# Characterisation of polysulfides in *Saccharomyces cerevisiae* cells and finished wine in cysteine-supplemented model grape must

Running title: Polysulfides in yeast and wine post-fermentation

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## ABSTRACT

Recently, wine polysulfides have attracted research interest, as their degradation can result in the release of hydrogen sulfide ($H_2S$) in model wine, imparting an unpleasant rotten-egg smell that is detrimental to wine quality. The presence of polysulfides in wine has now been demonstrated, but their biogenesis remains unclear. This study aimed to further investigate the role of the yeast, *Saccharomyces cerevisiae*, in polysulfide formation during fermentation with and without supplementation of 5 mM cysteine, a known source of $H_2S$. Using an established liquid chromatography-tandem mass spectrometry method, monobromobimane derivatives of several hydropolysulfides, such as CysSSSH, CysSSSSH and GSSSSH, and two oxidised polysulfides, GSSG and GSSSSG, were detected for the first time in *S. cerevisiae* cells that had completed fermentation in a grape juice-like medium. Polysulfide production by four single deletants (BY4743 Δ*cys3*, Δ*cys4*, Δ*met17* and Δ*tum1*) showed no significant differences in their polysulfide profiles compared to BY4743 wild type, suggesting the existence of unknown pathways maintaining cellular polysulfide homeostasis. Furthermore, while the addition of 5 mM cysteine increased the formation of shorter sulfur chain species, including GSS-bimane and GSSG, it did not result in greater production of longer sulfur chain species. Finally, most of the longer sulfur chain polysulfides detected within *S. cerevisiae* were symmetrical and not detected in the fermented medium, excepting CysSSS-bimane. This evidence could suggest that these polysulfides were either not stable in this wine-like environment, or not transported extracellularly. Collectively, our data illustrate the complexity of polysulfide metabolism in *S. cerevisiae* under fermentation conditions. Further investigation using more sensitive detection methods will be required to pinpoint specific genes involved in this process.

**KEYWORDS**

Fermentation; hydrogen sulfide; hydropolysulfides; oxidised polysulfides; *Saccharomyces cerevisiae*

## 1. Introduction

The undesirable, 'rotten-egg' odour of hydrogen sulfide ($H_2S$) produced by the yeast *Saccharomyces cerevisiae* during fermentation, is a common problem for the wine industry. According to results from the International Wine Challenge, the $H_2S$-derived 'rotten-egg' aroma, which also masks desirable fruity aromas, is responsible for around 29% of commercial wine faults (Goode and Harrop, 2008). If $H_2S$ production is left unchecked, it can result in the formation of further unwanted fermentation-derived volatile sulfur compounds (VSCs), imparting 'cabbage', 'cooked onion' and 'garlic' off-aromas, which are very difficult to remediate (Kinzurik et al., 2016). While much progress has been made towards understanding the mechanisms underlying $H_2S$ production, with formation stemming from the sulfate assimilation pathway (SAP) (Huang et al., 2017a; Kinzurik et al., 2017, 2020; Thomas and Surdin-Kerjan, 1997), cysteine degradation (Huang et al., 2017b; Winter et al., 2014), and application of elemental sulfur (Araujo et al., 2017; Kwasniewski et al., 2014), the pathways identified so far do not account for all $H_2S$ present in wine. Additionally, unknown pathways resulting in latent $H_2S$ release during wine storage can cause long-term wine quality issues (reference). Recently, $H_2S$ formation and release via a novel pathway involving the formation and degradation of polysulfides ($S_x^{2-}$; $x > 2$) and protein persulfides has been proposed (Huang et al., 2017a; Kreitman et al., 2019, 2017). Since this proposal, $H_2S$ release has been shown to occur during wine ageing due to the presence of copper ($Cu^{2+}$), introduced via copper sulfate fining (Bekker et al., 2018; Kreitman et al., 2017), as well as early in the winemaking process, catalysed by $Cu^{2+}$ from grapevine pesticides, initiating condensation reactions in thiols to form polysulfides, which then go on to form $H_2S$ (Dekker et al., 2020).

Recent advances in analytical techniques have allowed for the precise identification and semi-quantitation of a wide range of polysulfide compounds in wine (Bekker et al., 2018; Dekker et al., 2022, 2020; Jastrzembski et al., 2017; Kreitman et al., 2017; Nardin et al., 2020;

Starkenmann et al., 2016; van Leeuwen et al., 2020). Studies have shown that different amounts of thiolated polysulfides are present between unfermented must and fermented wine, strongly suggesting that yeast play a role in polysulfide production (Dekker et al., 2020). Furthermore, variation in the formation of TCEP-releasable $H_2S$ (polysulfides) has been observed amongst different commercial yeast strains, indicating that genetic differences could affect polysulfide production (Jastrzembski et al., 2017). Polysulfides, which appear to be ubiquitous within organisms for cell-to-cell signalling, are now known to be present in *S. cerevisiae* cells under various aerobic growth conditions (Hu et al., 2019; Pilkington et al., 2019; Ran et al., 2019), with different sulfur sources altering the intracellular polysulfide composition. *S. cerevisiae* single deletants in genes orthologous to mammalian polysulfide-producing enzymes, including *CYS3* (cystathionine γ-lyase (CSE)), *CYS4* (cystathionine β-synthase (CBS)), and *TUM1* (3-mercaptopyruvate sulfurtransferase (3MST) (Ida et al., 2014; Kimura, 2015), showed accumulation of different polysulfide species during aerobic growth, reiterating the idea that the biogenesis of polysulfides by yeast has a genetic component and is impacted by genes in the SAP (Pilkington et al., 2019). However, the formation of polysulfides within *S. cerevisiae* cells under fermentative conditions, and their degree of release into a wine-like matrix, has not been greatly explored.

An *in-house* LC-MS/MS method (Pilkington et al., 2019) was applied to measure the presence of the oxidised polysulfides and monobromobimane (mBBr) derivatives of hydropolysulfides within *S. cerevisiae* cells, and in finished synthetic wine, after alcoholic fermentation in a medium mimicking grape juice. To identify the role of yeast candidate genes in the SAP, these methods were applied to *S. cerevisiae* lab strain, BY4743 wild type, and four single deletants, BY4743 Δ*cys3*, BY4743 Δ*cys4*, BY4743 *Δmet17* and BY4743 Δ*tum1*, with the *CYS3*, *CYS4* and *TUM1* genes chosen based on their orthology to mammalian polysulfide-producing enzymes. The *MET17* gene was chosen due to the central role of Met17p in the SAP,

and because Δ*met17* mutants accumulate very high $H_2S$ when grown with the addition of exogenous cysteine (Huang et al., 2016). Finally, the addition of 5 mM exogenous cysteine to the fermentations was investigated to determine the contribution of $H_2S$ (generated via cysteine degradation) towards intracellular and extracellular polysulfides. These experiments aimed to provide evidence for the presence of polysulfides within *S. cerevisiae* cells and their release into the medium, under fermentation-specific conditions, to provide greater insights into polysulfide formation pathways.

## 2. Materials and methods

### 2.1 *S. cerevisiae* strains and culture

The *S. cerevisiae* parental BY4743 strain (*MATa/α, his3Δ1/his3Δ1, leu2Δ0/leu2Δ0, LYS2/lys2Δ0, met15Δ0/MET15, ura3Δ0/ura3Δ0*) and four single homozygous deletants, BY4743 Δ*cys3*, BY4743 Δ*cys4*, BY4743 Δ*met17* and BY4743 Δ*tum1*, were obtained from the EUROSCARF collection (Scientific Research and Development GmbH, Oberursel, Germany). The yeast strains, stored at -80 °C, were grown for two days at 28 °C on yeast extract-peptone-dextrose (YPD) plates (10 g $L^{-1}$ yeast extract, 20 g $L^{-1}$ peptone, 20 g $L^{-1}$ glucose and 20 g $L^{-1}$ agar). Strains with *kanMX* cassettes were selected on YPD plates containing 200 mg $L^{-1}$ geneticin (G418) sulfate (Sigma-Aldrich, Auckland, New Zealand) (Goldstein and McCusker, 1999).

### 2.2 Fermentations and $H_2S$ quantification

Yeast starter cultures were prepared by inoculating a single yeast colony into starter medium (2% sugar, non-sulfate synthetic grape must (SGM) plus 0.15 mM methionine (Huang et al., 2017b)) for 24 h at 28 °C. The starter culture was centrifuged, washed, and resuspended in

sterile water to inoculate 100 mL of medium (non-sulfate SGM ± 5 mM cysteine + 0.15 mM methionine) at $2.5 \times 10^6$ cells $L^{-1}$. The additional amounts of histidine (200 mg $L^{-1}$), leucine (300 mg $L^{-1}$) and uracil (100 mg $L^{-1}$) were added for auxotrophies (Harsch et al., 2010). Fermentations were conducted in triplicate in 250 mL flasks at 28 °C with constant shaking at 100 rpm. Fermentations were considered finished when weight loss was ≤ 0.1 g $day^{-1}$. $H_2S$ produced by yeast during fermentation was detected by silver nitrate $H_2S$ detector tubes (120SF: 1-1000 ppm; KITAGAWA, Japan) that were tightly fitted into the glass airlock of the flask (Park, 2008).

