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Antioxidant Action of Glutathione and the Ascorbic Acid/Glutathione Pair in a Model White Wine

Francesca Sonni,[†] Andrew C. Clark,[*,‡] Paul D. Prenzler,[‡] Claudio Riponi,[†] and Geoffrey R. Scollary[‡,§]

[†]Department of Food Science, University of Bologna, 40127 Bologna, Italy

[‡]National Wine and Grape Industry Centre, Charles Sturt University, Locked Bag 588, Wagga Wagga, NSW 2678, Australia

[§]School of Chemistry, The University of Melbourne, Melbourne, VIC 3010, Australia

Supporting Information

ABSTRACT: Glutathione was assessed individually, and in combination with ascorbic acid, for its ability to act as an antioxidant with respect to color development in an oxidizing model white wine system. Glutathione was utilized at concentrations normally found in wine (30 mg/L), as well as at concentrations 20-fold higher (860 mg/L), the latter to afford ascorbic acid (500 mg/L) to glutathione ratios of 1:1. The model wine systems were stored at 45 °C without sulfur dioxide and at saturated oxygen levels, thereby in conditions highly conducive to oxidation. Under these conditions the results demonstrated the higher concentration of glutathione could initially provide protection against oxidative coloration, but eventually induced color formation. In the period during which glutathione offered a protective effect, the production of xanthylium cation pigment precursors and *o*-quinone-derived phenolic compounds was limited. When glutathione induced coloration, polymeric pigments were formed, but these were different from those found in model wine solutions without glutathione. In the presence of ascorbic acid, high concentrations of glutathione were able to delay the decay in ascorbic acid and inhibit the reaction of ascorbic acid degradation products with the wine flavanol compound (+)-catechin. However, on depletion, the glutathione again induced the production of a range of different polymeric pigments. These results highlight new mechanisms through which glutathione can offer both protection and spoilage during the oxidative coloration of a model wine.

KEYWORDS: glutathione, oxidation, ascorbic acid, white wine, dihydroxybenzaldehyde, *o*-quinone, vinyl catechol

INTRODUCTION

The oxidative spoilage of white wine is a well-documented phenomenon. White wine is particularly sensitive to oxygen exposure, which can result in detrimental changes to the sensory color and aroma attributes of the wine.[1,2] After wine is bottled, it is oxygen trapped in the headspace of the bottle (i.e., between the bottle closure and the wine), oxygen dissolved within the wine, and oxygen that permeates through the closure that is available during bottle aging.[3,4]

The main preservative utilized in white wine to prevent oxidative spoilage is sulfur dioxide (SO_2). This sulfur(IV) species is in equilibrium with hydrogen sulfite (HSO_3^-, the dominant form at wine pH) and sulfite (SO_3^{2-}). These sulfur species are not efficient at directly scavenging oxygen, but instead react with the metal-catalyzed oxidation productions of oxygen and phenolic compounds, namely, hydrogen peroxide and *o*-quinone compounds.[5] Often sulfur dioxide is used in combination with ascorbic acid, because the latter can efficiently scavenge oxygen before reaction of oxygen with phenolic compounds (Scheme 1).[6]

If ascorbic acid is used without sulfur dioxide, then the hydrogen peroxide and dehydroascorbic acid degradation products can lead to the formation of spoilage pigments upon the near depletion of ascorbic acid (Scheme 1).[7] In model wine systems, these pigments have been identified as yellow xanthylium cations formed from the skin- and seed-derived flavonoid (+)-catechin.[8,9] In other model wine systems containing the grape pulp-derived, non-flavonoid caffeic acid in combination with (+)-catechin, the color of the system appeared to be more orange/brown rather than yellow.[10]

Although sulfur dioxide is efficient in its protective role against the oxidation of white wine, there are certain consumers that are sensitive to this antioxidant.[11] With this in mind, the tripeptide glutathione has shown some potential to assist sulfur dioxide in its role as an antioxidant. Indeed, glutathione is already known to be capable of performing the main antioxidant reactions of sulfur dioxide, although this has not always been in a wine-related matrix.[12−17] Glutathione is known to scavenge *o*-quinone compounds efficiently in wine/juice conditions (Scheme 2).[12,13] It can react with hydrogen peroxide and undergo addition reactions with aldehyde compounds,[14−16] although these reactions have not been studied in wine-like conditions. Cilliers and Singelton[17] examined the oxidation of caffeic acid in the presence of a variety of thiols, including glutathione, and showed a protective effect of the thiols against both caffeic acid loss and oxidative color production. However, this study was in alkaline aqueous solutions, without added ethanol, metal ions, or (+)-catechin. All three of these latter components have since been highlighted as critical factors in the oxidative coloration of model wine systems and/or the oxidation reactions of thiol compounds.[1,5,18−20]

