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# Measuring protection of aromatic wine thiols from oxidation by competitive reactions vs wine preservatives with *ortho*-quinones

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ABSTRACT

Quinones are central intermediates in wine oxidation that can degrade the quality of wine by reactions with varietal thiols, such as 3-sulfanylhexanol, decreasing desirable aroma. Protection by wine preservatives (sulphur dioxide, glutathione, ascorbic acid and model tannin, phloroglucinol) was assessed by competitive sacrificial reactions with 4-methyl-1,2-benzoquinone, quantifying products and ratios by HPLC–UV–MS. Regioselectivity was assessed by product isolation and identification by NMR spectroscopy. Nucleophilic addition reactions compete with two electron reduction of quinones by sulphur dioxide or ascorbic acid, and both routes serve as effective quenching pathways, but minor secondary products from coupled redox reactions between the products and reactants are also observed. The wine preservatives were all highly reactive and thus all very protective against 3-sulfanylhexanol loss to the quinone, but showed only additive antioxidant effects. Confirmation of these reaction rates and pathways in wine is needed to assess the actual protective action of each tested preservative.



## 1. Introduction

Maintaining white wine quality through the various stages of vinification, barrel or bottle aging and shelf storage remains an extensive challenge. While several attributes are used to establish overall wine quality, two aspects in particular have received considerable attention: colour and flavour stability. Colour and flavour stability in white wines is associated to the control of oxidative mechanisms (Escudero, Asensio, Cacho, & Ferreira, 2002; Nikolantonaki & Darriet, 2011). In wine, polyphenols and especially flavonoids such as the flavan-3-ols and their condensed products, the proanthocyanidins, represent a class of readily oxidizable compounds involved in browning and important varietal aroma components (i.e. 3-sulfanylhexan-1-ol (**6**)) instability during aging (Blanchard, Darriet, & Dubourdieu, 2004; Fernandez-Zurbano et al., 1995; Nikolantonaki et al., 2012; Rossi & Singleton, 1966).

The oxidation of *ortho*-diphenolic compounds (**1**) allows a cascade of reactions that result the formation of quinones (**2**), via a semi-quinone radical, while oxygen is initially reduced to hydrogen peroxide, mediated by redox cycling of iron(III)/iron(II) (Waterhouse & Laurie, 2006). The produced quinones have potential electrophilic properties, which makes them react with surrounding nucleophilic molecules. When the addition of the nucleophilic molecules occurs, the catechol structure is converted back to their reduced phenolic forms, albeit with a substituted nucleophilic group attached. The semi-quinones radicals are also capable of reacting with other radical species, including hydrogen atoms, to regenerate the *ortho*-diphenol structure. In wine, compounds such as, sulphur dioxide (**3**), ascorbic acid (**4**), glutathione (**5**) which are preservatives by virtue of their ability to act as quinones reductants and/or scavengers, considered key factors govern wine resistance to oxidative aging and varietal thiol stability (Brajkovich et al., 2005; Lavigne Cruège, Pons, Choné, & Dubourdieu, 2003; Ugliano et al., 2011).

Compound **3** is utilised in enology to limit the detrimental impact of any oxygen ingress into the wine. Its main virtue is to efficiently scavenge hydrogen peroxide, *ortho*-quinones and carbonyl compounds (Adachi et al., 1979; Danilewicz & Wallbridge, 2010). However, in wine science the efficiency of **3** as a preservative is frequently called into question. There is a general enological interest in lowering the levels of **3** because of real or imagined allergic reactions among some consumers, but it has been challenging to find suitable replacements or compounds having a syn-