2.3 Harvesting yeast cells and fermentations for analysis of polysulfides via LC-MS/MS

Yeast cells and 'wine' from the finished fermentations were harvested together when weight loss reached ≤ 0.1 g $day^{-1}$. Cells (~$2 \times 10^8$; estimated using a Neubauer haemocytometer) were washed twice with sterile water and incubated with 20 µL of Zymolyase®-20T (0.005 g Zymolyase powder (20,000 units $g^{-1}$; Amsbio, Abingdon, UK) in 1 mL of Zymolyase buffer) and 60 µL Zymolyase buffer (1.2 M sorbitol, 0.1 M $KH_2PO_4$, pH 7.2) for 10 min at 37 °C. Then, 200 µL of mBBr (ThermoFisher Scientific, Auckland NZ; 10 mmol $L^{-1}$: 27.2 mg in 10 mL acetonitrile) was added and incubated a further 10 min at 37 °C. Finally, cells were incubated for 10 min at 60 °C and then stored at -80 °C before semi-quantitative analysis of polysulfides using LC-MS/MS (Pilkington et al., 2019). Finished fermentations (1 mL) were centrifuged at 3000 *g* for 10 min to separate yeast cells and solids before being stored at -80 °C. Thawed finished fermentations (0.4 mL) were mixed with 0.1 mL of mBBr (10 mmol $L^{-1}$) prior to analysis.

2.4 LC-MS/MS method

Analysis of the polysulfides present in yeast lysates and finished fermentations was carried out using an Agilent 1290 Infinity liquid chromatography (LC) system (Agilent Technologies,

Santa Clara, CA, USA) fitted with a quaternary pump and coupled to an Agilent 6460 triple quadrupole mass spectrometer (Agilent Technologies, Santa Clara, CA, USA) as described in Pilkington *et al.* (2019). Briefly, samples were centrifuged and filtered through Phenex™-RC 4 mm syringe filters 0.2 μm (Phenomenex, Torrance, CA, USA). Samples (10 μL) were injected onto a Kinetex C18 column (100 × 3 mm ID, 100 Å, 2.6 μm particle size, Phenomenex, Torrance, CA, USA), with the column heater set at 25 °C. Mobile phase A was 0.1 % aqueous formic acid and mobile phase B was 100 % acetonitrile. Two methods were used in the analysis: one for oxidised polysulfides with a flow rate of 0.3 mL $min^{-1}$ and one for mBBr-derivatised hydropolysulfides with a flow rate of 0.5 mL $min^{-1}$. The same gradient was used for both methods with mobile phase B as follows: 5 % at 0 min, 5 % at 6 min, 15 % at 10 min, 80 % at 12 min, 100 % at 15 min, and then back to 5 % at 17 min, for a total run time of 20 min. All mass spectrometric data were obtained in positive-ion mode using an Agilent jet stream electrospray ionisation (ESI) probe. Nitrogen (BOC, Auckland, New Zealand) was used as the desolvation gas at 5 L $min^{-1}$ and 300 °C, the nebuliser was set at 45 psi, with the sheath gas temperature at 250 °C and a flow of 11 L $min^{-1}$. The ESI capillary, nozzle and fragmentor voltages were set at 3500, 500 and 100 V, respectively. Agilent MassHunter Workstation Software Quantitative Analysis (Version B.0700) was used to analyse and semi-quantitate the polysulfides present (Santa Clara, CA, USA). The multiple reaction monitoring (MRM) properties of polysulfide compounds synthesised during this project and utilised for LC-MS analysis are presented in Table S1. Retention times were determined by analysing both the oxidised polysulfides and mBBr-derivatised polysulfides synthesised at the University of Auckland (Pilkington et al., 2019).

### 2.5 Statistical measures and software

148 The mean, standard deviation (SD), analysis of variance (ANOVA) and post-hoc Tukey's
149 honestly significant difference (HSD) test were performed using JMP software (SAS Institute,
150 Cary, NC, USA). Statistical significance was assigned if $p < 0.05$ (95 % confidence interval).
151 Calculation of the t-test statistic was performed using R software (version 4.1.1; reference) and
152 the package ggplot2 (Wickham and Chang, 2016) was used for data visualisation.

## 3. Results and discussion

### 3.1 $H_2S$ production by *S. cerevisiae* BY4743 and BY4743 single deletants (Δ*cys3*, Δ*cys4*, Δ*met17* and Δ*tum1*) during fermentation in a grape juice-like medium supplemented with or without 5 mM cysteine

BY4743 wild type and BY4743 single homozygous deletants Δ*cys3*, Δ*cys4*, Δ*met17* and Δ*tum1* were fermented in SGM, with and without 5 mM cysteine supplementation, based on our previous experiments (Huang et al., 2016, 2017b). A minimal concentration (0.15 mM) of methionine was also supplemented to all fermentations to facilitate the yeast cell growth impeded by the *met17* auxotrophy of BY4743 Δ*met17*. No $H_2S$ was detectable when yeast strains were fermented in non-sulfate SGM plus 0.15 mM methionine, without the supplementation of cysteine (Table 1). The absence of cysteine also resulted in the lack of growth of the Δ*cys3* and Δ*cys4* mutants, which are cysteine auxotrophs (Thomas and Surdin-Kerjan, 1997). The addition of 5 mM cysteine to SGM resulted in detectable $H_2S$ production for the BY4743 wild type (160 ppm). Deletion of *CYS3*, *CYS4* and *MET17* in the BY4743 background resulted in an increase in $H_2S$ production from cysteine (~787 ppm, > 1000 ppm and >1000 ppm, respectively), while the deletion of *TUM1* reduced the amount of $H_2S$ generated by approximately half compared to the wild type (70 ppm) (Table 1). The elevated $H_2S$ production for the Δ*met17* deletant and the reduced production for Δ*tum1* from cysteine is consistent with our previous findings (Huang et al., 2016; Kinzurik et al., 2016). Both *CYS4* and *CYS3* are involved in cysteine biosynthesis (Fig. 1, Ono et al. (1999)) and the generation of $H_2S$ from cysteine (Hopwood et al., 2014; Singh et al., 2009; Tan et al., 2017). The increased $H_2S$ production from cysteine in Δ*cys3* and Δ*cys4* may be due to their inability to synthesise cysteine and glutathione downstream of the SAP; the excess sulfide generated from cysteine being released as $H_2S$ during fermentation. *CYS4* and *CYS3* are responsible for the first and the

second step of cysteine biosynthesis, respectively (Fig. 1). This may explain why Δ*cys3* produced less $H_2S$ than Δ*cys4* as the sulfide in a Δ*cys3* mutant could flow down one step further in the pathway to make cystathionine from homocysteine via Cys4p. While sulfide can also be used to synthesise methionine, it has been shown that homocysteine is primarily directed to the cysteine and glutathione route rather than towards methionine (Ono et al., 1999). Interestingly, the increased $H_2S$ production from the addition of 5 mM cysteine to the BY4743 Δ*cys3* and Δ*cys4* deletants was not observed in the same mutants in the commercial wine yeast strain AWRI1631 background (Huang et al., 2016).

3.2 Analysis of the intracellular polysulfides present within *S. cerevisiae* after fermentation in a grape juice-like medium supplemented with or without 5 mM cysteine

A semi-quantitative *in-house* LC-MS/MS method was employed to investigate the polysulfide species present at the end of fermentation within *S. cerevisiae* cells, using the BY4743 laboratory strain and four BY4743 single deletants in Δ*cys3*, Δ*cys4*, Δ*met17* and Δ*tum1* (Table 1). No polysulfide data is reported for the Δ*cys3* and Δ*cys4* deletants in the '0 cysteine' treatment group as the strains were unable to grow without cysteine. Polysulfides were detectable and semi-quantified in the BY4743 wild type and deletant cells that were able to grow and ferment in the SGM, with or without cysteine. CysS-bimane (cysteine) and GS-bimane (glutathione), plus five mBBr-derivatised hydropolysulfides (CysSS-bimane, CysSSS-bimane, CysSSSS-bimane, GSS-bimane and GSSSS-bimane), and two oxidised polysulfides (GSSG and GSSSSG), were detected for the first time within *S. cerevisiae* cells after fermentation. As expected, both the tripeptide glutathione (γ-glutamylcysteinylglycine, GSH, GS-bimane), and its oxidised form, GSSG, were detected in *S. cerevisiae*. This observation is in agreement with the intracellular detection of GS-bimane and GSSG in Pilkington et al.

(2019) with BY4743 under aerobic growth conditions using the same LC-MS/MS method. Glutathione is known to be abundantly present in *S. cerevisiae* (1–10 mM) and plays a critical role in the protection against oxidative stress (Powis et al., 1995). GSH is in equilibrium with its oxidised form GSSG, at a 50:1 (GSH:GSSG) ratio under reducing cellular conditions (Drakulic et al., 2005). Glutathione can be synthesised by yeast from either methionine or cysteine (Fig.1). The addition of cysteine (5 mM) significantly increased the formation of GSH and GSSG (Table 1), which is in agreement with a previous study (Alfafara et al., 1992). High concentrations of cysteine are toxic to yeast (Kumar et al., 2006), so excess cysteine is converted to both $H_2S$ and glutathione (Fig.1).

