

Additions of Glutathione or Specific Glutathione-rich Dry Inactivated Yeast Preparation (DYP) to Sauvignon *blanc* Must: Effect on Wine Chemical and Sensory Composition

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Although the addition of pure glutathione (GSH) is not allowed under current regulations, the concentration of this compound can be increased in wine through the addition of glutathione-enriched dry yeast preparations (DYP). These preparations have been observed to have antioxidant properties and could thus influence wine aroma and sensory characteristics. The main aim of this study was to investigate the effect of DYP and GSH juice additions on the sensory and chemical composition of Sauvignon blanc wine. Four juice additions were performed and compared against a control treatment: 5.5 mg/L of GSH; 0.4 g/L of DYP; 80 mg/L of GSH; 0.4 g/L of DYP plus 80 mg/L of GSH. After three months of bottling, the volatile and sensorial composition was investigated. The addition of DYP preparations to must increased the concentration of certain wine volatile compounds, with increased attributes of riper tropical fruit aromas, which was not always observed with the GSH additions. The addition of DYP influenced the concentrations of some volatile compounds, which modified the white wine aroma. The release of compounds other than GSH by the yeast products is proposed as the reason for these changes. The results observed in this study can assist winemakers to modify the aroma profile of Sauvignon blanc wines.

INTRODUCTION

The tripeptide L-glutamyl-L-cysteinyl-glycine, or glutathione (GSH), is the major low-molecular-mass thiol compound in plants and animals (Kritzing *et al.*, 2013a). GSH plays an important role in the biosynthesis of many aroma precursors present in the berries of several cultivars. An example of this is given by the varietal's thiol compounds that contribute to typical Sauvignon blanc aromas of flower, boxwood, blackcurrant (mercapto-4-methylpentan-2-one, 4MMP), citrus, guava and passion fruit (3-mercaptohexan-1-ol, 3MH, 3-mercaptohexyl acetate, 3MHA), which originate from the precursors [S-3-(hexan-1-ol)-L-cysteine, S-4-(4-methylpentan-2-one)-L-cysteine, [S-3-(hexan-1-ol)-glutathione and S-4-(4-methylpentan-2-one)-glutathione (Coetzee & Du Toit, 2012). GSH conjugates are thus found in the juice and can be a source of the aroma compounds in the wines (Fedrizzi *et al.*, 2009; Roland *et al.*, 2010). The concentration of GSH in grapes and juice depends on the cultivar and climatic conditions. Lavigne-Cruege and

Dubourdieu (2002) reported GSH levels in different white cultivars to be between 17 and 114 mg/kg (56 to 372 $\mu\text{mol/kg}$). The GSH content in grape juice is influenced by factors such as exposure to oxygen, tyrosinase activity and grape skin maceration during pre-fermentation (Cheynier *et al.*, 1989; Du Toit *et al.*, 2007), with reported ranges varying from 10 to 100 mg/L (Cheynier *et al.*, 1989). Additionally, several authors have reported conflicting results on the trend of GSH during alcoholic fermentation (Du Toit *et al.*, 2007; Andujar-Ortiz *et al.*, 2012; Kritzing *et al.*, 2013b). The concentration of GSH in wine is lower than in the juice and grapes, and values from 1 to 20 mg/L typically are found (Du Toit *et al.*, 2007). *Saccharomyces cerevisiae* can also affect GSH content in wine during alcoholic fermentation and lees ageing (Kritzing *et al.*, 2013b), with a maximum reported increase of around 20 mg/L for a particular yeast strain (Lavigne-Cruege *et al.*, 2007).

The biological importance of GSH is due to the

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nucleophilic and reducing properties of the thiol group in the cysteine residue (Elskens *et al.*, 1991). GSH is involved in many vital functions of *S. cerevisiae*, such as maintaining the integrity of mitochondrial membranes, responding to oxidative stress, xenobiotics and endogenous toxic metabolite detoxification, and responding to deficiencies in sulphur and nitrogen (Penninckx, 2002; Tominaga & Dubourdieu, 2003). Moreover, GSH plays an important role in the oxidative phenomena of white musts and wines. Enzymatic oxidation occurs in musts and is correlated with the amounts of hydroxycinnamic acid esters (caftaric acid and coutaric acid) and flavan-3-ols (Li *et al.*, 2008). Phenolic compounds, particularly those with an *ortho*-diphenol group, are mainly responsible for oxidative browning (Betés-Saura *et al.* 1996). The reaction involves the oxidation of catechols to form unstable quinones, which are responsible for the wine browning (Oliveira *et al.*, 2011). GSH limits this phenomenon through the trapping of the unstable quinone molecule (Cheynier *et al.*, 1990; Rigaud *et al.*, 1991). GSH has also been proposed as an alternative antioxidant to SO₂ that potentially could lead to a decrease in the required sulphur levels in finished wines (Makhotkina *et al.*, 2014).

In addition, exposure to oxygen during white wine ageing can profoundly change the colour and aromatic profile of a wine (Simpson, 1978). In a young wine, the oxidative degradation of volatile compounds leads to a loss in fruity and floral aromas. Moreover, it has been reported that, in cases where the concentration of GSH in white wine exceeds 6 to 10 mg/L, both colour and aroma were better preserved during ageing and storage (Lavigne-Cruege & Dubourdieu, 2004). A number of authors have reported a positive effect of GSH against the oxidation of varietal thiols during the ageing and storage of white wine (Blanchard *et al.*, 2004; Dubourdieu & Lavigne-Cruege, 2004; Ugliano *et al.*, 2011, Coetzee & Du Toit 2012; Coetzee *et al.*, 2013).