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ergic preservative effect (Walker, 1985). Inhibition of polyphenol oxidation through the use of **3** and **4** in combination has been observed in wines to varying degrees (Oliveira, Silva Ferreira, Guedes de Pinho, & Hogg, 2002). However, the utilisation of **4** has some risks to wine quality. According Barril, Clark, Prenzler, Karuso, and Scollary (2009) **4** is a highly unpredictable molecule, while its degradation products in the presence of catechin can eventually react farther to give coloured xanthylium cation pigments. Given the known inability of **3** to scavenge **4** oxidative degradation products, it is likely that **3** should not be to reduce degradation of **4** (Barril, Clark, & Scollary, 2012). Moreover, Pons, Lavigne, Landais, Darriet, and Dubourdieu (2010) underlined the potential of **4** to compromise wine flavour by linking it to the origin of sotolon (3-hydroxy-4,5-dimethyl-2(5H)-furanone) in dry white wines. In addition to **3** and **4**, the tripeptide glutathione (**5**) a native grape antioxidant, is now occasionally employed in winemaking. Past studies have shown that **5**, in combination with reduced levels of **3,** inhibit the loss of desirable aroma compounds such as mono-terpenes and esters, and second, delay the formation of oxidative browning, particularly yellow xanthylium cation pigments (Bouzanquet, Barril, Clark, Dias, & Scollary, 2012; Roussis, Lambropoulos, & Tzimas, 2007; Sonni, Clark, Prenzler, Riponi, & Scollary, 2011). Moreover, Lavigne and Dubourdieu (2002) first observed that **5** directly protected against loss of volatile thiols, such as **6**, during wine barrel aging. The same protective effect was recently confirmed by Ugliano et al. (2011), who showed greatly reduced loss of **6** at 6 months of bottle storage, when 20 mg/L of **5** was added to Sauvignon blanc wines at bottling. However, our understanding of glutathione usage in wine protection is in its infancy, and the complementary antioxidant actions of **3**, **4**, and **5** provide very promising options for wine oxidation management (Kritzinger, Bauer, & du Toit, 2012).

On the basis of our previous work in model wine system at room temperature, the odoriferous volatile thiols (i.e., **6**) showed lower reaction rates (*K*) than those of the wines preservation compounds (**3**, **4**, **5**) based on loss of the quinone chromophore (Nikolantonaki & Waterhouse, 2012). The results clearly demonstrated that each of these preservative compounds can provide a protective effect as sacrificial nucleophiles due to their fast reaction rates with the quinone, potentially suppressing varietal thiol consumption by the oxidised *ortho*-biphenols. The next step is to challenge this hypothesis with actual competitive reactions between **6** and the preservatives, and also establish what reactions have actually occurred by identifying the products. This study investigates the relative nucleophilic power of major wine preservatives (**3**, **4**, **5**) as well as a natural preservative (**7**) versus the varietal thiol **6**, in competitive reactions with a model quinone and determines the structure of the products. In addition, the potential existence of synergistic protective interactions between these preservatives is evaluated.

## 2. Material and Methods

### 2.1. Reagent and chemicals

Amberlyst® A-26(OH) ion-exchange resin, periodic acid, glutathione, 5,5′-dithiobis(2-nitrobenzoic acid) (DTNB), formic acid and 3-sulfanylhexan-1-ol were purchased from Sigma–Aldrich, Inc., St. Louis, MO. 4-Methylcatechol, phloroglucinol dihydrate, sodium bisulphite and formic acid, were purchased from Acros Organics, Morris Plains, NJ. Acetonitrile, methanol, L-tartaric acid and ascorbic acid were purchased from Fisher Bioreagents, Fisher Scientific, Fair Lawn, NJ. Tetrahydrofuran (THF), and anhydrous ethyl ether were from EMD Chemicals Inc., Gibbstown, NJ. Water was purified using a Milli-Q system (Millipore, Billerica, MA). All chemicals were of analytical grade or of the highest available purity.

### 2.2. Synthesis, isolation and characterisation of reaction products between 4-methyl-1,2-benzoquinone and wine relevant nucleophiles

