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Title: Sensorially important aldehyde production from amino acids in model wine systems: impact of ascorbic acid, erythorbic acid, glutathione and sulphur dioxide.

Journal: Food Chemistry

ISSN: 0308-8146 Year: 2013 Pages: 304 - 312

Volume: 141 Issue: 1

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URLs:

FT: http://dx.doi.org/10.1016/j.foodchem.2013.02.100

Sensorially important aldehyde production from amino acids in model wine systems:

Impact of ascorbic acid, erythorbic acid, glutathione and sulfur dioxide

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Running title: Wine aldehyde production from amino acids
ABSTRACT

The efficiency of different white wine antioxidant systems in preventing aldehyde production from amino acids by oxidative processes is not well understood. The aim of this study was to assess the efficiency of sulfur dioxide alone and in combination with either glutathione, ascorbic acid or its stereoisomer erythorbic acid, in preventing formation of the sensorially important compounds methional and phenylacetaldehyde from methionine and phenylalanine in model white wine.

UHPLC, GC-MS/MS, LC-MS/MS, flow injection analysis and luminescence sensors determined both compositional changes during storage, and sulfur dioxide-aldehyde apparent equilibrium constants. Depending on temperature (25 °C or 45 °C) or extent of oxygen supply, sulfur dioxide was equal or more efficient in impeding the production of methional compared to the other antioxidant systems. For phenylacetaldehyde, erythorbic acid or glutathione with sulfur dioxide provided improved inhibition compared to sulfur dioxide alone, in conditions of limited oxygen consumption. The results also demonstrate the extent to which sulfur dioxide addition can lower the free aldehyde concentrations to below their aroma thresholds.

*Keywords*: amino acid oxidation, ascorbic acid, glutathione, methional, phenylacetaldehyde
1. Introduction

Wines undergo detrimental changes to their appearance and flavour after reaction with excessive amounts of molecular oxygen, and these changes are often first observed in the aroma of the wine prior to chromatic changes (Ferreira, Oliveira, Hogg & de Pinho, 2003). The main compounds responsible for the oxidation aroma have been well researched (Culleré, Cacho & Ferreira, 2007), and those contributing significantly have been identified to be aldehyde compounds. Of the aldehyde compounds identified, phenylacetaldehyde and methional are reported to be particularly important, contributing ‘honey’ and ‘boiled vegetable/rotten potato’ aroma notes, respectively, for oxidised wines (Culleré et al., 2007; Escudero, Hernández-Orte, Cacho & Ferreira, 2000). These potent aroma compounds have reported aroma thresholds between 0.25-0.75 µg/L for methional (Escudero et al., 2000) and 1.0-25 µg/L for phenylacetaldehyde (Culleré et al., 2007; Ferreira, De Pinho, Rodrigues & Hogg, 2002). Interestingly, the aroma threshold for methional depended on the experience of the sensory panel; some sensory panellists could not detect it below 50 µg/L (Escudero et al., 2000). However, generally only low concentrations of methional or phenylacetaldehyde are required to exert a significant impact on white wine aroma.

The formation of these aldehyde compounds is suggested to occur from the parent amino acid via a Strecker degradation (Fig. 1) (Escudero et al., 2000; Ferreira et al., 2002; Pripis-Nicolau, de Revel, Bertrand & Maujean, 2000). This requires the amino acid to react with either a α-dicarbonyl or a β-quinone compound to produce the aldehyde via a range of intermediate compounds (Pripis-Nicolau et al., 2000; Rizzi, 2006). The role of oxygen is proposed (Escudero et al., 2000) to induce the
production of carbonyl compounds, either through oxidation reactions directly (i.e., o-quinone production) or through consumption of sulfur dioxide and subsequent release of bound α-dicarbonyl compounds. On the other hand, Nikolantonaki and Waterhouse (2012) have queried the importance of o-quinone for Strecker degradations in wine conditions, indicated that the rate of reaction between o-quinone compounds and amino acids is ‘essentially zero’. Escudero et al. (2000) provided evidence of an alternative mechanism to the Strecker reaction for methional, after showing higher concentrations of methional in wine supplemented with methionol. A similar pathway for phenylacetaldehyde production from phenylethanol (i.e. a compound known to contribute floral aroma in wine) was proposed by Jarauta, Cacho and Ferreira (2005) with a peroxidation mechanism being suggested for the conversion of the alcohol to the aldehyde. Finally, α-unsaturated aldehydes can also react with amino acids in a ‘Strecker-like’ reaction to also afford the corresponding Strecker aldehyde (Baert, De Clippeleer, Hughes, De Cooman & Aerts, 2012).

In studies conducted on the production of phenylacetaldehyde and methional in wines, both oxygen and temperature were found to enhance production, whilst sulfur dioxide impeded formation. For example, Ferreira et al. (2002) showed that in a white wine, higher storage temperatures (i.e., 45 °C vs. 15 °C) in combination with high oxygen (i.e., consumption of 10 mg/L oxygen) provided the highest concentrations of aldehydes. In low oxygen conditions (i.e., consumption of ~2-3 mg/L oxygen), 50 mg/L sulfur dioxide prevented formation of the aldehyde compounds.
As further evidence of the protective effect of sulfur dioxide, methional was only detected in model wines containing methionine and methyl glyoxal (a $\alpha$-dicarbonyl compound) when sulfur dioxide was absent (Ferreira et al. 2002). Although the impact of sulfur dioxide on the production of methional and phenylacetaldehyde has been reported, the ability of added sulfur dioxide to bind these specific aldehyde compounds as not been reported.