Despite the observed effects of GSH in wine, the addition of GSH prior to bottling was recently approved by the OIV, as long as doses are never higher than 20 mg/L (OIV, 2015). In addition, GSH can be added to the must or wine through the addition of other permitted additives (DYP), which allow the amount of GSH to be increased (Pozo-Bayón *et al.*, 2009; OIV, 2013). DYPs are manufactured from the thermal inactivation of *S. cerevisiae* cultivated under specific conditions in which the intra-cellular accumulation of GSH is stimulated (Kritzinger *et al.*, 2013a). In the work of Pozo-Bayón *et al.*, (2009), four classification categories for DYPs were proposed: inactive yeast, yeast autolysates, yeast hulls or walls, and yeast extracts. The products referred to as GSH-rich inactivated dry yeast preparations (DYPs) are added during alcoholic fermentation and are expected to increase the wine GSH content, either by the liberation of GSH into the wine, or by allowing the yeast to assimilate GSH precursors during alcoholic fermentation (Kritzinger *et al.*, 2012). The addition of DYPs has been recommended by the producers in order to prevent aroma and colour losses and to increase the availability of fermentative nutrients in wines (Pozo-Bayón *et al.*, 2009). Andujar-Ortiz *et al.* (2012) and Kritzinger *et al.* (2012) reported an increase in the concentration of GSH after the addition of DYPs to Grenache and Sauvignon blanc juice. Moreover, Andujar-

Ortiz *et al.* (2014) reported an increase in the intensity of typical fruity attributes (banana, strawberry) in young Grenache rosé wines made after the addition of DYPs. These authors suggest that the GSH released from DYP could have caused a protective effect against the oxidation of wine aroma compounds. Other authors have also observed the protection of white and rosé wines against oxidation after the addition of a specific GSH-enriched inactivated DYP during alcoholic fermentation. Better protection of the chromatic and organoleptic properties, as well as a higher presence of some of the particular volatile thiols, was observed (Aguera *et al.*, 2012).

The evolution of the GSH content in GSH- or DYP-supplemented must during the fermentation process currently is poorly investigated. It is also not well known whether changes brought about in the sensorial and volatile composition of wines treated with DYPs are due to the GSH contained in these products or to other constituents being released into the must or wine. The aim of this work was thus to study the levels of extracellular GSH during alcoholic fermentation after the addition of different dosages of pure GSH, or by adding varying concentrations of a specific glutathione-rich inactivated dry yeast preparation (DYP). Moreover, the study also reports and discusses the influence of GSH and DYP additions on the volatile composition and sensory characteristics of Sauvignon blanc white wines.

MATERIALS AND METHODS

Chemicals and reagents

The organic yeast rehydration nutrient (GO-FERM PROTECT EVOLUTION™) and the specific GSH-rich inactivated yeast (OPTI-MUM WHITE EVOLUTION™ (DYP)) were obtained from Lallemend Blagnac, France. N₂ gas and CO₂ gas were purchased from Afrox, Cape Town, South Africa. Ascorbic acid, sulphur dioxide as K₂S₂O₅, methanol, reduced GSH, formic acid, acetonitrile, tris-EDTA, meta-phosphoric acid, ethanol, sodium sulphate anhydrous, *p*-hydroxymercurybenzoate, butylated hydroxyanisole, dichloromethane and diethyl ether were purchased from Sigma Aldrich (St Louis, MI, USA). Standards for the quantification of esters, alcohols, fatty acids and terpenes were also obtained from Sigma Aldrich. HPLC-grade water was obtained from a Milli-Q system (Millipore Filter Corp., Bedford, MA).

Juice and winemaking treatments

Clear Sauvignon blanc juice (2013 vintage, 22.5°Brix, pH 3.3 and total acidity 5.5 g/L) was collected from Neil Ellis Wines, based in Stellenbosch, and divided into 10 L stainless steel canisters. Nitrogen in gas form was blown inside the canisters to replace oxygen before juice transfer. The juice was divided into five treatments: the control (C), and additions of 5.5 mg/L of GSH (GSH 5.5), 80 mg/L of GSH (GSH 80), 0.4 g/L of DYP (DYP), and 80 mg/L of GSH plus 0.4 g/L of DYP (DYP+GSH 80). DYP was added at levels according to Lallemend's recommendation (DYP treatment). Finally, the maximum dose of GSH was combined with DYP addition at 0.4 g/L to investigate a possible synergistic effect. An aliquot of the juice was used to ensure complete dissolution of the GSH or DYP additions into the juice matrix. Treatments

were supplemented just before yeast inoculation according to the supplier's recommendations (www.lallemand.com). Three fermentation replicates per treatment were performed. The yeast strain *Saccharomyces cerevisiae* LALVIN® QA23 (Lallemand) was rehydrated in GO-FERM PROTECT EVOLUTION™ at 0.3 g/L (Lallemand) and inoculated into the juice at 0.3 g/L. Fermentation took place under controlled temperature at 15°C. The progress of fermentation was monitored by measuring weight loss. At the end of fermentation, when no more weight loss had occurred for three consecutive days, 60 mg/L of sulphur dioxide was added to all the treatments and the wines were stored at -4°C for ten days to carry out tartrate stabilisation. The wine was then racked with N₂ from the yeast lees and bottled in green 750 mL screw-cap wine bottles under CO₂ gas.

Standard winemaking analyses

Routine wine analyses were performed after alcoholic fermentation (residual reducing sugar, total acidity (TA), alcohol, volatile acidity (VA), malic acid and pH) using a Grapescan™ FT 120 instrument (Foss Electric, Denmark).

Sampling procedure

Samples destined for UPLC-MS/MS analyses were taken just after the juice was collected in the 10 L stainless steel canisters. The sampling was carried out before and after the addition of DYP and GSH, and at difference stages throughout the fermentation, namely after 25%, 50%, 75% and 100% completion of alcohol fermentation. Twenty mL of juice were transferred from the canisters into 20 mL plastic vials. To completely inhibit residual phenol oxidase or laccase activity, SO₂ and ascorbic acid is raised to 1 000 mg/L and 500 mg/L respectively. The plastic vials were saturated with CO₂ before and after the juice was transferred into the vials. Additional CO₂ was blown over the headspace, and the vials were sealed hermetically. The vials were then frozen at -20°C until analysis. The analysis of aroma compounds and sensory analysis were performed three months after bottling.

Preparation of glutathione samples

Samples (4 mL) were thawed and centrifuged (Centrifuge 5415 D, Eppendorf, Hamburg, Germany) at 1 600 rpm for 5 min at 20°C. The supernatant was then diluted five times with a solution containing 1 000 mg/L of SO₂ and 500 mg/L of ascorbic acid. Finally, the samples were filtered through a 0.45 µm syringe filter and injected onto the UPLC-MS/MS instrument (Kritzing et al. 2012).

Quantification of reduced GSH released from the specific glutathione-rich inactivated yeast (DYP)

The GSH levels released from the DYP were evaluated in a model solution consisting of 5 g/L of tartaric acid adjusted to pH 3.3 with 1 M NaOH. In order to decrease oxygen in the model solution, N₂ gas was bubbled for 30 min prior to DYP addition, after which 0.4 g of DYP was added to 100 mL of model solution and stirred for 10 min. A sample was then drawn and injected within the next half an hour. The quantification was performed in triplicate.