For cysteine hydropolysulfides, there was an overall trend towards higher peak areas with the addition of 5 mM cysteine to the fermentations, with the exception of CysSSSS-bimane (Table 1). Although the standard errors for each species were too high to determine whether this general increase was significant using ANOVA with post-hoc Tukey's HSD, calculation of the t-test statistic, showed negative values for CysS-bimane, CysSS-bimane and CysSSS-bimane for BY4743 (Fig. 2). This means that there is a strong indication that higher cysteine levels induce cysteine hydropolysulfide formation in cell lysates. Higher polysulfide peak areas were expected in cells supplemented with 5 mM cysteine, given that this amino acid is an important precursor for polysulfide production (Hu et al., 2019). It is thought that extracellular cysteine enters yeast cells and is converted directly to cysteine persulfide/polysulfides (CysSSH/CysS–$(S)_n$–H) by cysteinyl-tRNA synthetases (CARSs), as reported in prokaryotes and mammals (Akaike et al., 2017) (Fig. 1). Yeast cysteinyl-tRNA synthetase (encoded by *CRS1*) has been proposed to be involved in mitochondrial energy metabolism which is linked to the persulfide function (Nishimura et al., 2019), but its role in the production of cysteine polysulfides still needs to be verified. In the fermentations without cysteine supplementation, the formation of polysulfides derived from cysteine are still possible, as cysteine can be made

by yeast through the addition of 0.15 mM methionine (Fig. 1). The presence of CysS-bimane (cysteine) in the yeast cells with 0 mM cysteine in the medium also supports this statement (Table 1). It is also clear that the addition of 5 mM cysteine to the fermentation medium greatly increases the amount of cysteine recovered back from cell pellets, as expected (Table 1). CysSSSSS-bimane was not detected within any of the yeast cells. The absence of CysSSSSS-bimane could also be explained by there being a degree of thermodynamic instability in having an odd-number of sulfur atoms, as CysSSS-bimane was found to have much lower peak areas than CysSS-bimane and CysSSSS-bimane. This idea is further supported by our data, with an absence of glutathionylated polysulfides with three or five S atoms, including GSSS-bimane, GSSSSS-bimane, GSSSG and GSSSSSG, within yeast cells (Table 1). In our previous study that looked at intracellular polysulfides present in yeast cells grown aerobically, polysulfides with odd numbers of S atoms, in this case including CysSSS-bimane, were also undetected (Pilkington et al., 2019). In this study we were able to detect the persulfide CysSS-bimane. In our previous study (Pilkington et al., 2019), CysSS-bimane was unexpectedly absent within yeast cells, given that the cystine provided as a sulfur source should result in the formation of CysSSH (Ida et al., 2014).

Surprisingly, there were no definitive or significant differences in cysteine hydropolysulfide levels (peak areas) detected in the BY4743 wild type compared to all four BY4743 single deletants (Δ*cys3*, Δ*cys4*, Δ*met17* and Δ*tum1*); Table 1). This result suggests that although these genes have key roles in the SAP and detoxification, with notable impacts on $H_2S$ and cysteine formation, there exist mechanisms within yeast cells to stabilise the levels of cysteine polysulfides. The level of total polysulfides in the fungus *Aspergillus nidulans* has been shown to be stable and not affected by differing levels of cysteine in the cell (Wróbel et al., 2009). The authors suggested that a stable level of polysulfides supports the idea that polysulfides play a critical role in cellular regulation, particularly for signalling, cell protection, and redox

253 homeostasis (Kasamatsu et al., 2016; Sawa et al., 2020; Wróbel et al., 2009) and excess levels
254 are associated with disease and cellular dysfunction (Kimura, 2015; Kunikata et al., 2017).
255 While this may partly explain the results observed, recent studies have revealed that the level
256 of total polysulfides changes during growth of *S. cerevisiae* in either synthetic defined (SD)
257 minimal medium using green-fluorescent-protein (GFP)-based probes (Hu et al., 2019), or in
258 YPD using high performance liquid chromatography (HPLC) with a fluorescence detector (Ran
259 et al., 2019). In general, the level of total polysulfides increased in the lag and early log phases
260 (~ 5-10 h), decreased at the end of log phase (~ 24 h), and then increased again in the stationary
261 phase (~ 48 h). Since all cells analysed in this research were taken at the stationary phase, there
262 could be growth-phase dependent homeostasis that is not impacted by the SAP. To prevent the
263 buildup of excess cysteine polysulfides, yeast may exchange the cysteine for glutathione, or
264 $H_2S$ may be regenerated from the polysulfides (Fig. 1). $H_2S$ has been widely regarded as the
265 precursor of polysulfides (Kharma et al., 2019) and can be generated from cysteine by Cys4p
266 (cystathionine $\beta$-synthase, CBS; Singh et al., 2009), Cys3p (cystathionine $\gamma$-lyase, CSE;
267 Hopwood et al., 2014) and Tum1p (sulfur transferase; Huang et al., 2016)) (Fig. 1). The
268 released $H_2S$ can then be transformed into polysulfides through several pathways: (1) sulfane
269 sulfur ($S^0$; sulfur-bonded sulfur atoms with six electrons but no charge) in cysteine polysulfides
270 generated by CARSs can transfer to $H_2S$ to yield other polysulfides (Kharma et al., 2019;
271 Shinkai and Kumagai, 2019); (2) sulfide:quinone oxidoreductase (SQOR) converts $H_2S$ to
272 thiosulfate, which is further converted to glutathione persulfide (GSSH) by
273 thiosulfate:glutathione sulfurtransferase (TST) (Melideo et al., 2014); (3) superoxide dismutase
274 (SOD) converts $H_2S$ to $H_2S_2$, $H_2S_3$ and $H_2S_5$ (Olson, 2018); and (4) nitric oxide (NO) interacts
275 with $H_2S$ to produce polysulfides ($H_2Sn$) (Kharma et al., 2019). Furthermore, it is possible that
276 cellular compartmentalisation, rather than the total abundance of each polysulfide species,
277 could be more important for cellular signalling, as suggested by studies on human cells and

tissues (Yuan et al., 2017). A recent study by Li et al. (2020) treated *S. cerevisiae* cells with cysteine and found that polysulfides did not change in peroxisomes but increased in the cytoplasm and mitochondria, supporting the idea of cellular compartmentalisation. Evidence from Dóka et al. (2016) has shown that the thioredoxin reductase-1 enzyme was able to reduce the amount of $H_2S_n$ in a polysulfide-dependent manner, plus glutathione (with NADPH and glutathione reductase) could decrease polysulfide levels (Liu et al., 2019). Further investigation into the yeast orthologs of these genes will be valuable to further understand polysulfide degradation and homeostasis within the cell.

For glutathione hydropolysulfides within BY4743 cells, there was a significant increase in peak areas with the addition of 5 mM cysteine for GS-bimane (p-value = 0.030) and GSS-bimane (p-value = 0.016) (Table 1 and Fig. 2). This increase was also seen for GSSSS-bimane but not significant (p-value = 0.405). As mentioned above, GSSS-bimane and GSSSSS-bimane were not detected within the yeast cells and were absent from cells in our previous study of intracellular polysulfides (Pilkington et al., 2019). There were no significant differences between intracellular glutathione levels in BY4743 compared to the Δ*tum1* and Δ*met17* deletants with no exogenous cysteine addition (as before, there was no growth for the Δ*cys3*, Δ*cys4* mutants). At 5 mM cysteine, Δ*cys4* had significantly lower amounts of glutathione than all strains except Δ*met17*, which has also been observed in the literature (Oluwatosin and Kane, 1997). The reason for the lower concentrations of glutathione in Δ*cys4* mutants is because these strains are unable to properly acidify their vacuoles, as the lack of Cys4p activity changes the redox state of the cytosol causing the inactivation of vacuolar ATPases (Oluwatosin and Kane, 1997). Glutathione is used to restore vacuolar acidification, and therefore restore yeast (V)-ATPase activity (Oluwatosin and Kane, 1997). For GSS-bimane and GSSSS-bimane, there were no significant differences at 0 mM or 5 mM cysteine in BY4743 compared to the Δ*cys3*, Δ*cys4*, Δ*met17* and Δ*tum1* deletants (Table 1). At 5 mM cysteine, the level of GSS-bimane in

303 Δ*cys4* did appear to be significantly lower than that of Δ*tum1*, which is likely due to the lower
304 glutathione levels in the Δ*cys4* mutant. Additionally, the t-test statistic demonstrates a very
305 strong negative value for the increase in GSS-bimane for Δ*tum1* upon 5 mM cysteine addition
306 (Fig. 2) which had a correspondingly low p-value (p-value = 0.001). Overall, the relative
307 consistency in polysulfide levels across the wild type and deletant yeast cells still supports the
308 notion that there is intracellular homeostasis of polysulfides within cells.
309 For oxidised polysulfides, none of the cysteine species were found within the BY4743 yeast
310 wild type cells (Table 1). This result is in agreement with our previous study (Pilkington et al.,
311 2019). For oxidised glutathione species, only GSSG and GSSSSG were found within yeast
312 cells. In BY4743 cells fermented in SGM, there was significantly more GSSG at 5 mM cysteine
313 compared to no addition of cysteine (p-value = 0.003). This trend was also observed for
314 GSSSSG, but the standard deviation was too high for this result to be significant. The t-test
315 statistics shown in Figure 3 also represent this, with a more negative value for GSSG than
316 GSSSSG, when comparing 0 vs 5 mM cysteine addition to BY4743. The higher GSSG at 5
317 mM cysteine would be due to the increased glutathione resulting from the intracellular
318 detoxification of excess cysteine, as discussed above. The single deletants also followed the
319 same trends for GSSG and GSSSSG (Table 1 and Fig. 3). These results further support the idea
320 that cells have mechanisms to maintain internal polysulfide homeostasis, despite the
321 differences between these mutants in sulfur regulation and the SAP.
322
323 3.3 Analysis of the extracellular polysulfides present in wines after *S. cerevisiae* fermentation
324 in a grape juice-like medium supplemented with or without 5 mM cysteine
325
326 The LC-MS/MS method was next applied to the supernatants, representing finished model
327 wines, after completion of fermentation with *S. cerevisiae* BY4743 wild type and four single

deletants in SGM with or without supplementation of 5 mM cysteine (Table 2). No hydropolysulfides, nor oxidised polysulfides, were detected in finished wines made by BY4743 or the BY4743 deletants without the supplementation of cysteine to the fermentation medium. With no cysteine addition, any polysulfides that are generated appear to remain within the yeast cells and are either not exported into the medium, are unstable, or are below the limit of detection. It is important to note that the 0 mM cysteine fermentations did not produce $H_2S$, which would likely limit the non-enzymatic formation of any polysulfides in the wine matrix.

With the addition of 5 mM cysteine to the SGM, CysS-bimane (cysteine) and GS-bimane (glutathione) were detected, along with three hydropolysulfides, CysSS-bimane, CysSSS-bimane, and GSS-bimane. All five of these species were shorter chain molecules and no polysulfides with greater or equal to four S atoms were detected in the finished wines.