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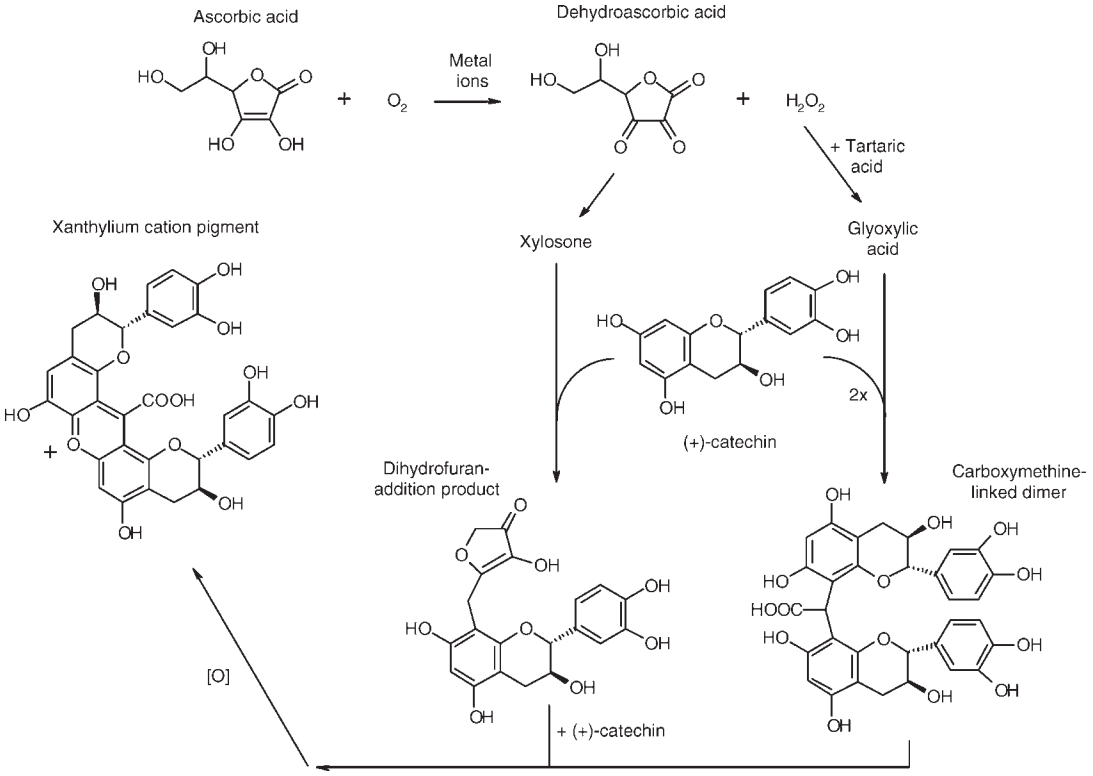
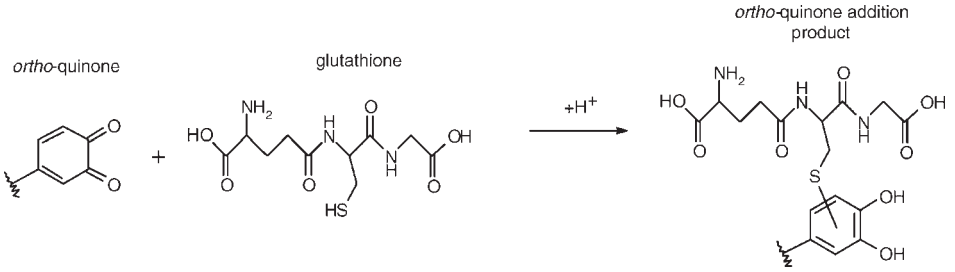
Scheme 1. Oxidation of Ascorbic Acid in Wine and Reaction of Its Degradation Products with (+)-Catechin To Afford Yellow Xanthylium Cation Pigments



Scheme 2. Reaction of Glutathione with *o*-Quinone Compounds[a]



[a] The site of addition depends on the type of phenolic compound involved.