Glutathione analysis

GSH concentrations were determined using an UPLC-MS/MS method described by Kritzing et al. (2012). A Waters Acquity UPLC (Milford, MA, USA) connected to a Waters Xevo triple-quadrupole mass spectrometer was used for GSH quantification. The separation was achieved using a Waters Acquity BEH phenyl column (100 mm x 2.1 mm x 1.7 µm). For solvents A and B, 0.4% trifluoroacetic acid and acetonitrile were used respectively. Solvent gradient has been reported elsewhere (Kritzing et al., 2012). A cone voltage of 18 V in combination with a multiple reaction monitoring transition of 308.1 > 179.1 at a collision energy of 17 eV was used as optimised setting. The injection volume was 3 µL and a dilution of five times in water was used to achieve the best accuracy.

Thiol analysis

Thiol compounds were analysed using the extraction method reported by Tominaga et al. (1998), with some modifications as reported by Coetzee et al. (2013). Initially, 5 mL of 1 mM *p*-hydroxymercurybenzoate (*p*-HMB), and then 0.5 mL of 2 nM butylated hydroxyanisole (BHA) solution, were added to 50 mL of wine. After stirring, 50 µL of a deuterated internal standard solution (supplied by Auckland University, New Zealand) containing 22 mM of 3-mercapto-hexanol (3MH), 2.8 mM of 3-mercapto-hexyl acetate (3MHA) and 2.5 nM of 4-methoxy-2-methyl-2-mercaptobutane (4M2M2SB) used to quantify 4-mercapto-4-methylpentan-2-one (4MMP) was incorporated into the wine. This was followed by the injection of 2 µL of a concentrated sample onto an Agilent Gas Chromatograph 6890N coupled to an Agilent 5973 mass-selective detector (Agilent, Santa Clara, CA, USA). A HP-Innowax column (60 m x 252 µm x 0.25 µm) with helium as carrier gas was used for compounds separation. Flow rates, oven temperatures and standard curves used for thiol quantification have been reported elsewhere (Coetzee et al., 2013).

Ester, alcohol, fatty acid and terpene analyses

Ester, alcohol and fatty acid concentrations were determined using the GC-Flame ionisation detector (FID) method described by Coetzee et al. (2013). The volatile compounds were extracted from the wine (5 mL) using a liquid-liquid extraction by sonicating the diethyl ether (1 mL) wine mixture for 5 min. 4-Methyl-2-pentanol (100 µL of 0.5 mg/l solution in a 12% ethanol–water mixture) was used as internal standard. The mixture was then centrifuged at 4 000 RPM for 3 min before the ether layer was removed and dried by adding Na₂SO₄. The extracts were injected in duplicate in a Agilent 6890 Plus GC (Little Falls, Wilmington, DE, USA) equipped with a split/splitless injector and FID detector using a J&W DB-FFAP capillary GC column (Agilent, Little Falls, Wilmington, DE, USA) with the following dimensions: 60 m x 0.32 mm x 0.5 µm. A 15:1 split ratio was used, with a split flow rate of 49.5 mL/min and a column flow rate of 3.3 mL/min using hydrogen as a carrier. Method validation, quantification as well as separation conditions have been reported elsewhere (Coetzee et al., 2013).

Monoterpene analysis was performed following a solid phase extraction in a Visiprep SPE vacuum manifold 20-

port model from Supelco (Bellefonte, Pennsylvania, USA). A volume of 50 µl of 2,6-dimethyl-6-hepten-2-ol (25 mg/L in ethanol) was used as internal standard and was added to 50 mL of wine. Dichloromethane (4 mL), methanol (4 mL) and, finally, a 12% ethanol–water solution (4 mL) were used to condition the cartridges (Strata SDB-L; Phenomenex, Torrance, CA, USA). Vacuum suction was used to rinse the wine through the cartridge, and 4 mL of Milli-Q-Water were later used to clean the cartridges. Samples were then dried under vacuum for 15 min. Finally, dichloromethane (2 mL) was used to elute the terpenic compounds from the cartridge. Sodium sulphate crystals were added to remove any traces of water. Each sample was injected in duplicate in splitless mode on a Hewlett-Packard (Palo Alto, CA, USA) 5890 Series II gas chromatograph equipped with a 60 m x 0.32 mm x 0.5 mm fused DB-FFAP capillary column (J&W Scientific, Folsom, CA, USA), and FID. Separation conditions have been reported elsewhere (Coetzee *et al.*, 2013) and quantification was done by comparison with a calibration curve of pure standards.

Sensory analysis

Wines corresponding to the different treatments were analysed by means of general descriptive sensory analysis (DA). A trained panel consisted of two males and eight females, with ages ranging from 22 to 65 years old (with an average age of 40.1). Panellists were trained three times a week. Each training session lasted two hours, with a 15-minute break. The first three training sessions were used to establish the lexicon needed to describe the wines. During the first training session, the five wines were presented and the panel was asked to generate sensory attributes to describe them. A list of attributes was compiled and the corresponding reference standards were prepared and presented in the following session. The list of attributes used to describe the set of wines was reduced from 21 attributes to 13 by means of discussion amongst the panellists during the following two training sessions (Table 1). The last four training sessions were used to establish consensus amongst the panellists with

regard to the attributes and the wines' ratings on a 10 cm line scale anchored at none on the left-hand side and intense on the right-hand side. Although a 10 cm line scale was used, the results of the data analysis are expressed out of 100. The wines were evaluated by the panellists in triplicate on the same day. Panellists were forced to take breaks longer than 10 minutes between replicates. The sensory evaluation was conducted in off-white tasting booths located in a sensory laboratory. The sensory laboratory was quiet, odour free and had controlled air conditioning ($20 \pm 2^\circ\text{C}$). Twenty-five millilitres of each wine sample were presented in black ISO tasting glasses covered with petri dishes coded with pseudo random three-digit codes generated by an online research randomiser. In order to limit biases, each panellist received the samples in a different order according to a balanced Williams Latin square design, computed using the design of experiments (DOE) function of the MX package of XLSTAT (Microsoft, Redmond, WA, USA). Data was captured on paper ballots, line scales were measured with rulers and data was captured using Microsoft Excel 2010 (Microsoft, Redmond, WA, USA).