Bekker et al. (2018) also observed higher levels of shorter sulfur chain species, including trisulfane (65.6%) ≫ tetrasulfane (22.5%) ≫ pentasulfane (10.1%) ≫ hexasulfane (1.4%) in model wine supplemented with 500 μM L-cysteine, 250 μM $Na_2S{\cdot}9H_2O$, 100 μM Fe(III) and 50 μM Cu(II). It could be hypothesised that the longer chain polysulfides were either not stable or their formation was less favourable. However, these observations are not in agreement with the results from Dekker et al. (2022), where longer chain polysulfides were detected later during fermentation, with no clear difference in abundance based on chain length, using a time course experiment. It is possible that the longer chain polysulfides were not stable in our particular synthetic wine matrix, or that the CysSSSSH and GSSSSG which had been formed by the BY4743 yeast (Table 1) were not exported out of the cell. The export of longer chain polysulfides into the wine shown by Dekker et al. (2022) could represent differences between lab yeast strains and commercial wine strains, as the Anchor VIN13 yeast was used in their fermentations.

Further investigation is required to understand the formation, export, and degradation of longer chain polysulfide species. So far, there is evidence that they disappear with wine ageing (Bekker et al., 2018; Nardin et al., 2020). Regardless, the appearance of the shorter chain polysulfides with the addition of 5 mM cysteine does support the hypothesis that $H_2S$ resulting from cysteine degradation can contribute to extracellular polysulfide formation (Huang et al., 2017b; Kreitman et al., 2017). There was significantly higher CysS-bimane (cysteine) in the BY4743 wild type compared to the four single deletants (Δ*cys3*, Δ*cys4*, Δ*met17* and Δ*tum1*). Higher cysteine present in BY4743 wines may be due to the differential requirement and higher consumption of exogenous cysteine in the SAP mutants compared to wild type, which can synthesise cysteine through its fully functional SAP. A flow-on effect of elevated cysteine in the wines produced by the BY4743 wild type compared to the deletants was also observed for the cysteine hydropolysulfide species, suggesting that cysteine is being converted into these hydropolysulfide species, with lower CysSS-bimane and CysSSS-bimane in all deletants (albeit not significantly different for CysSS-bimane in Δ*cys4* due to the high standard error). Glutathione and GSS-bimane also exhibited this same trend, with wines produced by the Δ*cys3*, Δ*cys4*, Δ*met17* and Δ*tum1* mutants also having much lower peak areas than those in the BY4743 wild type wine. This result also likely reflects the greater need for cysteine in the SAP mutants compared to wild type.

No oxidised polysulfide species were detected in finished wines produced by any of the BY4743 background strains. This result was particularly interesting, given that all wines with 5 mM cysteine had notable concentrations of $H_2S$ present, and that GSSG and GSSSG have previously been detected in red wines fermented with exogenous elemental sulfur (Jastrzembski et al., 2017), in young commercial white wines (Nardin et al., 2020; van Leeuwen et al., 2020), and in Chardonnay and synthetic grape musts (Dekker et al., 2022). However, a follow-up experiment by Jastrzembski et al. (2017), using a white must treated

with $S^0$ prior to fermentation did not have any GSSG or GSSSG, which was attributed to the formation of glutathione-S-sulfonate adducts, albeit with the caveat that free $SO_2$ was not measured. As discussed in Dekker et al. (2022), the wine matrix plays an important role in the accumulation of polysulfide species, with synthetic must, but not Chardonnay must, behaving differently based on the various treatment applications (control, $CuSO_4$, $S^0$, and $CuSO_4$ + $S^0$). While studies have confirmed the presence of polysulfides in young experimental wines made by researchers (Dekker et al., 2022; Jastrzembski et al., 2017; Nardin et al., 2020; van Leeuwen et al., 2020), the presence of polysulfides in commercial wine is still relatively unknown. To the best of our knowledge, there is only one study which has reported the presence of HSSH in commercial Swiss Chasselas wines (two out of 80 bottles in Starkenmann et al. (2016)). Therefore, further investigation into commercial wine would provide insight into the role of polysulfide species on wine aroma and the contribution of these molecules towards VSC-associated off-aromas and minerality.

## 4. Conclusions

In this work, we have shown that the bimane derivatives of hydropolysulfides e.g., CysSS-bimane, CysSSS-bimane, CysSSSS-bimane, GSS-bimane and GSSSS-bimane, and oxidised polysulfides GSSG as GSSSSG, were present within *S. cerevisiae* cells after fermentation in grape juice-like conditions, using a liquid chromatography-tandem mass spectrometry (LC-MS/MS) method. We then attempted to identify genes involved through studying the polysulfide production of the candidate gene deletants (BY4743 Δ*cys3*, Δ*cys4*, Δ*met17* and Δ*tum1*), chosen based on orthology to mammalian polysulfides-producing enzymes (*CYS3*, *CYS4* and *TUM1*) or the generation of large amounts of $H_2S$ from cysteine (*MET17*). Unexpectedly, no significant differences were observed between the deletants and the wild type,

indicating the existence of unknown pathways in yeast maintaining polysulfide homeostasis. Furthermore, while the addition of cysteine (5 mM) did increase the formation of shorter sulfur chain species such as GSS-bimane and GSSG in *S. cerevisiae*, there was no increase in the longer sulfur chain species. In addition, most of the longer sulfur chain polysulfides detected in *S. cerevisiae* were not detected in the finished fermentations, except for CysSSS-bimane, suggesting that the longer sulfur chain polysulfides were either not stable in a wine-like environment or their transportation was less favourable. There also appeared to be a stability issue for polysulfides with an odd number of S atoms, which were lower in abundance than even-numbered forms. These findings provide the first evidence for the presence of polysulfides within *S. cerevisiae* cells under fermentation conditions and highlight the complexity of their formation and release.

## Appendix A. Supplementary data

Supplementary data related to this article can be found at XXX (Table S1).

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## References


Akaike, T., Ida, T., Wei, F.-Y., Nishida, M., Kumagai, Y., Alam, Md.M., Ihara, H., Sawa, T., Matsunaga, T., Kasamatsu, S., Nishimura, Akiyuki, Morita, M., Tomizawa, K., Nishimura, Akira, Watanabe, S., Inaba, K., Shima, H., Tanuma, N., Jung, M., Fujii, S., Watanabe, Y., Ohmuraya, M., Nagy, P., Feelisch, M., Fukuto, J.M., Motohashi, H., 2017. Cysteinyl-tRNA synthetase governs cysteine polysulfidation and mitochondrial bioenergetics. Nature Communications 8, 1177. https://doi.org/10.1038/s41467-017-01311-y

Alfafara, C.G., Kanda, A., Shioi, T., Shimizu, H., Shioya, S., Suga, K., 1992. Effect of amino acids on glutathione production by Saccharomyces cerevisiae. Applied Microbiology and Biotechnology 36, 538–540. https://doi.org/10.1007/BF00170199

Araujo, L.D., Vannevel, S., Buica, A., Callerot, S., Fedrizzi, B., Kilmartin, P.A., du Toit, W.J., 2017. Indications of the prominent role of elemental sulfur in the formation of the varietal thiol 3-mercaptohexanol in Sauvignon blanc wine. Food Research International 98, 79–86. https://doi.org/10.1016/j.foodres.2016.12.023

Bekker, M.Z., Wilkes, E.N., Smith, P.A., 2018. Evaluation of putative precursors of key 'reductive' compounds in wines post-bottling. Food Chemistry 245, 676–686. https://doi.org/10.1016/j.foodchem.2017.10.123

Dekker, S., Fedrizzi, B., van Leeuwen, K.A., Nardin, T., Dell'Anna, C., Larcher, R., 2022. Time course accumulation of polysulfides in Chardonnay and model juice fermentations. Food Chemistry 371. https://doi.org/10.1016/j.foodchem.2021.131341

Dekker, S., Nardin, T., Mattana, M., Fochi, I., Larcher, R., 2020. Identification and characterisation of thiolated polysulfides in must and wine using online SPE UHPLC-HRMS. Analytical and Bioanalytical Chemistry 412, 5229–5245. https://doi.org/10.1007/s00216-020-02734-1

Dóka, É., Pader, I., Bíró, A., Johansson, K., Cheng, Q., Ballagó, K., Prigge, J.R., Pastor-Flores, D., Dick, T.P., Schmidt, E.E., Arnér, E.S.J., Nagy, P., 2016. A novel persulfide detection method reveals protein persulfide- and polysulfide-reducing functions of thioredoxin and glutathione systems. Science Advances 2, e1500968. https://doi.org/10.1126/sciadv.1500968

Drakulic, T., Temple, M.D., Guido, R., Jarolim, S., Breitenbach, M., Attfield, P.V., Dawes, I.W., 2005. Involvement of oxidative stress response genes in redox homeostasis, the level of reactive oxygen species, and ageing in Saccharomyces cerevisiae. FEMS Yeast Research 5, 1215–1228. https://doi.org/10.1016/j.femsyr.2005.06.001

Goldstein, A.L., McCusker, J.H., 1999. Three new dominant drug resistance cassettes for gene disruption in Saccharomyces cerevisiae. Yeast 15 (14), 1541–1553. https://doi.org/10.1002/(SICI)1097-0061(199910)15:14<1541::AID-YEA476>3.0.CO;2-K

Goode, J., Harrop, S., 2008. Wine faults and their prevalence: data from the world's largest blind tasting. In: Proceedings of Les XX$^{es}$ enttretiens scientifiques Lallemand ' Sulphur compounds production and sensory impact on wine. Horsens, Denmark (May 15, 2008).Harsch, M.J., Lee, S.A., Goddard, M.R., Gardner, R.C., 2010. Optimized fermentation of grape juice by laboratory strains of Saccharomyces cerevisiae. FEMS Yeast