A variety of complementary antioxidants are known to assist sulfur dioxide in protecting white wines from oxygen (Bradshaw, Barril, Clark, Prenzler & Scollary, 2011; Clark, Vestner, Barril, Maury, Prenzler & Scollary, 2010; Rousis, Lambropoulos & Tzimas, 2007). Though it is known that there are different mechanisms involved in oxygen consumption when antioxidants such as ascorbic acid or erythorbic acid are present, their effect on the production of methional and phenylacetaldehyde have not been reported. Ascorbic acid scavenges oxygen generating the primary products of dehydroascorbic acid and hydrogen peroxide with the ascorbate radical as an intermediate (Bradshaw et al., 2011). In the absence of ascorbic acid, the $\alpha$-quinone and hydrogen peroxide are the primary wine oxidation products, with semi-quinone radicals as intermediates (Danilewicz, Seccombe & Whelan, 2008). Sulfur dioxide can scavenge all these primary products (Danilewicz et al., 2008), although it would seem to be less efficient in scavenging dehydroascorbic acid and its degradation products, some of which are $\alpha$-dicarbonyl compounds (Barril, Clark & Scollary, 2012). Erythorbic acid acts in an equivalent manner to ascorbic acid (Clark et al., 2010).
Although the tripeptide glutathione (consisting of glutamate, cysteine and glycine) is not yet a permitted additive to wine, recent reports have shown that it has some antioxidant capabilities in wine conditions (Rousis et al. 2008). It is known to bind some wine-relevant aldehyde compounds (Sonni, Moore, Clark, Chinnici, Riponi & Scollary, 2011), $o$-quinone compounds (Cheynier & Van Hulst, 1988) and also form glutathionyl vinyl phenol adducts during oxidation in the presence of hydroxycinnamic acids (Bouzanquet, Barril, Clark, Dias & Scollary, 2013). However, in some cases, glutathione has also been shown to induce production of hydrogen sulfide concentrations (i.e., inducing a detrimental wine aroma) during wine ageing in low oxygen conditions (Ugliano, Kwiatkowski, Vidal, Capone, Siebert, Dieval et al., 2011).

The purpose of this study was firstly to confirm the ability of the aldehyde compounds (i.e. phenylacetaldehyde and methional) to be produced in a simplified oxygenated model wine system without added $\alpha$-dicarbonyl compounds (i.e., methyl glyoxal) and in the absence of other precursors to the aldehydes other than the amino acids (phenylalanine and methionine). Furthermore, this study was designed to establish the binding of the aldehydes at typical wine sulfur dioxide concentrations, and assess the impact of various antioxidant systems in either unlimited or defined oxygenated conditions, and with residual free sulfur dioxide in the latter.

2. Materials and methods
2.1. Reagents

All glassware and plastic ware were soaked for at least 16 hours in 10\%(v/v) nitric acid (BDH, AnalaR, Poole, UK) and then rinsed with copious amounts of Grade 1 water (ISO 3696). Solutions and dilutions were prepared using Grade 1 water. Chemicals were obtained from Sigma-Aldrich (St. Louis, USA) (potassium hydrogen tartrate, L(+)-tartaric acid, caffeic acid, (+)-catechin monohydrate, methionine, phenylalanine, methional, and phenylacetaldehyde), BDH (Poole, UK) (copper(II) sulfate pentahydrate (AnalaR)), and AJAX (Taren Point, Australia) (iron(II) sulfate heptahydrate (AR)).

2.2. Preparation of model wine systems and storage conditions for high oxygenated systems

A model wine system comprising 12\%(v/v) aqueous ethanol and buffered to pH 3.2 with 0.011 M potassium hydrogen tartrate and 0.008 M tartaric acid (George, Clark, Prenzler & Scollary, 2006) was used for all experiments. Caffeic acid (200 mg/L) was added to model the non-flavonoid phenolic compounds in white wine, and catechin (50 mg/L) to model the lower concentration of skin-derived phenolic compounds. To this the amino acids phenylalanine (90 mg/L) and methionine (30 mg/L) were added at concentrations reported in white wines (Ali, Pätzold & Brücker, 2010). The metal ions, copper(II) and iron(III), were added to the model system at concentrations of 0.2 mg/L and 5.0 mg/L, respectively, as these are typical concentrations of these metal ions in white wine (Danilewicz, 2007). Sulfur dioxide was added at 45 mg/L and either ascorbic acid, erythorbic acid or glutathione at 100 mg/L, 100 mg/L and 174 mg/L, respectively. This concentration of glutathione was
utilised to enable a 1:1 molar ratio with ascorbic acid or erythorbic acid, and thereby enable a direct comparison between the three antioxidants. It is important to note that this concentration of glutathione is above the levels reported in white wines (maximum concentration of 35 mg/L (du Toit, Lisjak, Stander & Prevoo, 2007; Janeš, Lisjak & Vanzo, 2010)). Samples of 300mL were prepared in triplicate, stored in 1000mL bottles, and saturated with pure oxygen gas to achieve ~30mg/L dissolved oxygen. The saturation was achieved by sparging the solution with oxygen gas for 10 min. The headspace was also thoroughly flushed with oxygen gas (10 min). The samples were then stored at either 25 ºC or 45 ºC for seven days with preparation of triplicate samples for each temperature.