Panel performance was assessed using the workflow suggested by Tomic *et al.* (2010). Repeatability (p^* MSE plots and F -plots), discriminability and panel consensus (Tucker-1) were tested using PanelCheck 1.4 (Nofima Mat, Tromsø, Norway). DA data was analysed by means of two-way ANOVA with panellists and wine samples as main effects. Wine sample effect was tested by the regular F test to identify significant ($p < 0.05$) attributes. Only significant attributes were used for further statistical analysis. Principal component analysis (PCA) was conducted on the correlation matrix of the average intensity scores to obtain a multivariate sensory map of the DA data.

Data analysis

After quantification, descriptive statistical measurements, including mean and standard deviation, were calculated using Microsoft Excel 2007 (Microsoft Corporation, www.microsoft.com). ANOVA was performed with

TABLE 1
Sensory attributes and corresponding reference standards used during general descriptive sensory analysis training.

Attribute	Reference standard composition
Fresh pineapple ^{a,c}	three 1 cm ² pieces of fresh pineapple
Ripe pineapple ^{a,c}	three 1 cm ² pieces of canned pineapple (Koo)
Fresh tropical ^b	30 mL of tropical fruit juice (Ceres), 5 mL fresh homogenised pineapple supernatant
Canned tropical ^b	30 mL tropical fruit juice (Ceres), 5 mL canned guava syrup (Koo)
Green guava ^b	one 2 cm ² piece of guava with a green skin
Ripe guava ^b	one 2 cm ² piece of guava with a yellow/pink skin
Grapefruit ^{a,c}	one 3 cm ² grapefruit peel, one 3 cm ² grapefruit flesh
Passion fruit ^{a,c}	one 2 cm ² piece of skin and four pips of fresh passion fruit
Banana ^{a,c}	one 3 cm ² piece of fresh banana (without the peel)
Stonefruit ^b	one 3 cm ² piece of fresh yellow cling peach, 20 mL apricot juice (Liqui-Fruit)
Cooked vegetables ^a	2 mL asparagus brine (Koo), 5 mL green bean brine (Koo)

^a These quantities were added to 100 mL of neutral white wine and soaked for 18 hours.

^b Standards were presented fresh in amber glass bottles covered with foil.

^c All fresh products were purchased from local grocers, Checkers, Spar or Woolworths.

Statistica 10 (Statsoft Inc., www.statsoft.com), followed by a post-hoc Fisher's least significant difference (LSD) analysis, to determine significant differences in the chemical and sensory analyses ($p < 0.05$). Principal component analysis (PCA) was performed with the same software for sensory data analysis.

RESULTS AND DISCUSSION

Wine fermentations followed similar performance in the assayed treatments, being completed within 16 days, with residual sugar levels lower than 4 g/L. The standard oenological parameters (alcohol, volatile acidity (mg/L), pH, total acidity (mg/L) and residual sugar) showed non-significant differences between treatments (results not shown).

GSH released from DYP

The release of reduced GSH from DYP was performed in a synthetic wine as reported by Andujar-Ortiz *et al.* (2012). The amount of reduced GSH released from 0.4 g/L of GSH-rich inactivated yeast preparation into a model solution under our conditions was found to be 5.48 ± 0.42 mg/L. Based on this result, 5.5 mg/L of GSH was added to the juice in one of the studied treatments. Kritzinger *et al.* (2012) reported a GSH release of between 1.45 and 2.53 mg/L for a 0.3 g/L addition of DYP. Moreover, the results observed in this study are close to those reported by Andujar-Ortiz *et al.* (2012), in which 0.4 g/L of eight different DYPs were tested. The reduced GSH release was found to be between 2.03 and 0.46 mg/L. The differences found in the levels of GSH could be attributed to variations in the manufacturing processes. The availability of different nitrogen sources and other nutrients during the growth of the yeast culture (Andujar-Ortiz *et al.*, 2012), or possible batch differences, could explain the reported results. Furthermore, Li *et al.* (2004) and Wen *et al.* (2004) reported cysteine level as a limiting factor for GSH biosynthesis. In addition, Tirelli *et al.* (2010) and Andujar-Ortiz *et al.* (2012) reported a decrease in the levels of GSH present in DYPs, possibly due to the thermal treatment involved in the drying process.

GSH concentrations during alcoholic fermentation

The assayed treatments presented different trends in GSH levels during alcoholic fermentation. Before GSH and/or DYP additions (ba), GSH concentrations in the juices ranged from 2.4 to 3.2 mg/L (Fig. 1), but these were non-significant. After additions (aa, Fig. 1), the treatments GSH 80, which was selected based on the highest GSH concentration reported in a South African grape juice (Du Toit *et al.*, 2007), and DYP + GSH 80 showed the highest levels of GSH, whereas, as expected, the control (C) wines showed the lowest levels. After addition (aa), the GSH 5.5 and DYP treatments showed similar levels of GSH, with 8.8 and 8.3 mg/L respectively. The difference in GSH levels between the control (C) and the DYP treatment (5.7 mg/L) was consistent with the results reported by Andujar-Ortiz *et al.* (2012). During alcoholic fermentation, the GSH levels decreased until 50% completion of the fermentation was reached, and from this point onwards an increase was observed until the end of the fermentation. Several authors have observed contradictory results, with

either an increase (Park *et al.*, 2000; Fracassetti *et al.*, 2010; Kritzinger *et al.*, 2013a) or a decrease in GSH concentrations (Du Toit *et al.*, 2007; Coetzee *et al.*, 2013) during alcoholic fermentation. The GSH 5.5 and DYP treatments showed similar levels of GSH during the alcoholic fermentation, with no significant differences at the end of this process (11.8 and 13.2 mg/L respectively). Moreover, the GSH 80 and DYP+GSH 80 wines showed similar concentrations (67.3 and 69.8 mg/L respectively), which were significantly higher when compared to the other treatments.

The GSH assimilation during alcoholic fermentation might be carried out by cellular transporters, which have already been characterised by Miyake *et al.* (1998) and Bourbouloux *et al.* (2000). GSH could be assimilated and secreted by yeast during alcoholic fermentation; therefore, the amount of GSH might be affected by factors such as yeast strain, oxygen level, initial GSH concentrations and nutriment status of the juice (Kritzinger *et al.*, 2013b). Kritzinger *et al.* (2013b) found that the addition of glutathione-enriched inactive dry yeast preparations led to increased levels of GSH at the end of alcoholic fermentation. However, it is not clear whether these results are due to increases in GSH levels due to GSH contained in the DYP, GSH synthesis by the yeast from free amino acids, peptides, etc. in the DYP, or preferential uptake of nutrients supplied by the DYP over those supplied by native GSH (Kritzinger *et al.*, 2013b). One of the aims of this study was to investigate whether increased GSH levels in the wine are due to the production of GSH from constituents in DYP, or simply due to the addition of GSH contained in the DYP. After addition (aa), the DYP additions led to increases in GSH levels in the juice, but this higher trend did not occur at the latter stages of alcoholic fermentation, with no significant differences between the DYP and corresponding GSH additions. This work could not provide a clear answer to this question, which requires further investigation.

