutathione levels in South African white grape juices and wines made with different levels of oxygen," *Journal of Agricultural and Food Chemistry*, vol. 55, no. 8, pp. 2765–2769, 2007.

[34] D. Giustarini, I. Dalle-Donne, A. Milzani, P. Fanti, and R. Rossi, "Analysis of GSH and GSSG after derivatization with *N*-ethylmaleimide," *Nature Protocols*, vol. 8, no. 9, pp. 1660–1669, 2013.

[35] X. Sun, R. S. Berger, P. Heinrich et al., "Optimized protocol for the in situ derivatization of glutathione with *N*-ethylmaleimide in cultured cells and the simultaneous determination of glutathione/glutathione disulfide ratio by HPLC-UV-QTOF-MS," *Metabolites*, vol. 10, no. 7, p. 292, 2020.

[36] R. B. Allison and G. L. Sacks, "Brine-releasable hydrogen sulfide in wine: mechanism of release from copper complexes and effects of glutathione," *Journal of Agricultural and Food Chemistry*, vol. 69, no. 44, pp. 13164–13172, 2021.

[37] S. Millan, D. W. Jeffery, S. Dall'Acqua, and A. Masi, "A novel HPLC-MS/MS approach for the identification of biological thiols in vegetables," *Food Chemistry*, vol. 339, Article ID 127809, 2021.

[38] J. R. Winther and C. Thorpe, "Quantification of thiols and disulfides," *Biochimica et Biophysica Acta (BBA) - General Subjects*, vol. 1840, no. 2, pp. 838–846, 2014.

[39] S.A. Schmidt, S. Dillon, R. Kolouchova, P.A. Henschke, and P.J. Chambers, "Impacts of variations in elemental nutrient concentration of Chardonnay musts on Saccharomyces cerevisiae fermentation kinetics and wine composition," *Applied Microbiology and Biotechnology*, vol. 91, no. 2, pp. 365–375, 2011.

[40] M. Thompson, S.L.R. Ellison, and R. Wood, "Harmonized guidelines for single-laboratory validation of methods of analysis (IUPAC Technical Report)," *Pure and Applied Chemistry*, vol. 74, no. 5, pp. 835–855, 2002.

[41] B.C. Dukes and C.E. Butzke, "Rapid determination of primary amino acids in grape juice using an ophthaldialdehyde/N-acetyl-L-cysteine spectrophotometric assay," *American Journal of Enology and Viticulture*, vol. 49, no. 2, pp. 125–134, 1998.

[42] H. U. Bergmeyer and H.-O. Beutler, *Methods of Enzymatic Analysis*, H. U. Bergmeyer, Ed., pp. 454–461, Verlag Chemie, Weinheim, 3rd edn. edition, 1985.

[43] H.-J. Hohorst, "d-Glucose-6-phosphate and d-fructose-6-phosphate: Determination with glucose-6-phosphate dehydrogenase and phosphoglucose isomerase," in *Methods of Enzymatic Analysis*, H.-U. Bergmeyer, Ed., pp. 134–138, Academic Press, New York, 1965.

[44] S. Vermeir, B.M. Nicolai, K. Jans, G. Maes, and J. Lammertyn, "High-throughput microplate enzymatic assays for fast sugar and acid quantification in apple and tomato," *Journal of Agricultural and Food Chemistry*, vol. 55, no. 9, pp. 3240–3248, 2007.

[45] B. Chauve, D. Guillarme, P. Cléon, and J.-L. Veuthey, "Evaluation of various HILIC materials for thefast separation of polar compounds," *Journal of Separation Science*, vol. 33, no. 6-7, pp. 752–764, 2010.

[46] D. Fracassetti, N. Lawrence, A. G. J. Tredoux, A. Tirelli, H. H. Nieuwoudt, and W. J. Du Toit, "Quantification of glutathione, catechin and caffeic acid in grape juice and wine by a novel ultra-performance liquid chromatography method," *Food Chemistry*, vol. 128, no. 4, pp. 1136–1142, 2011.

[47] L. Janes, K. Lisjak, and A. Vanzo, "Determination of glutathione content in grape juice and wine by high-performance liquid chromatography with fluorescence detection," *Analytica Chimica Acta*, vol. 674, no. 2, pp. 239–242, 2010.

[48] S. Marsit, A. Mena, F. Bigey et al., "Evolutionary advantage conferred by an eukaryote-to-eukaryote gene transfer event in wine yeasts," *Molecular Biology and Evolution*, vol. 32, no. 7, pp. 1695–1707, 2015.

[49] A. R. Borneman, A. H. Forgan, R. Kolouchova, J. A. Fraser, and S. A. Schmidt, "Whole genome comparison reveals high levels of inbreeding and strain redundancy across the spectrum of commercial wine strains of *Saccharomyces cerevisiae*," *G3 Genes|Genomes|Genetics*, vol. 6, no. 4, pp. 957–971, 2016.

[50] M. Bely, J. M. Sablayrolles, and P. Barre, "Automatic detection of assimilable nitrogen deficiencies during alcoholic fermentation in oenological conditions," *Journal of Fermentation and Bioengineering*, vol. 70, no. 4, pp. 246–252, 1990.

[51] S. J. Bell and P. A. Henschke, "Implications of nitrogen nutrition for grapes, fermentation and wine," *Australian Journal of Grape and Wine Research*, vol. 11, no. 3, pp. 242–295, 2005.

[52] J.-M. Aurand, P. Wegmann-Herr, S. Ullrich, H.-G. Schmarr, and D. Durner, "Use of glutathione during white wine production–impact on S-off-flavors and sensory production," in *Proceedings of the BIO Web of Conferences 39th World Congress of Vine and Wine*, Bento Gonçalves, Brazil, October 2016.

[53] C. Huang, M. Roncoroni, and R. C. Gardner, "MET2 affects production of hydrogen sulfide during wine fermentation," *Applied Microbiology and Biotechnology*, vol. 98, no. 16, pp. 7125–7135, 2014.