2.3. Determination of hydrogen sulfite-aldehyde apparent dissociation constants and time of equilibration

Standards of phenylacetaldehyde (255 µg/L) and methional (116 µg/L) were prepared in a simplified model wine system, consisting of 12 %(v/v) aqueous ethanol buffered to pH 3.20 with tartaric acid. These concentrations were chosen as they are within the ranges encountered for the aldehyde compounds in subsequent experiments (Table 1). Triplicate samples were prepared from this bulk solution containing 0, 40 and 80 mg/L sulfur dioxide (added as sodium metabisulfite) and samples were left to equilibrate overnight (17 h at 25 ºC) and analysed by GCMS for ‘free’ methional and phenylacetaldehyde.

Apparent equilibrium constants, $K_{app}$, were determined as the product of the measured remaining ‘free’ aldehyde compound multiplied by the free sulfur dioxide concentration, and divided by the calculated ‘bound’ form of the relevant carbonyl
compound (i.e., \( K_{\text{app}} = \frac{[\text{free SO}_2][R-\text{CHO}]}{[R-\text{CH(OH)-HSO}_3]} \)) where R = -CH\(_2\)(C\(_6\)H\(_5\)) or -(CH\(_2\))\(_2\)-SCH\(_3\) for phenylacetaldehyde or methional, respectively).

The mean \( K_{\text{app}} \) values were calculated from each aldehyde to free sulfur dioxide ratio. The \( K_{\text{app}} \) differs from the equilibrium constant (K) as it does not take into account hydration equilibria of the aldehyde compound. Furthermore, the hydrogen sulfite concentration utilised in the true equilibrium constant, is approximated by the ‘free sulfur dioxide’ concentration in the \( K_{\text{app}} \).

To access the reversibility of the hydrogen sulfite addition product at 25 °C, the above experiment was repeated at sulfur dioxide concentrations of 0 and 40 mg/L, and 16 h after the sulfur dioxide addition, acetaldehyde, concentrated acid or hydrogen peroxide were each added to samples containing sulfur dioxide.

Acetaldehyde and hydrogen peroxide were added at a 1:1 molar ratio to sulfur dioxide concentration whilst for acidification, hydrochloric acid was added to adjust the pH to 1.0. Samples were analysed for phenylacetaldehyde and methional at 1 h and 25 h intervals after acetaldehyde, hydrochloric acid or hydrogen peroxide additions.

2.4. Preparation of model wine systems and storage conditions for consumption of 15 mg/L oxygen

The same model wine system utilised in Section 2.2 was supplemented with sulfur dioxide, ascorbic acid, erythorobic acid and glutathione to concentrations of 120-150 mg/L, 200mg/L, 200mg/L and 390mg/L, respectively. These higher concentrations ensured that some portion of each of these components would remain by the end of
the experiment, and the mole ratio of the latter three were around 1:1. An additional erythorbic acid sample (i.e., 200 mg/L) was prepared with a lower sulfur dioxide concentration of 12 mg/L. Samples were stored in gas tight containers such that the dissolved oxygen concentration was around 20 mg/L after sparging with pure oxygen gas, and the decay in oxygen concentration was monitored whilst storing the sample at 45 ºC until its concentration reached ~5 mg/L (i.e. consumption of ~15 mg/L oxygen, Table 3). This amount of oxygen consumption was chosen to maximise the likelihood of aldehyde production whilst in the presence of sulfur dioxide or the other antioxidants. The suitability of the gas tight containers was determined by measuring the oxygen ingress into the argon-degassed containers over ~two days (or exactly 2640 min) at 45ºC, and the ingress was determined to be 0.24 ± 0.05 mg/L (n=3, standard error) or 0.13 mg/L/day.

2.5. Analytical instrumental analysis

Phenolic compounds, ascorbic acid and erythorbic acid were quantified via UHPLC (Sonni, Clark, Prenzler, Riponi & Scollary, 2011), sulfur dioxide by the FOSS FIAstar™ 5000 wine analyser (Barril et al., 2012), and oxygen by luminescence with the a PreSens Fibox 3 trace v3 oxygen meter in combination with Pst3 oxygen sensors. The sensors were utilised with the calibration data as supplied by the manufacturer. Glutathione was quantified by LC-MS/MS using a modified version of duToit et al. (2007). This utilized an Agilent 1200 series triple-quadrupole (6410) with a Waters Acquity BEH C18 (1.7µm, 2.1 x 50 mm) column; injection volume 4 µL; column temperature 45°C; flow rate 0.40 mL/min and elution solvents that consisted of 0.1 % formic acid in water (solvent A), and acetonitrile (solvent B). The
elution gradient was as follows (expressed as solvent A followed by cumulative
time): 100%, 0 min; 100%, 0.5 min; 20%, 6.5 min; 20%, 7.5 min; 100%, 8.5 min;
100%, 10.5 min. The MS component was operated at 350°C, gas flow of 9 L/min,
nebulizer at 40 psi, and capillary at 4 kV. MS/MS analyses were carried out in the
positive ion mode with the fragmentor at 100 V and a collision energy of 10 V. The
parent ion was m/z 308 and the detected product ion m/z 179.