Volatile compounds

Table 2 shows the levels of volatile compounds quantified in the Sauvignon blanc wines. Esters, fatty acids and higher alcohols have a fermentative origin, while terpenes and certain volatile thiols are classed as varietal aromas (Tominaga & Dubourdieu, 2003; Swiegers *et al.*, 2005; Polášková *et al.*, 2008). Concentrations found in the wines made in this study are in accordance with previously reported research (Francis & Newton, 2005; Benkowitz *et al.*, 2012; Jouanneau *et al.*, 2012). Moreover, differences were found for the aroma composition between the different wine treatments (Table 2). The volatile compounds ethyl butyrate, ethyl hexanoate, ethyl octanoate, ethyl decanoate, isoamyl acetate and 2-phenylethyl acetate were all found above their perception thresholds (p.t.) and were influenced to a different extent in some treatments compared to the control wines. The ethyl decanoate levels were also significantly higher in the DYP+GSH 80 wines compared to the control. Ethyl acetate levels were also increased by the DYP addition. Levels of hexyl acetate were increased in all the treatments – except the GSH80 treatment – when compared to the control. The increases in certain esters found in some of the treated wines could be related to the extra supplementation of nitrogenous

TABLE 2

Concentration of esters (mg/L), fatty acids (mg/L), alcohols (mg/L), terpenes ($\mu\text{g/L}$) and thiols (ng/L) (mean \pm standard deviation) detected in the control wines (C) and in the wines supplemented with GSH-DYP and GSH. The quantification of aroma compounds was performed at the end of alcoholic fermentation. Letters indicate significant difference ($p < 0.05$). Data are reported as mean values ($n = 3$) \pm standard deviation.

Aroma compounds	Odour quality	Perception threshold	GSH and GSH-SIY treatments			
			Control	GSH 5.5	GSH 80	DYP
Ethyl acetate	Pineapple	12.3 ^a	64.4 \pm 1.1 ^b	68.0 \pm 2.6 ^{ab}	65.1 \pm 1.2 ^b	69.4 \pm 3.9 ^a
Ethyl butyrate †	Apple	0.020 ^a	1.02 \pm 0.01 ^b	1.11 \pm 0.04 ^a	1.06 \pm 0.01 ^{ab}	1.12 \pm 0.05 ^a
Isomyl acetate †	Banana	0.030 ^a	4.80 \pm 0.13 ^b	5.76 \pm 0.25 ^a	5.46 \pm 0.22 ^{ab}	5.90 \pm 0.74 ^a
Ethyl hexanoate †	Apple-peel-fruit	0.014 ^a	0.80 \pm 0.07 ^b	1.42 \pm 0.07 ^a	1.35 \pm 0.01 ^{ab}	1.41 \pm 0.10 ^a
Hexyl acetate	Fruit-herb	0.67 ^a	0.12 \pm 0.01 ^b	0.20 \pm 0.02 ^a	0.17 \pm 0.02 ^{ab}	0.21 \pm 0.05 ^a
Ethyl lactate	Fruit	155 ^b	11.25 \pm 0.16 ^{ab}	11.26 \pm 0.23 ^{ab}	11.08 \pm 0.13 ^b	11.52 \pm 0.12 ^a
Ethyl octanoate †	Fruit-fat	0.005 ^a	1.54 \pm 0.04 ^b	1.90 \pm 0.05 ^a	1.74 \pm 0.04 ^{ab}	1.87 \pm 0.20 ^a
Ethyl-3-hydroxybutanoate	-	67 ^a	0.51 \pm 0.01 ^b	0.53 \pm 0.01 ^{ab}	0.52 \pm 0.02 ^b	0.53 \pm 0.02 ^{ab}
Ethyl decanoate †	Apple-peel-fruit	0.20 ^a	0.68 \pm 0.03 ^b	0.75 \pm 0.75 ^{ab}	0.70 \pm 0.03 ^{ab}	0.75 \pm 0.05 ^{ab}
Diethyl succinate	Wine-fruit	200 ^b	0.34 \pm 0.01 ^b	0.35 \pm 0.01 ^b	0.34 \pm 0.01 ^b	0.36 \pm 0.01 ^a
Ethyl phenylacetate	Fruit-sweet	-	0.01 \pm 0.00 ^a	0.10 \pm 0.06 ^a	0.02 \pm 0.03 ^a	0.07 \pm 0.02 ^a
2-Phenylethyl acetate †	Fruit-sweet	0.25 ^a	0.68 \pm 0.02 ^b	0.74 \pm 0.01 ^a	0.73 \pm 0.01 ^{ab}	0.76 \pm 0.05 ^a
Acetic acid †	Vinegar	200 ^a	420 \pm 26 ^b	463 \pm 22 ^a	419 \pm 14 ^b	454 \pm 21 ^{ab}
Propionic acid	Soy-pungent-rancid	8.1 ^a	1.70 \pm 0.13 ^{ab}	1.89 \pm 0.15 ^a	1.61 \pm 0.02 ^b	1.72 \pm 0.08 ^{ab}
Isobutyric acid	Rancid-butter-cheese	2.3 ^a	1.27 \pm 0.09 ^c	1.51 \pm 0.06 ^{ab}	1.38 \pm 0.06 ^{bc}	1.52 \pm 0.04 ^a
Butyric acid †	Rancid-sweat-cheese	0.17 ^a	1.86 \pm 0.06 ^c	2.17 \pm 0.05 ^{ab}	2.02 \pm 0.03 ^b	2.18 \pm 0.06 ^{ab}
Isovaleric acid †	Sweat-fat-rancid	0.033 ^b	1.04 \pm 0.06 ^c	1.14 \pm 0.03 ^{ab}	1.09 \pm 0.04 ^{bc}	1.19 \pm 0.03 ^a
Valeric acid	-	-	0.46 \pm 0.04 ^a	0.51 \pm 0.02 ^a	0.34 \pm 0.06 ^b	0.50 \pm 0.02 ^a
Hexanoic acid †	Resin-flower-green	0.42 ^a	5.05 \pm 0.15 ^b	6.10 \pm 0.25 ^a	5.72 \pm 0.14 ^{ab}	5.99 \pm 0.5 ^a
Octanoic acid †	Sweat-cheese	0.50 ^a	8.44 \pm 0.21 ^b	9.78 \pm 0.36 ^a	9.31 \pm 0.37 ^{ab}	9.47 \pm 0.82 ^{ab}
Decanoic acid †	Rancid-fat	1.0 ^a	3.05 \pm 0.21 ^a	3.21 \pm 0.20 ^a	3.08 \pm 0.11 ^a	3.10 \pm 0.24 ^a
						3.11 \pm 0.32 ^a