Sulphur dioxide – 4-methyl-1,2-benzoquinone major mono adduct (**8a**). **2** (1.6 mM) prepared in acetonitrile by periodate resin according to the procedure described by Nikolantonaki and Waterhouse (2012), was dissolved in a phosphate buffer solution (pH = 3.5, 0.1 M) (200 mL) containing **3** (2.4 mM). This reaction mixture was stirred for 10 min at ambient temperature after which time HPLC-MS monitoring, using the analytical conditions described in Section 2.4, indicated the completion of the reaction. All reaction products were identified by comparison of their UV–*Vis* and mass spectral properties (Nikolantonaki & Waterhouse, 2012). Afterwards, 200 mg of sodium bisulphite was added to stop the reaction and the mixture was then evaporated under vacuum. The residue was dissolved in 100 mL of water, and the aqueous solution was frozen and freeze-dried to give 13.87 g crude mixture. Part of the crude mixture (1.27 g) was purified using an Agilent 1100 series Prep-LC and a LiChrospher 100 RP-18 end-capped column (100 mm × 4.6 mm, 5 μm particle diameter) with 4.0 mL $min^{-1}$ gradient elution of 1% aqueous acetic acid and methanol. The flow rate was set at 4 mL $min^{-1}$, and the injection volume was set to 100 μL of each crude reaction mixture. Acquisitions were performed using Chemstation software and, data was collected at 280 nm. The gradient was as follows: 0 min, 0% B; 3 min, 10% B; 15 min, 53% B; 17 min, 100% B, followed by washing and reconditioning of the column. Collected fractions were concentrated and then freeze-dried to furnish pure **8a** (20.3 mg) as a white amorphous powder: ESI-MS (*m/z*) $[M-H]^-$, calcd for $C_7H_7O_5S$, 203.0008; found, 203.0019 (Nikolantonaki & Waterhouse, 2012). NMR spectra of **8a**, collected from semi-preparative purification, were run at 295 K on a Bruker DRX 600 spectrometer (600 and 150 MHz for $^1H$ and $^{13}C$ observations, respectively). Carbon multiplicities were determined by DEPT135 experiments, whereas proton and carbon NMR signals were assigned on the basis of their diagnostic correlations observed on 2D NMR spectra (*i.e.*, HMBC and $^1H$–$^1H$ COSY). $^1H$ NMR (DMSO, 600 MHz) $\delta$ 2.33 (3H, s, $CH_3$), 6.36 (1H, d, $J$ = 7.9 Hz, H-5), 6.55 (1H, d, $J$ = 7.9 Hz, H-4), 8.32 (br s, OH-3), 11.60 (br s, OH-2); $^{13}C$ NMR (DMSO, 150 MHz), $\delta$ 20.1 ($CH_3$), 115.6 (C-4), 120.8 (C-5), 126.7 (C-6), 128.8 (C-1), 143.0 (C-2), 143.9 (C-3).

Glutathione – 4-methyl-1,2-benzoquinone major mono adduct (**10a**). **10a** was prepared and characterised according to the general procedure described above for **8a**. A solution of **2** (1.6 mM) and **5** (2.4 mM) in a phosphate buffer solution (pH = 3.5, 0.1 M) (200 mL) was stirred for ten minutes at ambient temperature and then treated as above to give 6.16 g crude mixture. Part of the crude mixture (2.65 g) was purified using preparatory scale liquid chromatography as above to isolate the thiol adduct (**10a**). The gradient was as follows: 0 min, 30% B; 6 min, 49% B; 12 min, 50% B; 14 min, 52% B; 15 min, 100% B, followed by washing and reconditioning of the column. Collected fractions were concentrated and then freeze-dried to furnish pure **10a** (12.81 mg) as a white amorphous powder: ESI-MS (*m/z*) $[M-H]^-$, calcd for $C_{17}H_{22}N_3O_8S$, 428.1122; found, 428.1102. $^1H$ NMR ($D_2O$, 600 MHz) $\delta$ 1.96 (2H, m, H-8′), 2.07 (3H, s, $CH_3$), 2.29 (2H, m, H-7′), 3.08 (1H, dd, $J$ = 14.5, 8.2 Hz, H-1′a), 3.22 (1H, dd, $J$ = 14.5, 4.7 Hz, H-1′b), 3.69 (3H, m, H-9′,4′), 4.31 (1H, dd, $J$ = 8.2, 4.7 Hz, H-2′), 6.63 (1H, br s, H-4), 6.72 (1H, br s, H-6); $^{13}C$ NMR ($D_2O$, 150 MHz), $\delta$ 19.5 ($CH_3$-5), 25.9 (C-8′), 31.9 (C-7′), 34.5 (C-1′), 41.9 (C-4′), 53.3 (C-2′), 53.9 (H-9′), 117.2 (C-4), 118.6 (C-1), 125.8 (C-6), 131.6 (C-5), 142.3 (C-2), 143.9 (C-3), 171.9 (C-3′), 173.7 (C-5′), 174.3 (C-10′), 174.5 (C-6′). NMR signals identified as for **8a**.