The aldehydes were measured by the derivatisation-SPE-GCMS method adapted
from Ferreira, Culleré, Loscos and Cacho (2006). A LiChrolutEN solid phase-
extraction cartridge (200 mg) was conditioned with 4 mL dichloromethane, 4 mL
methanol and 4 mL of 12% (v/v) ethanol aqueous solution. A 50 mL aliquot of
sample was spiked with 50 µL of an internal standard consisting of 121 mg/L 3-tert-
butyl-4-hydroxyanisole (BHA). After which the sample was loaded onto the
extraction cartridge. Acetaldehyde, an interfering agent for the technique, was
removed by cleanup of the cartridge with 10 mL of an aqueous solution containing
1% NaHCO₃. A 2 mL solution of 5g/L o-(2,3,4,5,6-pentafluorobenzyl)-
hydroxylamine hydrochloride (PFBHA) aqueous solution was applied to derivatize
the retained carbonyls in the cartridge and allowed to imbibe on the cartridge for 15
min at room temperature. Excess reagent was removed by a 10 mL aliquot of 0.05 M
sulfuric acid, and derivatized analytes eluted with 2 mL dichloromethane.

Analyses were carried out on a Varian CP-3800 gas chromatography system
equipped with a CombiPAL autosampler and a split/splitless injection port type
1079, interfaced to a Varian Saturn 2000 ion trap mass spectrometer detector. A
polar capillary column (Varian, CP-Wax 52 CB, 60 m x 0.25 mm i.d. 0.25 mm film
thickness) was used for qualitative and quantitative analyses. The injection volume was 2 µL, which was injected into the column at a temperature of 40°C. The injector temperature was held at 40°C for 0.25 min, then heated at 200 ºC/min to 250ºC. The injector split ratio was set to 100:1 for 0.25min. The split valve closed for 2.75 min and opened to 30:1. The oven temperature program was 5 min at 40°C and then increased to 210°C at a rate of 2 ºC/min, and held for 20 min. The flow rate of the helium carrier gas was constant at 1.5 mL/min. The ion trap, manifold and transfer line temperatures were 180ºC, 40°C and 230ºC respectively. Compounds were detected by electron ionisation in selective ion storage (SIS) mode with the segments as indicated: 0-71 min delay; 71-78 min (methional-PFBHA) over m/z 178-302; 78-86 min (phenylacetaldehyde-PFBHA) over m/z 178-319; and 86-95 min (BHA) over m/z 134-183. Quantification was performed with m/z 252.1 and m/z 297.2 for methional and phenylacetaldehyde, respectively, with the BHA signal at m/z 165.1 as the internal standard. A five point calibration curve was prepared in triplicate for methional (0-134 µg/L) and phenylacetaldehyde (0-450 µg/L) were derivatized (as described above), in triplicate, and analysed by GCMS/MS. The limits of detection (three times the standard deviation of the blank) for methional and phenylacetaldehyde were 1.8 µg/L and 0.5 µg/L, respectively, and the limits of quantification (ten times the standard deviation of the blank) were 4.7 and 1.6 µg/L, respectively.

2.6 Statistics

All statistical analyses were conducted using Matlab version 7.4 R2007a (The MathWorks Inc., Natick). A two-way analysis of variance (ANOVA) of the concentrations of methional and phenylacetaldehyde to test the effects of reaction
temperature (25°C and 45°C) and sample (Control, Ascorbic acid, Erythorbic acid and Glutathione) with interaction were conducted. Multiple comparison differences between means were tested using a Bonferroni post hoc analysis. To test for differences between the consumption of oxygen, free and total sulfur dioxide, complementary antioxidant and remaining caffeic acid and catechin concentrations following storage, a one-way ANOVA was conducted. Again multiple comparison of mean differences were evaluated using a Bonferroni post hoc analysis.