TABLE 2 (CONTINUED)

Aroma compounds	Odour quality	Perception threshold	GSH and GSH-SIY treatments			
			Control	GSH 5.5	GSH 80	DYP
Methanol	Ethanol-alcohol	670 ^μ	73.9 ± 4.5 ^c	82.4 ± 3.6 ^b	89.4 ± 0.2 ^a	76.9 ± 3.6 ^{bc}
Propanol	Alcohol-pungent	306 ^α	36.5 ± 1.2 ^{ab}	35.5 ± 0.8 ^{ab}	35.7 ± 0.4 ^{ab}	38.0 ± 3.8 ^a
Isobutanol	Wine-solvent-bitter	40 ^Ω	16.5 ± 0.4 ^a	16.5 ± 0.6 ^a	16.7 ± 0.4 ^a	17.5 ± 1.4 ^a
Butanol	Medicine-fruit	150 ^β	1.28 ± 0.05 ^a	1.31 ± 0.05 ^a	1.29 ± 0.02 ^a	1.36 ± 0.08 ^a
Isamyl alcohol †	Sweet, fusel	30 ^Ω	142.9 ± 5.8 ^c	168.8 ± 7.2 ^a	153.9 ± 2.5 ^{bc}	168.0 ± 9.0 ^{ab}
Hexanol †	Resin-flower-green	1.1 ^α	1.17 ± 0.02 ^c	1.31 ± 0.03 ^a	1.23 ± 0.01 ^{bc}	1.28 ± 0.05 ^{ab}
3-Ethoxy-1-propanol	-	50 ^μ	1.39 ± 0.04 ^b	1.58 ± 0.04 ^a	1.54 ± 0.01 ^{ab}	1.54 ± 0.12 ^{ab}
2-Phenylethanol †	Lilac-spice-rose-honey	14 ^Ω	20.2 ± 1.3 ^c	22.5 ± 1.4 ^{abc}	20.9 ± 0.9 ^{bc}	23.7 ± 1.4 ^a
Linalool-oxide 1	Flower	3000-5000 [‡]	6.24 ± 0.26 ^a	6.52 ± 0.36 ^a	4.94 ± 0.53 ^a	5.11 ± 0.50 ^a
Linalool-oxide 2	Flower	3000-5000 [‡]	7.74 ± 0.76 ^{ab}	6.92 ± 0.39 ^{ab}	6.27 ± 0.72 ^b	7.98 ± 0.46 ^a
Linalool	Flower-lavender	25 ^β	2.73 ± 0.77 ^{ab}	2.18 ± 0.15 ^b	2.84 ± 0.60 ^a	3.27 ± 0.30 ^a
Linalyl acetate	Sweet-fruit	-	11.39 ± 0.12 ^c	11.50 ± 0.58 ^c	12.22 ± 1.14 ^{cb}	13.78 ± 0.39 ^a
Citronellol	Rose	25 ^β	2.9 ± 0.07 ^b	3.00 ± 0.24 ^b	3.36 ± 0.54 ^b	4.08 ± 0.16 ^a
Nerol	Sweet	400 [‡]	84.87 ± 0.93 ^c	82.23 ± 3.97 ^c	100.19 ± 16.16 ^b	118.80 ± 1.05 ^a
α-Ionone	Wood-violet	5 ^Ω	4.47 ± 1.92 ^a	4.24 ± 1.92 ^a	3.14 ± 1.96 ^a	1.91 ± 0.33 ^a
β-Farnesol 1	Floral	2400 [‡]	31.75 ± 1.66 ^b	28.81 ± 0.91 ^b	33.78 ± 3.53 ^b	44.17 ± 2.60 [‡]
β-Farnesol 3	Floral	2400 [‡]	15.84 ± 1.85 ^b	14.30 ± 0.54 ^b	14.77 ± 1.64 ^b	17.47 ± 1.32 ^b
3MHA †	Grapefruit-black currant-mango- passion fruit-guava	35.49 ± 2.04 ^c	35.49 ± 2.04 ^c	40.36 ± 2.83 ^{abc}	38.91 ± 4.62 ^{bc}	43.71 ± 1.93 ^{ab}
3MH †	Passion fruit- grapefruit-tropical fruits	328.00 ± 5.44 ^b	328.00 ± 5.44 ^b	340.70 ± 9.42 ^b	328.22 ± 11.82 ^b	380.53 ± 3.92 ^a
						383.21 ± 1.77 ^a

^Ω Francis and Newton (2005); [‡] Dragone *et al.* (2009); ^β Gómez-Míguez *et al.* (2007); [‡] Ribéreau-Gayon *et al.* (2006); [∞] Plotto *et al.* (2006);

^α Peinado *et al.* (2006); [‡] Mateo and Jimenez (2006); ^μ Moreno *et al.* (2005).

† Compounds occurring in the wine in concentrations higher than their perception thresholds.