Research 10 (1), 72–82. https://doi.org/10.1111/j.1567-1364.2009.00580.x

Hopwood, E.M.S., Ahmed, D., Aitken, S.M., 2014. A role for glutamate-333 of Saccharomyces cerevisiae cystathionine γ-lyase as a determinant of specificity. Biochimica et Biophysica Acta (BBA) - Proteins and Proteomics 1844, 465–472. https://doi.org/10.1016/j.bbapap.2013.11.012

Hu, X., Li, H., Zhang, X., Chen, Z., Zhao, R., Hou, N., Liu, J., Xun, L., Liu, H., 2019. Developing polysulfide-sensitive gfps for real-time analysis of polysulfides in live cells and subcellular organelles. Analytical Chemistry 91, 3893–3901. https://doi.org/10.1021/acs.analchem.8b04634

Huang, C., Walker, M.E., Fedrizzi, B., Gardner, R.C., Jiranek, V., 2017a. Hydrogen sulfide and its roles in Saccharomyces cerevisiae in a winemaking context. FEMS Yeast Research 17 (6), fox058. https://doi.org/10.1093/femsyr/fox058

Huang, C., Walker, M.E., Fedrizzi, B., Gardner, R.C., Jiranek, V., 2017b. Yeast genes involved in regulating cysteine uptake affect production of hydrogen sulfide from cysteine during fermentation. FEMS Yeast Research 17 (5), fox046. https://doi.org/10.1093/femsyr/fox046

Huang, C., Walker, M.E., Fedrizzi, B., Roncoroni, M., Gardner, R.C., Jiranek, V., 2016. The yeast TUM1 affects production of hydrogen sulfide from cysteine treatment during fermentation. FEMS Yeast Research 16 (8), fow100. https://doi.org/10.1093/femsyr/fow100

Ida, T., Sawa, T., Ihara, H., Tsuchiya, Y., Watanabe, Y., Kumagai, Y., Suematsu, M., Motohashi, H., Fujii, S., Matsunaga, T., Yamamoto, M., Ono, K., Devarie-Baez, N.O., Xian, M., Fukuto, J.M., Akaike, T., 2014. Reactive cysteine persulfides and S-polythiolation regulate oxidative stress and redox signaling. Proceedings of the National Academy of Sciences USA 111, 7606. https://doi.org/10.1073/pnas.1321232111

Jastrzembski, J.A., Allison, R.B., Friedberg, E., Sacks, G.L., 2017. Role of Elemental Sulfur in Forming Latent Precursors of $H_2S$ in Wine. Journal of Agricultural and Food Chemistry 65, 10542–10549. https://doi.org/10.1021/acs.jafc.7b04015

Kasamatsu, S., Nishimura, A., Morita, M., Matsunaga, T., Abdul Hamid, H., Akaike, T., 2016. Redox Signaling Regulated by Cysteine Persulfide and Protein Polysulfidation. Molecules 21, 1721. https://doi.org/10.3390/molecules21121721

Kharma, A., Grman, M., Misak, A., Domínguez-Álvarez, E., Nasim, M.J., Ondrias, K., Chovanec, M., Jacob, C., 2019. Inorganic Polysulfides and Related Reactive Sulfur–Selenium Species from the Perspective of Chemistry. Molecules 24(7), 1359. https://doi.org/10.3390/molecules24071359

Kimura, H., 2015. Signaling molecules: hydrogen sulfide and polysulfide. Antioxid Redox Signal 22, 362–376. https://doi.org/10.1089/ars.2014.5869

Kinzurik, M.I., Deed, R.C., Herbst-Johnstone, M., Slaghenaufi, D., Guzzon, R., Gardner, R.C., Larcher, R., Fedrizzi, B., 2020. Addition of volatile sulfur compounds to yeast at the early stages of fermentation reveals distinct biological and chemical pathways for aroma formation. Food Microbiology 89, 103435. https://doi.org/10.1016/j.fm.2020.103435

Kinzurik, M.I., Herbst-Johnstone, M., Gardner, R.C., Fedrizzi, B., 2016. Hydrogen sulfide production during yeast fermentation causes the accumulation of ethanethiol, S-ethyl thioacetate and diethyl disulfide. Food Chemistry 209, 341–347. https://doi.org/10.1016/j.foodchem.2016.04.094

522 Kinzurik, M.I., Ly, K., David, K.M., Gardner, R.C., Fedrizzi, B., 2017. The GLO1
523 Gene Is Required for Full Activity of O-Acetyl Homoserine Sulfhydrylase
524 Encoded by MET17. ACS Chemical Biology 12, 414–421.
525 https://doi.org/10.1021/acschembio.6b00815
526 Koike, S., Ogasawara, Y., Shibuya, N., Kimura, H., Ishii, K., 2013. Polysulfide
527 exerts a protective effect against cytotoxicity caused by t-
528 buthylhydroperoxide through Nrf2 signaling in neuroblastoma cells. FEBS
529 Letters 587, 3548–3555. https://doi.org/10.1016/j.febslet.2013.09.013
530 Kolluru, G.K., Shen, X., Bir, S.C., Kevil, C.G., 2013. Hydrogen sulfide chemical
531 biology: Pathophysiological roles and detection. Nitric Oxide - Biology and
532 Chemistry 35, 5–20. https://doi.org/10.1016/j.niox.2013.07.002
533 Kreitman, G.Y., Danilewicz, J.C., Jeffery, David.W., Elias, R.J., 2017. Copper(II)-
534 Mediated Hydrogen Sulfide and Thiol Oxidation to Disulfides and Organic
535 Polysulfanes and Their Reductive Cleavage in Wine: Mechanistic
536 Elucidation and Potential Applications. Journal of Agricultural and Food
537 Chemistry 65, 2564–2571. https://doi.org/10.1021/acs.jafc.6b05418
538 Kreitman, G.Y., Elias, R.J., Jeffery, D.W., Sacks, G.L., 2019. Loss and formation
539 of malodorous volatile sulfhydryl compounds during wine storage. Critical
540 Reviews in Food Science and Nutrition 59, 1728–1752.
541 https://doi.org/10.1080/10408398.2018.1427043
542 Kumar, A., John, L., Alam, M.M., Gupta, A., Sharma, G., Pillai, B., Sengupta, S.,
543 2006. Homocysteine- and cysteine-mediated growth defect is not
544 associated with induction of oxidative stress response genes in yeast.
545 Biochemical Journal 396, 61–69. https://doi.org/10.1042/BJ20051411
546 Kunikata, H., Ida, T., Sato, K., Aizawa, N., Sawa, T., Tawarayama, H.,
547 Murayama, N., Fujii, S., Akaike, T., Nakazawa, T., 2017. Metabolomic
548 profiling of reactive persulfides and polysulfides in the aqueous and
549 vitreous humors. Scientific Reports 7, 41984.
550 https://doi.org/10.1038/srep41984
551 Kwasniewski, M.T., Sacks, G.L., Wilcox, W.F., 2014. Persistence of Elemental
552 Sulfur Spray Residue on Grapes during Ripening and Vinification.
553 American Journal of Enology and Viticulture 65, 453.
554 https://doi.org/10.5344/ajev.2014.14027
555 Li, Z., Wang, Q., Xia, Y., Xun, L., Liu, H., 2020. A Red Fluorescent Protein-Based
556 Probe for Detection of Intracellular Reactive Sulfane Sulfur. Antioxidants
557 (Basel) 9, 985. https://doi.org/10.3390/antiox9100985
558 Liu, H., Radford, M.N., Yang, C.-T., Chen, W., Xian, M., 2019. Inorganic
559 hydrogen polysulfides: chemistry, chemical biology and detection. British
560 Journal of Pharmacology 176, 616–627.
561 https://doi.org/10.1111/bph.14330
562 Melideo, S.L., Jackson, M.R., Jorns, M.S., 2014. Biosynthesis of a central
563 intermediate in hydrogen sulfide metabolism by a novel human
564 sulfurtransferase and its yeast ortholog. Biochemistry 53, 4739–4753.
565 https://doi.org/10.1021/bi500650h
566 Nardin, T., Larcher, R., van Leeuwen, K.A., Pilkington, L.I., Roman, T.,
567 Malacarne, M., Fedrizzi, B., 2020. Effect of antioxidant supplementation on
568 the polysulfides of white wines. LWT 134, 110132.
569 https://doi.org/10.1016/j.lwt.2020.110132
570 Nishimura, A., Nasuno, R., Yoshikawa, Y., Jung, M., Ida, T., Matsunaga, T.,
571 Morita, M., Takagi, H., Motohashi, H., Akaike, T., 2019. Mitochondrial
572 cysteinyl-tRNA synthetase is expressed via alternative transcriptional
573 initiation regulated by energy metabolism in yeast cells. Journal of