3.1 Results and discussion

3.1 Production of phenylacetaldehyde and methional with unlimited oxygen: impact of temperature and antioxidant systems

The initial experiment examined the ability of methional and phenylacetaldehyde to be generated in the model wine under conditions of high oxygen (i.e., saturation of solutions with pure oxygen and headspace over the samples with pure oxygen gas), and at temperatures of 25 °C and 45 °C, over a seven day period. The only precursors to the aldehydes were methionine and phenylalanine and the only source of dicarbonyl compounds for Strecker degradations (Fig. 1) would be those generated in situ. The antioxidants, ascorbic acid (100 mg/L), erythorbic acid (100 mg/L) or glutathione (174 mg/L), were added to the separate model wine systems in order to assess their impact on the production of the aldehyde compounds. Within the seven days of storage, the free sulfur dioxide concentration was depleted in all samples, as was the concentrations of ascorbic acid, erythorbic acid and glutathione (data not shown).
At 25 °C, phenylacetaldehyde and methional were highest in the ascorbic acid and erythorbic acid samples (Table 1) and lowest in the control and glutathione samples, albeit with a non-significant difference for methional in the glutathione sample compared to the ascorbic acid and erythorbic acid samples. In the equivalent samples stored at 45 °C (Table 1), the concentrations of methional and phenylacetaldehyde were higher for all samples than those at 25 °C. At 45 °C, there was no significant difference (P=0.05) for phenylacetaldehyde concentration in the control and ascorbic acid samples. For methional concentration, the control, ascorbic acid and erythorbic acid samples were not significantly different to each other. Glutathione, on the other hand, had the highest concentrations of both methional and phenylacetaldehyde at 45 °C.

These results showed that in this simplified oxygenated model wine system both phenylacetaldehyde and methional could be generated. For all samples, apart from the control at 25 °C, methional reached concentrations above the published aroma threshold ranges 0.25-0.50 µg/L (Escudero et al., 2000), whilst for phenylacetaldehyde, all samples were at least within the reported aroma threshold range 1.0-25 µg/L (Culleré et al., 2007; Ferreira et al., 2002). It is likely that at 25 °C, ascorbic acid and erythorbic acid accelerated production of the aldehydes, compared to the control, due to their ability to accelerate consumption of oxygen (Bradshaw et al., 2011), thereby leading to rapid depletion of sulfur dioxide and enhanced carbonyl compound production. The degradation products of both ascorbic acid and erythorbic acid are known to include a variety of α-dicarbonyl compounds (Barril, Clark, Karuso & Prenzler, 2009; Bradshaw et al., 2011). In typical bottled wine conditions,
where access to oxygen is limited (and will be discussed below), the absolute
depletion of sulfur dioxide as induced by ascorbic acid/erythorbic acid would be less
likely, but in any case it is interesting to note that in high oxygen conditions they can
induce the formation of these aldehyde compounds compared to the control.

Previously it has been shown that glutathione can act as an antioxidant, but its
degradation products can induce oxidative colouration in model wine systems stored
at elevated temperatures after depletion of glutathione (Sonni, Clark, et al., 2011). A
similar result is observed here whereby higher concentrations of the aldehydes were
produced in the glutathione sample compared to the control, when at the higher
storage temperatures and high oxygen concentrations. In this case, it is likely that
depletion of glutathione in high oxygenated/temperature systems (i.e., with low
sulfur dioxide) is capable of promoting the formation of some product that can
induce the conversion of the amino acids to their corresponding aldehydes. As
discussed below (Section 3.3), accelerated oxygen decay in the presence of
 glutathione would not be responsible for the high aldehyde production at 45°C as
glutathione does not increase oxygen consumption compared to the control sample.

Of interest are the minimal changes in amino acid concentration during the
experiment (Fig. 2) that did not reflect the relative amounts of methional and
phenylacetaldehyde concentrations generated in the samples (Table 1). Fig. 2 shows
negligible change in the concentration of the amino acids for samples stored at 25
°C, apart from the control sample, despite their different aldehyde concentrations
(Table 1). At 45°C there is an increased number of samples with a significant
decrease in amino acid concentration, but again the larger decrease in amino acid did
not equate to the highest amounts of aldehyde compounds. Given the maximum
amount of molar aldehyde generated to the initial molar amino acid concentration was less than 0.4% for phenylacetaldehyde and less than 0.7% for methional, it is not surprising that the change in amino acid concentration did not match the amounts of aldehydes generated. Apart from the Strecker degradation, the participation of the amino acids in side-reactions is also likely (Stadtman & Levine, 2003). The small change in amino acid concentrations in Fig. 2 is consistent with the rate of reaction between o-quinones and amino acids (conducted at 10 °C), being described as ‘essentially zero’ by Nikolantonaki and Waterhouse (2012). However, it is not certain if the spectrometric measurement of the o-quinone by Nikolantonaki and Waterhouse (2012) would detect a loss in the quinone that would still generate low µg/L quantities of the Strecker aldehyde, especially at 10 °C.

3.2. Binding of sulfur dioxide to phenylacetaldehyde and methional

To allow determination of the total concentration of aldehyde compounds in samples that may have free sulfur dioxide present (i.e., important for subsequent experiments), the apparent dissociation constants ($K_{app}$) for the sulfur dioxide-aldehyde addition products were determined in model wine conditions. Following 17 hours of equilibration with sulfur dioxide, the concentrations of methional and phenylacetaldehyde decreased with sulfur dioxide concentration (Table 2). Based on these data the $K_{app}$ was calculated for these aldehyde compounds to be $5.5 \times 10^{-4}$ M and $8.0 \times 10^{-4}$ M for phenylacetaldehyde and methional, respectively.