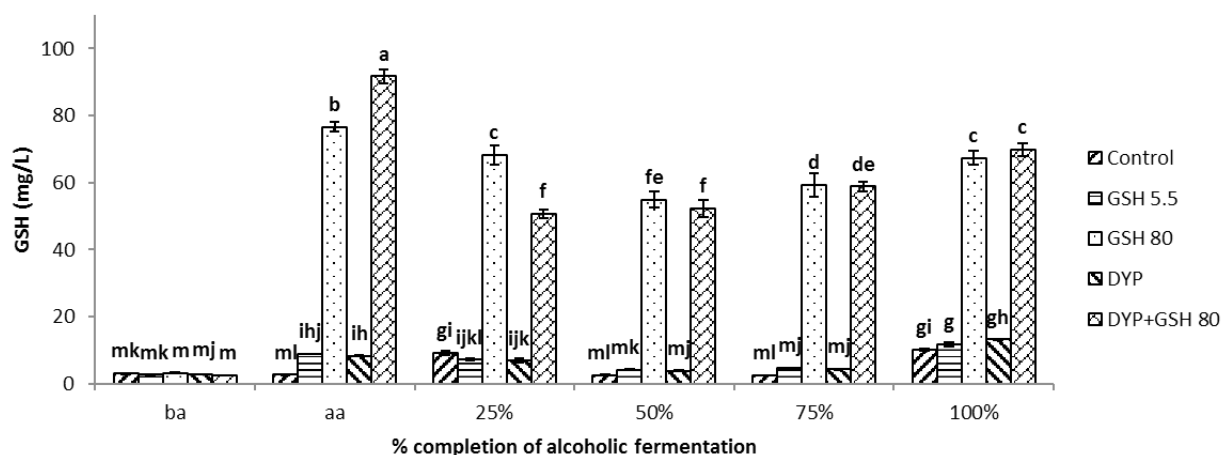


FIGURE 1

Extracellular GSH trend in Sauvignon blanc wine during alcoholic fermentation. Letters indicate significant difference ($p < 0.05$). ba: before additions of GSH and/or DYP, aa: after additions of GSH and/or DYP. Mean values ($n = 3$) and standard deviation (vertical bars) are reported.

compounds in DYP, as GSH and alpha amino acids released from GSH and/or DYP might have increased the production of these compounds during the alcoholic fermentation.

Fatty acids have been described as having fruity, fatty, cheese, sweat, butter and rancid notes (Francis & Newton, 2005). The treatments also led to changes in the fatty acid concentrations compared to the control wines, depending on the treatment and compound investigated (Table 2). In the case of butyric acid, for instance, all GSH and DYP additions led to higher levels of this fatty acid compared to the control wines, with varying results observed for hexanoic, octanoic and isobutyric acids. Regarding hexanoic acid, significantly higher concentrations were found in the GSH 5.5, DYP and DYP+GSH 80 wines when compared to the control. Moreover, isovaleric acid showed higher levels in the DYP and DYP+GSH 80 compared to the GSH 5.5 wines, in which the levels were also significantly higher than in the control wines. Finally, variable results were observed for octanoic and valeric acids.

Higher alcohols and esters are formed during alcoholic fermentation and play an important role in wine flavour (Valero *et al.*, 2002). The higher alcohols are characterised by the aromas of rose, spice, lilac, alcohol, ripe fruit resin, herbaceous and whiskey (Francis & Newton, 2005). At concentrations above 400 mg/L they are considered as negative quality factors (Rapp & Versini, 1991), while at low concentration (up to 300 mg/L) they are thought to increase the aromatic complexity of the wine (Ribéreau-Gayon *et al.*, 2006).

The amounts of total higher alcohols observed in this study ranged from 220 mg/L (C) to 251 mg/L (DYP). Hexanol, isoamyl alcohol and 2-phenylethanol were detected at concentrations higher than their perception thresholds, and at higher levels in the DYP-treated wines than those observed in the control wines. Hexanol and isoamyl alcohol levels were also significantly higher in the wines treated with GSH 5.5, DYP and DYP+GSH 80 than in the control wines. Moreover, the levels of 2-phenyl ethanol were significantly

higher in DYP and DYP+GSH 80 when compared to the control wines. Interestingly, methanol levels were slightly higher in the wines made from juice to which GSH had been added at different levels (GSH 5.5 and GSH 80). Finally, no clear trends were observed in the assayed wines for propanol levels. Andújar-Ortiz *et al.* (2014) also found increased levels of isoamyl acetate, hexyl acetate, 2-phenylethyl acetate and certain long-chain ethyl esters, as well as hexanoic acids to which DYP had been added, which corroborates our results. Limited data is available on the effect of only GSH additions on ester, fatty acid and higher alcohol production during alcoholic fermentation, which this work investigated.

The monoterpene alcohols feature prominently in the aroma of many wine varieties, such as Muscat, Gewürztraminer and Weißer Riesling (Kritzinger *et al.*, 2013a). Terpenes have been described as having rose, lavender, geranium, lychee and green notes (Francis & Newton, 2005). Due to the oxidation phenomena during wine ageing, monoterpene alcohols, such as geraniol and linalool, decrease to form dihydric alcohols and terpene dioxides, which have higher perception threshold (Simpson, 1978; Simpson & Miller, 1983). The volatile compound citronellol, the sesquiterpene β -farnesol 1, linalyl acetate and nerol were significantly increased by DYP addition (DYP and DYP+GSH 80 treatments). Moreover, the levels of nerol were also significantly higher in the GSH 80 wines compared to the control and GSH 5.5 treatments. Other researchers have also found higher levels of certain terpenes in wines exposed to DYP treatments (Andújar-Ortiz *et al.*, 2012; Rodríguez-Bencomo *et al.*, 2014). As the wines in this study were analysed only three months after bottling, the anti-oxidant activities of DYP in preserving certain terpenes, as reported by Rodríguez-Bencomo *et al.* (2014), probably also had such an effect.

Sauvignon blanc wines are characterised by the presence of highly odorous thiol compounds, such as 4-mercapto-4-methylpentan-2-one (4MMP), 4-mercapto-4-methylpentan-2-ol (4MMPOH), 3-mercaptohexan-1-ol (3MH) and

TABLE 3

Wine sensory data (out of 100) generated with descriptive analysis for the different treatments. Letters indicate significant differences ($p < 0.05$).