574 Biological Chemistry 294, 13781–13788.
575 https://doi.org/10.1074/jbc.RA119.009203
576 Olson, K.R., 2018. H2S and polysulfide metabolism: Conventional and
577 unconventional pathways. Biochemical Pharmacology 149, 77–90.
578 https://doi.org/10.1016/j.bcp.2017.12.010
579 Olson, K.R., Straub, K.D., 2016. The role of hydrogen sulfide in evolution and
580 the evolution of hydrogen sulfide in metabolism and signaling. Physiology
581 31, 60–72. https://doi.org/10.1152/physiol.00024.2015
582 Oluwatosin, Y.E., Kane, P.M., 1997. Mutations in the CYS4 gene provide
583 evidence for regulation of the yeast vacuolar H+-ATPase by oxidation and
584 reduction in vivo. Journal of Biological Chemistry 272, 28149–28157.
585 https://doi.org/10.1074/jbc.272.44.28149
586 Ono, B.-I., Hazu, T., Yoshida, S., Kawato, T., Shinoda, S., Brzvwczy, J.,
587 Paszewski, A., 1999. Cysteine biosynthesis in Saccharomyces cerevisiae :
588 a new outlook on pathway and regulation. Yeast 15, 1365–1375.
589 https://doi.org/10.1002/(SICI)1097-0061(19990930)15:13<1365::AID-
590 YEA468>3.0.CO;2-U
591 Park, S.K., 2008. Development of a method to measure hydrogen sulfide in wine
592 fermentation. Journal of Microbiology and Biotechnology 18, 1550–1554.
593 Pilkington, L.I., Deed, R.C., Parish-Virtue, K., Huang, C., Walker, M.E., Jiranek,
594 V., Barker, D., Fedrizzi, B., 2019. Iterative synthetic strategies and gene
595 deletant experiments enable the first identification of polysulfides in
596 Saccharomyces cerevisiae. Chemical Communications 55, 8868-8871.
597 https://doi.org/ 10.1039/c9cc03020d
598 Powis, G., Briehl, M., Oblong, J., 1995. Redox signalling and the control of cell
599 growth and death. Pharmacology & Therapeutics 68, 149–173.
600 https://doi.org/10.1016/0163-7258(95)02004-7
601 Ran, M., Wang, T., Shao, M., Chen, Z., Liu, H., Xia, Y., Xun, L., 2019. Sensitive
602 Method for Reliable Quantification of Sulfane Sulfur in Biological Samples.
603 Analytical Chemistry 91, 11981–11986.
604 https://doi.org/10.1021/acs.analchem.9b02875
605 Sawa, T., Motohashi, H., Ihara, H., Akaike, T., 2020. Enzymatic Regulation and
606 Biological Functions of Reactive Cysteine Persulfides and Polysulfides.
607 Biomolecules 10, 1245. https://doi.org/10.3390/biom10091245
608 Schaedler, T.A., Thornton, J.D., Kruse, I., Schwarzländer, M., Meyer, A.J., Van
609 Veen, H.W., Balk, J., 2014. A conserved mitochondrial ATP-binding
610 cassette transporter exports glutathione polysulfide for cytosolic metal
611 cofactor assembly. Journal of Biological Chemistry 289, 23264–23274.
612 https://doi.org/10.1074/jbc.M114.553438
613 Shinkai, Y., Kumagai, Y., 2019. Sulfane Sulfur in Toxicology: A Novel Defense
614 System Against Electrophilic Stress. Toxicological Sciences 170, 3–9.
615 https://doi.org/10.1093/toxsci/kfz091
616 Singh, S., Padovani, D., Leslie, R.A., Chiku, T., Banerjee, R., 2009. Relative
617 contributions of cystathionine β-synthase and γ-cystathionase to $H_2S$
618 biogenesis via alternative trans-sulfuration reactions. Journal of Biological
619 Chemistry 284, 22457–22466. https://doi.org/10.1074/jbc.M109.010868
620 Starkenmann, C., Chappuis, C.J.-F., Niclass, Y., Deneulin, P., 2016.
621 Identification of Hydrogen Disulfanes and Hydrogen Trisulfanes in $H_2S$
622 Bottle, in Flint, and in Dry Mineral White Wine. Journal of Agricultural and
623 Food Chemistry 64, 9033–9040.
624 https://doi.org/10.1021/acs.jafc.6b03938

Tan, B., Jin, S., Sun, J., Gu, Z., Sun, X., Zhu, Y., Huo, K., Cao, Z., Yang, P., Xin, X., Liu, X., Pan, L., Qiu, F., Jiang, J., Jia, Y., Ye, F., Xie, Y., Zhu, Y.Z., 2017. New method for quantification of gasotransmitter hydrogen sulfide in biological matrices by LC-MS/MS. Scientific Reports 7, 46278. https://doi.org/10.1038/srep46278

Thomas, D., Surdin-Kerjan, Y., 1997. Metabolism of sulfur amino acids in Saccharomyces cerevisiae. Microbiology and Molecular Biology Reviews 61, 503–532. https://doi.org/10.1128/mmbr.61.4.503-532.1997

van Leeuwen, K.A., Nardin, T., Barker, D., Fedrizzi, B., Nicolini, G., Larcher, R., 2020. A novel LC-HRMS method reveals cysteinyl and glutathionyl polysulfides in wine. Talanta 218, 121105 https://doi.org/10.1016/j.talanta.2020.121105

Wickham, H., Chang, W., 2016. ggplot2: create elegant data visualisations using the grammar of graphics.https://ggplot2.tidyverse.org

Winter, G., Cordente, A.G., Curtin, C., 2014. Formation of hydrogen sulfide from cysteine in Saccharomyces cerevisiae BY4742: Genome wide screen reveals a central role of the vacuole. PLoS ONE 9 (12), e113869. https://doi.org/10.1371/journal.pone.0113869

Wróbel, M., Lewandowska, I., Bronowicka-Adamska, P., Paszewski, A., 2009. The level of sulfane sulfur in the fungus Aspergillus nidulans wild type and mutant strains. Amino Acids 37, 565–571. https://doi.org/10.1007/s00726-008-0175-x

Yuan, S., Shen, X., Kevil, C.G., 2017. Beyond a Gasotransmitter: Hydrogen Sulfide and Polysulfide in Cardiovascular Health and Immune Response. Antioxid Redox Signal 27, 634–653. https://doi.org/10.1089/ars.2017.7096

652 **Table 1.** Production of $H_2S$ and intracellular levels (peak areas) of polysulfide species in cellular lysates of BY4743 and BY4743 Δ*cys3*, Δ*cys4*,
653 Δ*met17* and Δ*tum1* single deletants when fermented in grape juice-like conditions (non-sulfate synthetic grape must (SGM) + 0.15 mM
654 methionine), with and without the supplementation of 5 mM cysteine.

| | **BY4743** | | **BY4743 Δ*cys3*** | | **BY4743 Δ*cys4*** | | **BY4743 Δ*met17*** | | **BY4743 Δ*tum1*** | |
|---|---|---|---|---|---|---|---|---|---|---|
| **Compound** | 0 cysteine[+] | 5 mM cysteine | 0 cysteine[†] | 5 mM cysteine | 0 cysteine[†] | 5 mM cysteine | 0 cysteine | 5 mM cysteine | 0 cysteine | 5 mM cysteine |
| $H_2S$ (ppm) | nd | 160.0$^{b}$ | ng | 787.0 ± 49.0$^{a}$ | ng | >1000.0[*] | nd | >1000.0[*] | nd | 70.0 ± 20.0$^{c}$ |
| **mBBr-derivatised hydropolysulfides** | | | | | | | | | | |
| CysS-bimane | 631.0$^{ab}$ | 11826.7 ± 5133.7$^{a}$ | ng | 12772.3 ± 5121.1$^{a}$ | ng | 12019.7 ± 5092.0$^{a}$ | 608.3 ± 167.8$^{b}$ | 10088.3 ± 2100.8$^{ab}$ | 431.0 ± 41.4$^{b}$ | 13908.7 ± 2041.7$^{a}$ |
| CysSS-bimane | 16.0$^{b}$ | 561.0 ± 291.4$^{b}$ | ng | 1277.0 ± 453.9$^{a}$ | ng | 508.3 ± 274.5$^{b}$ | 16.3 ± 5.9$^{b}$ | 457.0 ± 106.6$^{b}$ | 18.0 ± 6.0$^{b}$ | 565.0 ± 185.3$^{b}$ |
| CysSSS-bimane | 5.0$^{a}$ | 11.7 ± 3.2$^{a}$ | ng | 7.7 ± 5.5$^{a}$ | ng | 14.7 ± 6.7$^{a}$ | 7.0 ± 1.0$^{a}$ | 13.0 ± 3.6$^{a}$ | 5.3.0 ± 0.58$^{a}$ | 16.3 ± 4.7$^{a}$ |
| CysSSSS-bimane | 178.0$^{a}$ | 122.0 ± 12.5$^{ab}$ | ng | 99.7 ± 36.7$^{ab}$ | ng | 68.3 ± 20.8$^{b}$ | 109.0 ± 7.9$^{ab}$ | 123.0 ± 22.9$^{ab}$ | 119.0 ± 23.0$^{ab}$ | 71.3 ± 11.0$^{b}$ |
| CysSSSSS-bimane | nd | nd | ng | nd | ng | nd | nd | nd | nd | nd |
| GS-bimane | 2169.0$^{c}$ | 89530.7± 26921.1$^{a}$ | ng | 99426.7 ± 21288.4$^{a}$ | ng | 41398.3 ± 13741.1$^{bc}$ | 1826.0 ± 361.5$^{c}$ | 67441.3 ± 10612.0$^{ab}$ | 2154.0 ± 433.9$^{c}$ | 93348.7 ± 17469.9$^{a}$ |
| GSS-bimane | 454.0$^{cd}$ | 1740.0 ± 284.4$^{ab}$ | ng | 1933.0 ± 301.6$^{ab}$ | ng | 1323.3 ± 364.6$^{bc}$ | 497.0 ± 24.3$^{d}$ | 1493.0 ± 95.5$^{ab}$ | 412.0 ± 20.5$^{d}$ | 1979.0 ± 97.8$^{a}$ |
| GSSS-bimane | nd | nd | ng | nd | ng | nd | nd | nd | nd | nd |
| GSSSS-bimane | 795.0$^{ab}$ | 1242.7 ± 740.8$^{ab}$ | ng | 2323.7 ± 1199.0$^{a}$ | ng | 764.3 ± 628.0$^{ab}$ | 418.0 ± 109.1$^{b}$ | 1085.7 ± 486.4$^{ab}$ | 541.0 ± 147.7$^{ab}$ | 1369.7 ± 203.1$^{ab}$ |
| GSSSSS-bimane | nd | nd | ng | nd | ng | nd | nd | nd | nd | nd |
| **Oxidised polysulfides** | | | | | | | | | | |
| CysSSCys | nd | nd | ng | nd | ng | nd | nd | nd | nd | nd |
| CysSSSCys | nd | nd | ng | nd | ng | nd | nd | nd | nd | nd |
| CysSSSSCys | nd | nd | ng | nd | ng | nd | nd | nd | nd | nd |

| | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| CysSSSSSCys | nd | nd | ng | nd | ng | nd | nd | nd | nd | nd |
| GSSG | 19.0[b] | 339.3 ± 31.2[a] | ng | 402.7 ± 103.5[a] | ng | 151.3 ± 24.7[b] | 9.0 ± 3.0[b] | 338.0 ± 36.5[a] | 24.0 ± 4.0[b] | 382.3 ± 81.2[a] |
| GSSSG | nd | nd | ng | nd | ng | nd | nd | nd | nd | nd |
| GSSSSG | 38.0[a] | 484.3 ± 475.2[a] | ng | 142.3 ± 110.8[a] | ng | 327.7 ± 83.9[a] | 992.7 ± 602.3[a] | 898.3 ± 711.9[a] | 492.0 ± 155.6[a] | 2976.5 ± 3297.3[a] |
| GSSSSSG | nd | nd | ng | nd | ng | nd | nd | nd | nd | nd |

655

656 * $H_2S$ was measured by silver nitrate $H_2S$ detector tubes with maximum detection limit of 1000 ppm.