The determined $K_{app}$ for methional and phenyacetaldehyde binding to hydrogen sulfite places them on par with keto acids and oxidised sugars for their potential to be
bound by sulfur dioxide in wine conditions (Burroughs & Sparks, 1973). It is also similar to the $K_{\text{app}}$ determined for other volatile wine aldehydes, such as $E$-pent-2-en-1-al and $E$-oct-2-en-1-al, and the wine ketones heptan-2-one and decan-2-one (De Azevedo, Reis, Motta, Da Rocha, Silva & De Andrade, 2007). Based on the $K_{\text{app}}$ values, it is apparent that once these volatile aldehyde compounds are generated it is particularly difficult to lower their concentration below the reported aroma thresholds under the solution conditions used here. For instance, the maximum total concentration of phenylacetaldehyde in white wine that would co-exist with 30 mg/L of free sulfur dioxide and still be below the aroma threshold (i.e. 1.0-25 µg/L) would be 1.9-46 µg/L. For methional, whose aroma threshold is 0.25-0.75 µg/L, the maximum concentration would be 0.4 – 1.2 µg/L. The ability to achieve high free sulfur dioxide concentrations in red wine is more difficult than white wine due to the anthocyanins of red wine binding sulfur dioxide. Consequently, it will be more difficult to lower the contribution of methional and phenylacetaldehyde aroma in red wines, than it would for white wines, by sulfur dioxide addition. This is also consistent with the higher concentrations of methional and phenylacetaldehyde that are generally reported in red wines compared to white wines (Culleré, Cacho & Ferreira, 2004; Culleré et al., 2007).

The reversibility of the binding of hydrogen sulfite to the aldehyde compounds was investigated. The addition of either acetaldehyde (to bind), concentrated acid (to convert hydrogen sulfite to molecular sulfur dioxide), or hydrogen peroxide (to oxidise) are well known to release sulfur dioxide from carbonyl compounds or even anthocyanins in the case of red wine (Iland, Bruer, Edwards, Weeks & Wilkes, 2004). Usually these agents are often combined with elevated temperature and/or
aspiration conditions (Iland et al. 2004), but this was not possible due to the volatile nature of methional and phenylacetaldehyde that were to be measured. Fig. 3 shows that on addition of these agents to the model wine solution with the aldehyde compounds and 40 mg/L sulfur dioxide, little change occurred to the aldehyde concentrations after one hour. However, 25 hours after the addition of acetaldehyde and hydrogen peroxide led to significant increases in quantified phenylacetaldehyde (P=0.05). A general increase in methional was also observed for these treatments but the magnitude of increase was not significant (P=0.05).

During the aging of wine, decreases in sulfur dioxide concentration may result in a similar release of methional and phenylacetaldehyde from their hydrogen sulfite addition products and once their aroma threshold is reached they will become sensorially perceptible. The results from Fig. 3 infer that the rate of establishing a new equilibrium distribution between hydrogen sulfite and the aldehydes, after loss of the majority of the hydrogen sulfite, occurred in the order of days rather than hours when conducted at 25 ºC. Given that wines are often stored for months to years, this time required to re-establish equilibrium after loss of sulfur dioxide is relatively negligible.

3.3. The production of phenylacetaldehyde and methional with consumption of 15 mg/L oxygen: impact of different antioxidant systems

Further experiments were conducted on the production of methional and phenylacetaldehyde in the presence of the different antioxidant systems, under defined oxygen consumption conditions (i.e., ~15 mg/L), and with residual sulfur
dioxide being present at the end of the experiment for all but one of the samples. For the latter, the erythorbic acid sample was selected to have both low (12 mg/L) and high (140 mg/L) sulfur dioxide concentrations. It was also the aim of this experiment to note how the different antioxidants impacted on the rate of oxygen decay, particularly glutathione, and consequently related aldehyde production. The storage temperature of 45 °C was utilised to induce rapid oxygen consumption and thereby test the antioxidant systems in severe circumstances.

Fig. 4 shows the oxygen consumption in the samples. The erythorbic acid sample with low sulfur dioxide had the fastest oxygen consumption rate (17.1 mg/L/h), followed by the erythorbic acid sample, with elevated sulfur dioxide (12.7 mg/L/h), and then the ascorbic acid sample (10.1 mg/L/h). For the latter two samples, there was no significant difference (P=0.05) in the concentrations of oxygen throughout the experiment. A significantly slower rate of oxygen consumption (P=0.05) was evident for the control sample (1.0 mg/L/h) followed by the glutathione sample (0.7 mg/L/h). For the samples with residual sulfur dioxide, the rates of oxygen consumption did not correlate with the amounts of sulfur dioxide consumed (Table 3), but instead depended on the type of antioxidant system. For instance, the glutathione sample had the least amount of free sulfur dioxide consumed whilst the control sample had the most (Table 3), despite both having the slowest rates of oxygen consumption. In terms of antioxidant consumption, ascorbic acid was consumed the most followed by erythorbic acid and then glutathione (Table 3). There was no significant difference in the amount of erythorbic acid consumed for the samples with high and low initial sulfur dioxide concentrations. Also, no differences
in the amount of consumed (+)-catechin or caffeic acid was evident between samples (Table 3).