Sensory descriptor	C	GSH 5.5	GSH 80	DYP	DYP+80 GSH
Cooked vegetable	1.3 ± 4.0 ^a	1.4 ± 4.4 ^a	2.5 ± 5.9 ^a	3.3 ± 7.0 ^a	2.5 ± 5.3 ^a
Overall tropical	35.7 ± 14.8 ^a	43.6 ± 12.2 ^b	47.7 ± 12.7 ^b	56.7 ± 10.1 ^c	56.2 ± 8.5 ^c
Canned tropical	25.9 ± 10.4 ^a	34.8 ± 18.0 ^b	49.4 ± 13.6 ^c	62.3 ± 10.6 ^d	63.6 ± 10.9 ^d
Banana	2.2 ± 7.2 ^a	11.0 ± 20.7 ^b	23.2 ± 15.4 ^c	32.1 ± 15.9 ^d	33.6 ± 19.3 ^d
Grapefruit	26.3 ± 18.4 ^b	30.1 ± 18.9 ^b	12.1 ± 15.6 ^a	11.7 ± 9.8 ^a	15.7 ± 15.4 ^a
Green guava	11.5 ± 15.6 ^b	11.3 ± 15.0 ^b	3.1 ± 7.5 ^a	2.4 ± 10.4 ^a	2.9 ± 9.8 ^a
Ripe guava	1.0 ± 3.9 ^a	5.0 ± 13.0 ^a	12.8 ± 16.2 ^b	19.6 ± 15.6 ^{bc}	21.1 ± 16.7 ^c
Fresh pineapple	36.7 ± 15.3 ^c	29.53 ± 19.9 ^b	6.3 ± 17.3 ^a	0.0 ± 0.0 ^a	1.2 ± 5.0 ^a
Canned pineapple	0.8 ± 4.6 ^a	11.8 ± 22.2 ^b	29.5 ± 19.5 ^c	41.4 ± 15.4 ^d	39.4 ± 17.1 ^d
Passion fruit	8.8 ± 10.9 ^{bc}	11.5 ± 9.8 ^c	3.0 ± 6.9 ^a	4.2 ± 6.7 ^a	5.9 ± 9.8 ^{ab}
Stone fruit	4.1 ± 9.4 ^a	6.5 ± 12.4 ^a	14.73 ± 10.0 ^b	19.6 ± 11.5 ^b	14.3 ± 11.6 ^b
Fresh tropical	74.1 ± 10.4 ^d	65.2 ± 18.0 ^c	50.6 ± 13.6 ^b	37.7 ± 10.6 ^a	36.4 ± 10.9 ^a

3-mercaptopentyl acetate (3MHA) (Darriet *et al.*, 1995; Tominaga *et al.*, 1998). 3MHA and 3MH are characterised by the mixed aroma of boxwood flowers, grapefruit and passion fruit (Tominaga *et al.*, 2006). 3MHA (p.t. 4 ng/L) and 3MH (p.t. 60 ng/L) were always present at concentrations higher than their perception thresholds (Table 2). 3MH and 3MHA levels were significantly higher in the wines treated with the DYP. Surprisingly, the addition of GSH, even at relatively high concentrations of 80 mg/L, did not increase the levels of these two important volatile thiols (Table 2). Glutathione-rich inactivated yeast (DYPs) might stimulate the production of varietal thiols through the release of GSH or/and cysteine, although the former mechanism seems unlikely, since the additions of pure GSH did not lead to higher levels of these compounds. In addition, higher levels of glutamic acid released from DYP can lead to higher levels of 3MH (Šuklje *et al.*, 2016). Contradictory results are found in the literature regarding the influence of GSH on thiol release, with some authors reporting an increase and others finding decreases (Patel *et al.*, 2010; Roland *et al.*, 2010; Makhotkina *et al.*, 2014). Moreover, GSH has been used as an antioxidant at harvest, complementing a moderate SO₂ addition. Higher thiol levels were observed, likely through an antioxidant effect (Makhotkina *et al.*, 2014). Should GSH or the DYPs support a more reductive environment and greater H₂S levels, the production of 3MH, and subsequently 3MHA, might also be favoured, although this might be a minor route under normal winemaking conditions. However, Harsch *et al.* (2013) demonstrated that supplying grape juice with an external source of hydrogen sulphide drastically increases thiol formation in the finished wines.

GSH-enriched DYPs seemed to influence higher alcohols, fatty acids and ester levels, but this effect was less pronounced in the GSH-treated wines. These results point to a possible positive effect of GSH on these specific volatile compounds that might be related to its activity as a strong antioxidant. However, these mechanisms seem unlikely, as the addition of pure GSH at the same level as that found in the DYP, as well as a high addition (80 mg/L), often did not

lead to the same increases. In the specific case of terpenes and volatile thiols, only DYP additions showed higher levels of these volatile compounds. Multiple reasons, such as the modification of by-products during fermentation, the release of volatiles from DYPs or the interaction of wine volatiles with compounds released by DYPs, could explain the differences observed in this study; and these need further investigation.

Descriptive analysis

In order to determine which sensory attributes were influenced by the addition of GSH or GSH-DYP, descriptive analysis was performed on the Sauvignon blanc wines. The results of the descriptive analysis are reported in Table 3. The statistical analysis (ANOVA) of each sensory descriptor showed significant differences between treatments (Table 3). The DYP and DYP+GSH 80 treatments were described by the panel as having higher levels of overall tropical, canned tropical, banana, stone fruit, ripe guava and canned pineapple attributes, whereas the GSH 80 sample presented significantly higher levels of the abovementioned compounds when compared against the control wines, but at lower intensities (except for the banana and stone fruit attributes) than those found for the DYP additions. The aroma of the control wine (C) was characterised by a sensory profile associated with fresh tropical and fresh pineapple notes. The GSH 5.5 wines had similar intensities of grapefruit, passion fruit and green guava compared to the control, and these intensities were higher than those observed in the other three treatments. The DYP additions thus influenced the volatile profile through increases of notes associated with ripe tropical fruits. On the other hand, adding 5.5 mg/L GSH to the must before fermentation did not seem to be sufficient to alter the sensorial profile of the wine compared to that of the control wine. However, our sensory results also correlate with those of Andújar-Ortiz *et al.* (2014), who also found increases in banana character in the DYP-treated wines, with higher levels of peaches. The sensorial differences observed in this study, along with those of Andújar-Ortiz *et al.* (2014)

and Šuklje *et al.* (2016), are thus probably not due to the GSH released from the DYP products, but due to the yeast forming different volatiles, brought about by the amino acids and peptides released from these products..

CONCLUSION

The addition of GSH or DYP to Sauvignon blanc grape must has been shown to increase the concentrations of certain volatile compounds, which included thiols and some monoterpenes in the case of the DYP-added treatments. The addition of DYP can also influence the sensory characteristics of the wine, leading to a change in the wine's aroma profile. The amount of GSH released into the wines when DYP was added, however, seems to be limited, and the increases in GSH, volatile thiols and other compounds are probably due to a change in the chemical composition of the must. Nitrogen-based compounds and other components present in the DYP preparations are more likely responsible for the changes observed than the GSH added to the must due to DYP addition. However, this needs to be investigated further using other cultivars and DYP preparations.

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