On the basis of previous work[10] in model wine systems stored at saturated oxygen levels and elevated temperature conditions (i.e., 45 °C), the key components of oxidative coloration in model white wine are ascorbic acid, caffeic acid, (+)-catechin, and tartaric acid. Each of these compounds can undergo reactions that generate pigments and/or their precursors. Therefore, upon introducing a further component to this system (i.e., glutathione), it is important to understand how this component interacts with those already identified as important. In particular, because ascorbic acid has a dual role in coloration reactions (protective vs enhancing), it is necessary to examine the impact of glutathione on pigment production in model wines both with and without ascorbic acid. This study provides baseline data on the reactions of two antioxidants, glutathione and ascorbic acid, which are essential before future work under conditions of typical wine storage (i.e., added sulfur dioxide, reduced temperature, and lower oxygen concentrations) can be undertaken.

MATERIALS AND METHODS

General. L-Ascorbic acid (99%), L-(+)-tartaric acid (>99.5%), (+)-catechin hydrate (98%), potassium bitartrate (99%), caffeic acid (99%), dihydroxybenzaldehyde (97%), glutathione (99%), and copper(II) sulfate pentahydrate (98%) were purchased from Sigma-Aldrich. Iron(II) sulfate heptahydrate (>98%) was purchased from Ajax Fine Chemicals. Ethanol (AR grade, > 99.5%, Ajax Fine Chemicals), methanol (AR grade, > 99.9%, Mallinckrodt Chemicals), and glacial acetic acid (AR grade, > 99.7%, APS Ajax Fine Chemicals) were used without further purification.

Absorbance measurements (440 and 500 nm) were recorded as per Clark et al.[21] and UPLC analyses conducted as per Barril et al.[9] LC-MS studies were conducted on an Agilent 1200 series triple-quadrupole (6410) LC-MS. The column and LC conditions were as described for the UPLC,[9] except for an injection volume of 20 μL. The MS component of the LC-MS was operated at 350 °C, gas flow of 9 L/min, nebulizer at 40 psi, and capillary at 4 kV. MS analyses were carried out in the negative

ion mode with the fragmentor at 80 V and scanning from m/z 100 to 1000. LC-MS^2 analyses were conducted on specific parent ions with the fragmentor at 80 V, a collision energy of either 10 or 20 eV, and scanning from m/z 100 to m/z 20 above the parent ion m/z value.

Reactions under Various Model Wine Conditions. A control model wine solution (5 L) was prepared consisting of 0.011 M potassium hydrogen tartrate, 0.008 M tartaric acid, and 0.20 M glycerol in 12% v/v aqueous ethanol that was stirred overnight at room temperature. To this solution were then added 1.11 mM (200 mg/L) caffeic acid and 0.17 mM (50 mg/L) (+)-catechin. Such levels of phenolic compounds are within the range quoted for their respective flavonoid and non-flavonoid groups in white wine[22,23] and the flavanol level more indicative of a white wine produced from heavier pressings. After the phenolic compounds were dissolved, with 2 h of stirring at room temperature, 3.1 μM (0.20 mg/L) Cu(II) and 91 μM (5.0 mg/L) Fe(II) were also added and are within the typical ranges quoted for these metal ions in wine.[24] The pH of the model wine solution was 3.2 ± 0.1.

Using the control model wine, samples were prepared with either no glutathione or low (0.10 mM, 30 mg/L) and high (2.80 mM, 860 mg/L) concentrations, in the absence and presence of ascorbic acid (2.80 mM, 500 mg/L). The ascorbic acid concentration was higher than that typically used in white wine (100−200 mg/L), but previous studies have demonstrated this concentration would aid assessment of ascorbic acid's role in the oxidizing storage conditions and enhance the identification of products.[10] Furthermore, Bradshaw et al.[7] reported little impact of ascorbic acid concentration on the range of degradation products produced. The low concentration of glutathione corresponded to the levels observed in wines for which the juice had been treated reductively.[25] The high glutathione concentration of 2.80 mM was included to assess glutathione when at a 1:1 ratio with ascorbic acid.

Samples (150 mL) were placed in 250 mL Schott Duran reagent bottles with a measured headspace volume of 170 mL. These were opened and stirred vigorously to vortex for 3.0 min at the start of the experiment and twice each day to replenish the oxygen in the bottles. The samples were otherwise held in darkness at 45 °C. All samples were prepared in triplicate, and the plotted data are the mean of the replicates. Error bars, confidence limits, and significant differences reported in the text are at the 95% level.[26]

Identification and/or Quantification of Model Wine Components. (+)-Catechin (m/z 289), caffeic acid (m/z 179), and dihydroxybenzaldehyde (m/z 137) were all identified via comparison of UPLC retention times, UV−vis spectra extracted from the UPLC data, and LC-MS data (negative ion mode) with those of their respective purchased standards. (+)-Catechin, ascorbic acid, and dihydroxybenzaldehyde were quantified via an external calibration curve based on their peak area at 280 nm. The quantification of caffeic acid was complicated by coeluting species on the UPLC and was monitored semiquantitatively by LC-MS in the negative ion mode (m/z 179). Glutathione and its disulfide form were detected by LC-MS in the negative ion mode at m/z 306 and 611.