The slower oxygen decay in the control sample compared to the ascorbic acid and erythorbic acid samples is as expected (Bradshaw et al., 2011), as is the similar rates of oxygen consumption for the ascorbic acid and erythorbic acid samples. The increased consumption of ascorbic acid compared to erythorbic acid (Table 3) is in contrast to results in other model wine systems (Clark et al., 2010). However, the past study (Clark et al., 2010) utilised much lower metal ion concentrations (0.1 mg/L iron(II) and 0 mg/L copper(II) versus 5.0 mg/L iron(II) and 0.2 mg/L copper(II)) which may have impacted the relatively efficiency of the antioxidants. Of interest, was the slowest oxygen decay in the sample with glutathione. This ability of glutathione to slow oxygen decay has been observed in other media and was attributed to glutathione binding copper(I) and thereby slowing the catalytic action of copper(I) to reduce oxygen (Jiménez & Speisky, 2000; Speisky, Gómez, Carrasco-Pozo, Pastene, Lopez-Alarcón & Olea-Azar, 2008). The results of Fig. 4 suggest that such a copper(I)-glutathione interaction may also occur in wine. For the erythorbic acid samples, the ability of sulfur dioxide to slow the oxygen consumption (Fig. 3a), whilst not impacting on the amount of erythorbic acid consumed (Table 3), suggests that sulfur dioxide scavenging hydrogen peroxide may prevent other side reactions capable of consuming oxygen. This is in contrast to wine systems without complementary antioxidants (i.e., ascorbic acid, erythorbic acid) whereby the presence of sulfur dioxide accelerates molecular oxygen consumption (Danielwicz, 2007).
Coupled with the slow rate of oxygen consumption was the ability of glutathione to limit the loss of free and total sulfur dioxide compared to the control. This effect was possibly due to glutathione aiding sulfur dioxide in the binding of oxidised phenolic compounds (i.e., o-quinones) and/or carbonyl compounds (Cheynier et al., 1988; Sonni, Moore, et al., 2011). The increase in free and total sulfur dioxide consumption in the control compared to the ascorbic acid was consistent with reported results (Barril et al., 2012).

Fig. 5 shows the measured free aldehyde concentration and the calculated bound concentration, the latter based on $K_{\text{app}}$ and the measured free sulfur dioxide concentration in the wines. The concentrations of the free aldehyde compounds in this experiment at 45 °C were far below those obtained for the experiments conducted with unlimited oxygen supply (and over a longer time frame) as shown in Table 1. The highest free concentration of both aldehydes was reached in the erythorbic acid sample that had low sulfur dioxide. However, methional and phenylacetaldehyde were detected (LOD of 1.8 and 0.5 µg/L, respectively) in all the samples regardless of the presence of residual sulfur dioxide. The concentration of free methional in the control was below the limit of quantification (i.e., < 4.7 µg/L), as was the concentration of free phenylacetaldehyde (< 1.8 µg/L) in the glutathione and erythorbic acid (with sulfur dioxide) samples. For the samples with residual sulfur dioxide there was a trend towards higher concentrations of free methional for the ascorbic acid, erythorbic acid and glutathione samples compared to the control sample, although the difference was only significant (P=0.05) for the control versus the ascorbic acid sample. For phenylacetaldehyde, the free concentrations in the control and ascorbic acid samples were higher than those in the erythorbic acid and
glutathione samples. The results in Fig. 5 demonstrate that the Strecker degradation of amino acids can occur for all antioxidant systems in the presence of sulfur dioxide, albeit at a significantly reduced yield, and that a component of the generated aldehyde can exist in a free form. It must be noted that the storage temperature and oxygen concentrations utilised for the results of Fig. 5 are higher than found in typical conditions for wine in bottle, but the conditions adopted do indeed demonstrate the capability of the different antioxidant systems in a worse case scenario.

The ratios for the total mmol/L aldehyde generated (i.e., combination of measured free and calculated bound aldehyde (Fig. 5)) to the mol/L oxygen consumed are shown in Fig. 5 to allow direct comparison of the results despite the minor differences in consumed oxygen between the different samples. For the control, no significant difference (P=0.05) is apparent in the efficiency of either methional or phenylacetaldehyde production from a given amount of oxygen consumed. However, in the remaining samples of Fig. 5, there is a general trend to increased efficiency in methional production compared to phenylacetaldehyde, albeit only significantly (P=0.05) for the glutathione sample, and the erythorbic acid sample without sulfur dioxide. This suggests that the range of carbonyl compounds that is generated in these latter samples may favour the Strecker degradation of methional in preference to phenylacetaldehyde. Indeed, \( \text{\textit{o}} \)-quinones have been shown to favour phenylacetaldehyde production over methional production (Rizzi, 2006), and both ascorbic acid and erythorbic acid would limit the production of \( \text{\textit{o}} \)-quinones due to their ability to scavenge oxygen before its reaction with phenolic compounds (Bradshaw et al., 2011; Sonni, Clark, et al., 2011). Furthermore, ascorbic or
erythorbic acids would introduce a range of novel α-dicarbonyl degradation products that would otherwise not be present in the wine media. For example, L-Xylosone and D-xylosone are suggested α-dicarbonyl products of ascorbic acid and erythorbic acid, respectively (Barril et al., 2009; Bradshaw et al., 2011; Clark et al., 2010), and the former is known to bind sulfur dioxide less efficiently than acetaldehyde and o-quinones (Barril et al., 2012; Bradshaw et al., 2011). Hence xylosone would readily available for Strecker reactions with amino acids despite the presence of sulfur dioxide. The relative rates of oxygen consumption (Fig. 4) and/or amounts of sulfur dioxide consumed (Table 3) did not appear to be linked to the relative amounts of aldehyde generated.