657 + Data are means ± SD ($n$ = 3), except for BY4743 (0 cysteine) as two samples were lost during the filtration step; Samples with the same
658 superscript letter for each polysulfide are not significantly different (ANOVA and Tukey's HSD, $p < 0.05$); ng: denotes no growth; nd: denotes
659 not detected.

660 † Both Δ*cys3* and Δ*cys4* strains are cysteine auxotrophs.

661 **Table 2.** Production of $H_2S$ and extracellular levels (peak areas) of polysulfide species present in wine-like media from BY4743 and BY4743

662 Δ*cys3*, Δ*cys4*, Δ*met17* and Δ*tum1* deletants when fermented in grape juice-like conditions (non-sulfate synthetic grape must (SGM) + 0.15 mM

663 methionine), with and without the supplementation of 5 mM cysteine.

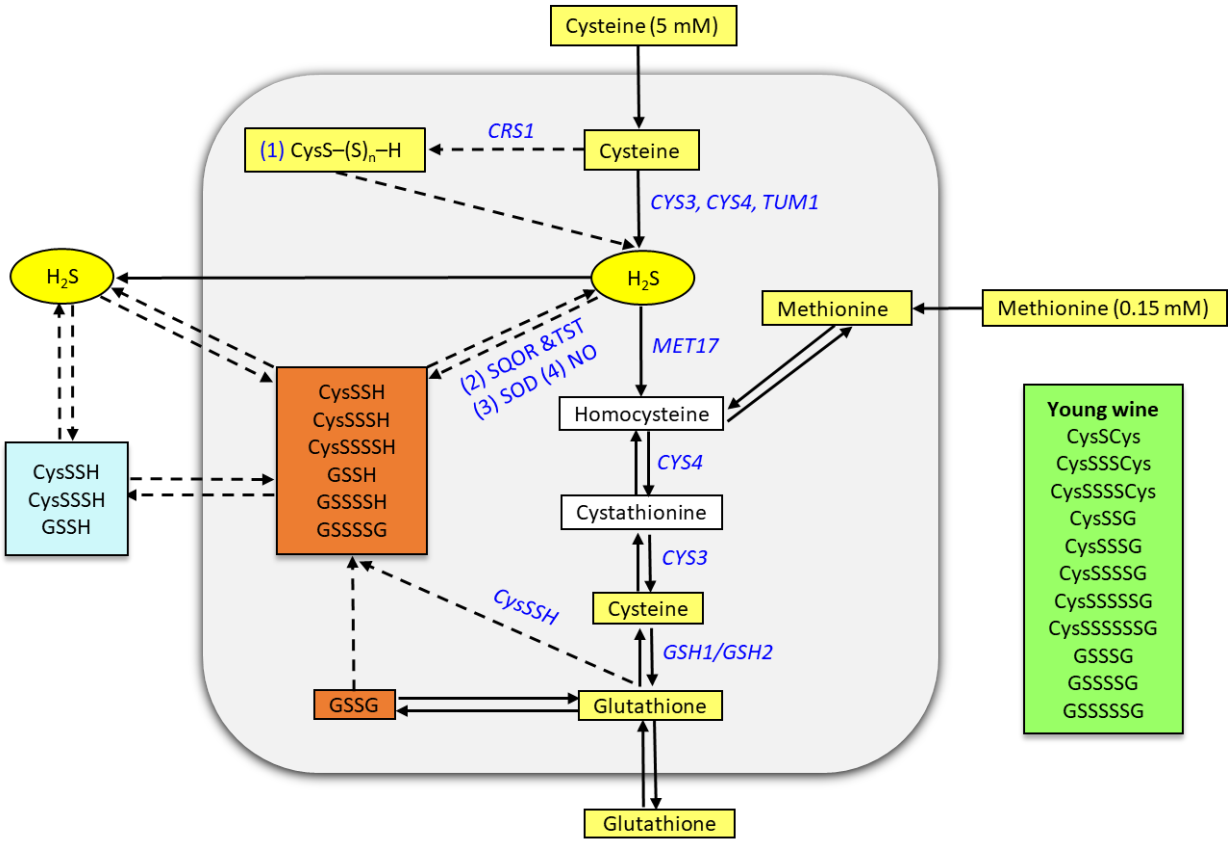
| | **BY4743** | | **BY4743 *Δcys3*** | | **BY4743 *Δcys4*** | | **BY4743 *Δmet17*** | | **BY4743 *Δtum1*** | |
|---|---|---|---|---|---|---|---|---|---|---|
| **Compound** | 0 cysteine[+] | 5 mM cysteine | 0 cysteine[†] | 5 mM cysteine | 0 cysteine[†] | 5 mM cysteine | 0 cysteine | 5 mM cysteine | 0 cysteine | 5 mM cysteine |
| $H_2S$ (ppm) | nd | 160.0[b] | ng | 787.0 ± 49.0[a] | ng | >1000.0[*] | nd | >1000.0[*] | nd | 70 ± 20.0[c] |
| **mBBr-derivatised polysulfides** | | | | | | | | | | |
| CysS-bimane | nd | 495476.0 ± 17083.7[a] | ng | 265020.3 ± 12332.4[b] | ng | 252101.3 ± 82595.0[b] | nd | 197279.0 ± 76687.1[b] | nd | 254182.3 ± 5652.3[b] |
| CysSS-bimane | nd | 72476.0 ± 5921.3[a] | ng | 42669.3 ± 3415.9[b] | ng | 49282.3 ± 7192.0[ab] | nd | 29476.5 ± 17182[b] | nd | 42527.0 ± 5165.7[b] |
| CysSSS-bimane | nd | 530.0 ± 38.4[a] | ng | 134.3 ± 31.3[b] | ng | 121.7 ± 31.3[b] | nd | 244.0 ± 38.4[b] | nd | 147.0 ± 31.3[b] |
| CysSSSS-bimane | nd | nd | ng | nd | ng | nd | nd | nd | nd | nd |
| CysSSSSS-bimane | nd | nd | ng | nd | ng | nd | nd | nd | nd | nd |
| GS-bimane | nd | 38775.0 ± 2825.6[a] | ng | 8074.7 ± 612.3[c] | ng | 14501.0 ± 838.5[b] | nd | 9762.0 ± 411.5[c] | nd | 10991.7 ± 760.5[c] |
| GSS-bimane | nd | 3034.5 ± 119.7[a] | ng | 941.3 ± 97.7[b] | ng | 1392.0 ± 97.7[b] | nd | 918.0 ± 119.7[b] | nd | 1209.0 ± 97.7[b] |
| GSSS-bimane | nd | nd | ng | nd | ng | nd | nd | nd | nd | nd |
| GSSSS-bimane | nd | nd | ng | nd | ng | nd | nd | nd | nd | nd |
| GSSSSS-bimane | nd | nd | ng | nd | ng | nd | nd | nd | nd | nd |
| **Oxidised polysulfides** | | | | | | | nd | | nd | |
| CysSSCys | nd | nd | ng | nd | ng | nd | nd | nd | nd | nd |
| CysSSSCys | nd | nd | ng | nd | ng | nd | nd | nd | nd | nd |
| CysSSSSCys | nd | nd | ng | nd | ng | nd | nd | nd | nd | nd |
| CysSSSSSCys | nd | nd | ng | nd | ng | nd | nd | nd | nd | nd |
| GSSG | nd | nd | ng | nd | ng | nd | nd | nd | nd | nd |
| GSSSG | nd | nd | ng | nd | ng | nd | nd | nd | nd | nd |

| | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| GSSSSG | nd | nd | ng | nd | ng | nd | nd | nd | nd | nd |
| GSSSSSG | nd | nd | ng | nd | ng | nd | nd | nd | nd | nd |

* $H_2S$ was measured by silver nitrate $H_2S$ detector tubes with maximum detection limit of 1000 ppm.

+ Data are means ± SD ($n$ = 3), except for BY4743 (0 cysteine) as two samples were lost during the filtration step; Samples with the same superscript letter for each polysulfide are not significantly different (ANOVA and Tukey's HSD, $p < 0.05$); ng: denotes no growth; nd: denotes not detected.

† Both Δ*cys3* and Δ*cys4* strains are cysteine auxotrophs.




669

670 **Fig. 1. Potential pathways for polysulfide production in the yeast *S. cerevisiae* under the**
671 **grape juice-like, cysteine-supplemented, fermentation conditions.** Extracellular cysteine is
672 taken up by *S. cerevisiae* to generate $H_2S$ and polysulfides. Cysteinylated (Cys) polysulfides
673 can be exchanged for glutathione (G). $H_2S$ may be regenerated from the polysulfides. Orange
674 background = polysulfides detected in *S. cerevisiae* (this study). Blue background =
675 polysulfides detected in finished fermentations (this study). Green background = polysulfides
676 identified in wine by other researchers but not in this study (Akaike et al., 2017; Dekker et al.,
677 2022; Kharma et al., 2019; Koike et al., 2013; Kolluru et al., 2013; Nardin et al., 2020; Olson
678 and Straub, 2016; Ono et al., 1999; Schaedler et al., 2014; van Leeuwen et al., 2020). Dashed
679 arrows indicate proposed pathways yet to be demonstrated. Only the genes relevant to the
680 present work are included; they are indicated via the blue text.

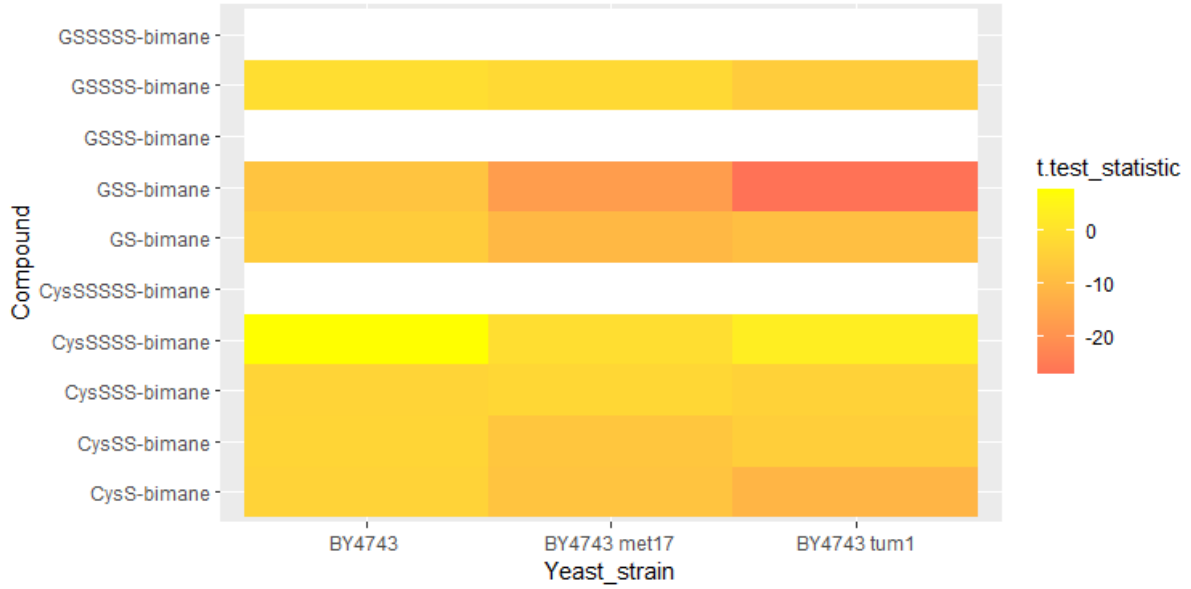



**Fig. 2. Heatmap representing the t-test statistic when comparing mBBr-derivatised hydropolysulfide levels (peak areas) with and without the supplementation of 5 mM cysteine to cellular lysates.** Negative t-test statistics are indicative of higher levels of hydropolysulfide when 5mM cysteine is added, whereas positive t-test statistics indicate lower levels of hydropolysulfide with 5mM cysteine, the magnitude being the strength of the evidence for the difference. A two-sample t-test was used, except when only one data replicate was available (noted in Tables 1 and 2) when a one sample t-test was used.

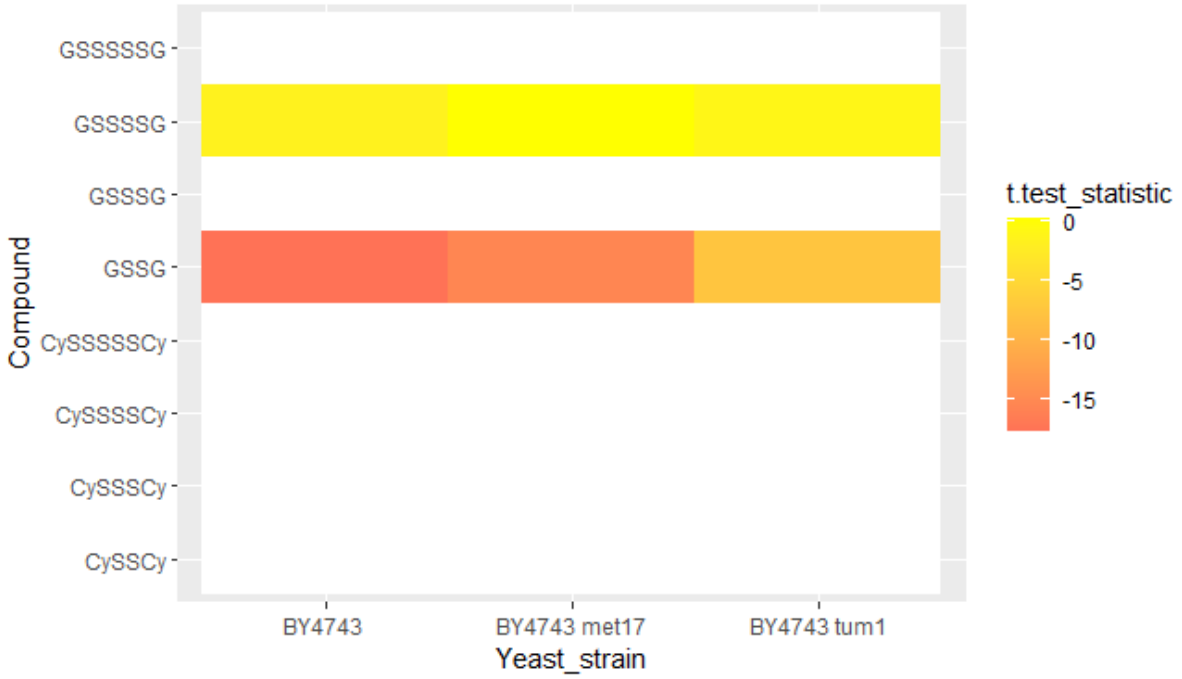



**Fig. 3. Heatmap representing the t-test statistic when comparing oxidised polysulfide levels (peak areas) with and without the supplementation of 5 mM cysteine to cellular lysates.** Negative t-test statistics are indicative of higher levels of oxidised polysulfide when 5mM cysteine is added, whereas positive t-test statistics indicate lower levels of oxidised polysulfide with 5mM cysteine, the magnitude being the strength of the evidence for the difference. A one sample t-test was used when only one data replicate was available (noted in Tables 1 and 2) and a two-sample t-test otherwise.

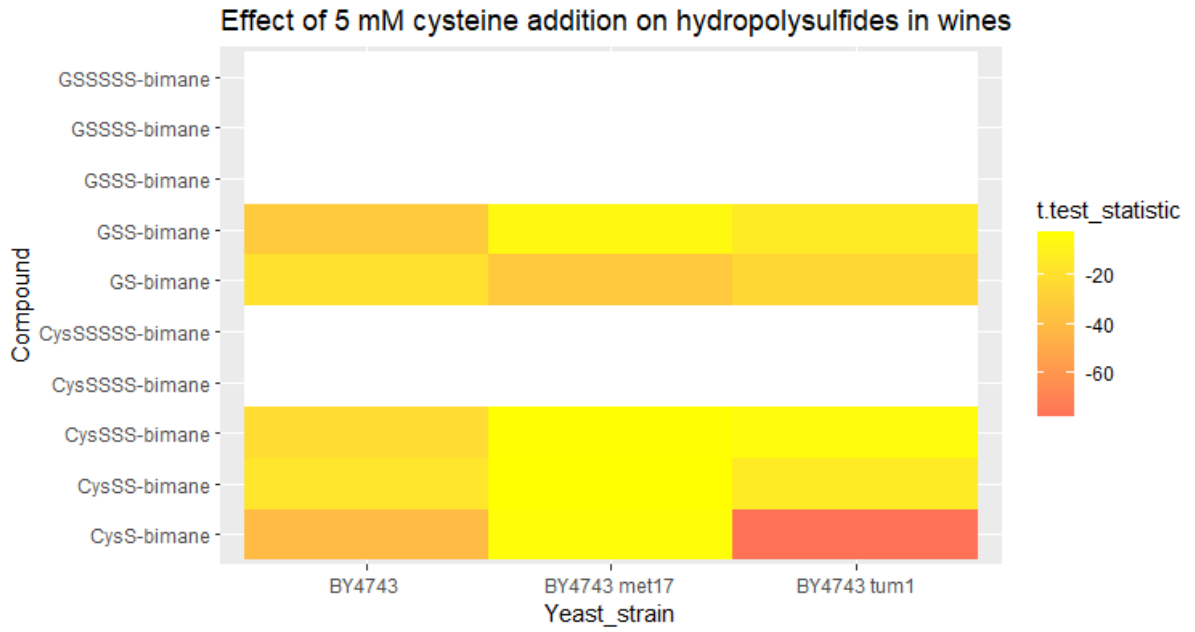



**Fig. 4. Heatmap representing the t-test statistic when comparing mBBr-derivatised hydropolysulfide levels (peak areas) with and without the supplementation of 5 mM cysteine in finished wines.** Negative t-test statistics are indicative of higher levels of hydropolysulfide when 5mM cysteine is added, whereas positive t-test statistics indicate lower levels of hydropolysulfide with 5mM cysteine, the magnitude being the strength of the evidence for the difference. A one sample t-test was used when only one data replicate was available (noted in Tables 1 and 2) and a two-sample t-test otherwise.