In comparing the results between ascorbic acid and erythorbic acid (Fig. 5b), it is evident that the phenylacetaldehyde concentrations are significantly higher for the samples containing ascorbic acid, whilst little difference is evident for methional (Fig. 5a). The comparative result for phenylacetaldehyde is consistent with the increased oxidative colouration observed for ascorbic acid compared to erythorbic acid in model wine systems after the depletion of the antioxidant (Clark et al., 2010). In particular, the concentration of the pigment precursor formed from the reaction between (+)-catechin with xylosone was higher when the xylosone was derived from ascorbic acid rather than erythorbic acid, implying that L-xylosone is more reactive than D-xylosone. A similar impact of xylosone stereochemistry may be impacting the Strecker degradation of phenylalanine.

The inhibitory role of glutathione in phenylacetaldehyde production compared to the control may be due to glutathione limiting the availability of o-quinone compounds
for Strecker degradation reaction with the amino acid (Cheynier et al., 1988; Sonni, Moore, et al., 2011) or due to alternate Strecker reactions between the amino groups of glutathione and $\alpha$-dicarbonyl compounds. The latter reaction has been described under high-temperatures (Lee, Jo & Kim, 2010) and can generate an alternate suite of volatile compounds, and further work is required to investigate the likelihood of such a mechanism in wine conditions.

4. Conclusion

This study showed the ability of sensory aroma compounds, relevant to ‘honey’ and ‘potato’ notes, to be generated from parent amino acids in wine-like conditions. Under high oxygen, and low sulfur dioxide conditions, higher temperatures favoured the formation of the aldehydes, as did erythorbic acid and ascorbic acid. Samples with added glutathione had the highest concentrations of the aldehydes when stored at high temperature and high oxygen conditions.

After consumption of 15 mg/L oxygen, with residual free sulfur dioxide, ascorbic acid and erythorbic acid offered no added protection against the production of methional compared to sulfur dioxide alone. However, erythorbic acid did inhibit phenylacetaldehyde formation compared to the control. Glutathione, provided similar inhibition to phenylacetaldehyde production as erythorbic acid, but it did so at a reduced consumption of free sulfur dioxide compared to the control. On the otherhand, under unlimited oxygen supply and higher storage temperatures, glutathione induced the highest concentrations of aldehyde formation compared to the other antioxidant systems.
The dissociation constant for the binding of sulfur dioxide to the aldehydes was determined and they were found to have similar binding strength as keto acids and certain oxidised sugars. The release of the bound forms of the aldehydes was also confirmed and found to occur in the order of days at room temperature. The results show that once formed, the aroma impact of these particular aldehyde compounds is difficult to remove with free sulfur dioxide concentrations typically used in wines.

Acknowledgments

This project was supported by Australia’s grapegrowers and winemakers through their investment body the Australian Grape and Wine Research and Development Corporation. The National Wine and Grape Industry Centre is a partnership between Charles Sturt University, DPI NSW and the NSW Wine Industry Association.
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Figure Captions

**Fig. 1.** The production of phenylacetaldehyde and methional from phenylalanine and methionine by Strecker degradation.

**Fig. 2.** Concentrations of amino acids after 7-days in high oxygen conditions and at temperatures 25 and 45°C. Sample numbers indicate the temperature. Box dimensions represent the 25th to 75th percentile; with whiskers extending to 1.5 times the interquartile range. Horizontal line within the box is the median value and means are shown as a target. Commencing amino acid concentration (mean and 95% confidence interval) is illustrated with horizontal lines across the boxes.

**Fig. 3.** The concentration of methional and phenylacetaldehyde in the absence (C) and the presence of 40 mg/L sulfur dioxide (S) and the impact of various treatments on the reversibility of the interactions. The treatments were acetaldehyde (Ald), acidic conditions (H+) and hydrogen peroxide (Hp) and measurement after one hour or 25 hours. The control is designated by ‘C’. The error bars indicate the standard error (n=3).

**Fig. 4.** Oxygen decay in samples over 100 min (A), and 1000 min (B). The absolute dissolved oxygen concentrations for each sample can be seen in Table 3. The error bars indicate the standard error (n=3) and where not evident are smaller than the size of the data symbol.
Fig. 5. Concentration of methional and phenylacetaldehyde in samples after depletion of ~15 mg/L oxygen during storage at 45°C. The error bars indicate the standard error (n=3) of the measured free aldehyde concentration. The horizontal dashed line indicates the limit of quantification.