Verification of the retention time of glutathionyl−phenolic compound addition products (Scheme 2) was achieved by comparison of their retention time, UV−vis spectra, and LC-MS^2 data to the equivalent addition products formed enzymatically (mushroom tyrosinase, T3824-25KU Sigma). The enzymatic preparation was based on methods by Cheynier et al.[13] for caffeic acid and Moridani et al.[27] for (+)-catechin. The LC-UV-MS^2 data for glutathionyl-(+)-catechin addition product showed two isomers with parent ion signals at m/z 594, both with fragment ions indicative of cleavage around sulfur (i.e., m/z 321 for catechin sulfide and m/z 272 for $[(\text{glutathione}-\text{HS}) - 2\text{H}^+]^-$) and absorbance maxima at 290 nm. For the glutathionylcaffeic acid addition product, a single isomer was detected with a parent ion signal at m/z 484 and fragmentation indicative of decarboxylation (m/z 440), cleavage around sulfur (i.e., m/z 211 for caffeic acid sulfide, and m/z 272 for $[(\text{glutathione-HS})\text{-}2\text{H}^+]^-$), and a combination of decarboxylation and cleavage around sulfur (i.e., m/z 167 for $[\text{caffeic acid sulfide} - \text{CO}_2]^-$). The UV−vis absorbance maxima were at 280 and 320 nm.

The (+)-catechin-derived pigment precursors consisting of the carboxymethine-linked (+)-catechin dimer and the dihydrofuran−(+)-catechin addition product, both shown in Scheme 1, were identified by matching their retention times, UV−vis spectra, and mass data (negative ion mode, m/z 635 and 401, respectively) to those previously reported.[9,28] The xanthylium cation pigments in the acid (m/z 617) and ester (m/z 645) forms were identified by matching their retention time, UV−vis spectra, and mass data (positive ion mode) as per ref 21.

RESULTS AND DISCUSSION

Data from two model systems are described below, one containing glutathione alone and one containing both glutathione and ascorbic acid. In each model system, the change in 440 nm absorbance was monitored as a measure of oxidative color change of the system during the experiment. Measurements at higher wavelengths (i.e., 500 nm) were also conducted (Supporting Information Figure S1), but given the similarity of results to those at 440 nm, they were not discussed further. The change in 440 nm absorbance is related to the impact of the antioxidants (glutathione and/or ascorbic acid) on previously reported pigment precursors (within Scheme 1), on glutathione adducts (Scheme 2) and on pigments.

Coloration of Model Wine Samples without Added Ascorbic Acid. *Color Formation.* Figure 1 shows the 440 nm absorbance of the model white wine over the 14 days of the experiment. In the solution with 0 mM glutathione (control), some color formation is evident in the first 2.5 days of reaction (Figure 1a). With 0.10 mM glutathione there is a trend to higher absorbance values, in contrast to solutions with 2.80 mM glutathione in which almost no coloration occurs in this first 2.5 day period. This illustrates the protective effect of high levels of glutathione against oxidative coloration. However, after 2.5 days, there was a rapid acceleration in coloration of the 2.80 mM glutathione sample to the extent that it had developed the most color by day 3.5 (Figure 1a). This sample subsequently continued to undergo oxidative coloration at an enhanced rate compared to the control and 0.10 mM glutathione samples (Figure 1b).

Model Wine Components. The presence of glutathione was assessed by LC-MS at day 2, during the period when 2.80 mM glutathione showed a protective effect against color formation and just before the rapid increase in color in this model wine. In the sample with 0.10 mM glutathione, no glutathione was detected, whereas it was still present in the sample with 2.80 mM glutathione sample (i.e., ion current 1.2×10^6). By day 14, however, no glutathione could be detected in any sample. Glutathione disulfide was also monitored by LC-MS and was detected in both samples at days 2 and 14, indicating that glutathione was oxidized during the reaction. The low initial color observed in the 2.80 mM glutathione sample (Figure 1) can now be related to a protective effect of residual glutathione.

The change in (+)-catechin concentration throughout the experiment is shown in Figure 2. In the first 2 days of the reaction, samples with 0 and 2.80 mM glutathione retained significantly higher (+)-catechin concentrations than the 0.10 mM glutathione sample. There was no significant difference ($p = 0.05$) for the decrease in (+)-catechin concentration within the first 2 days of the experiment for the 2.80 mM glutathione and control

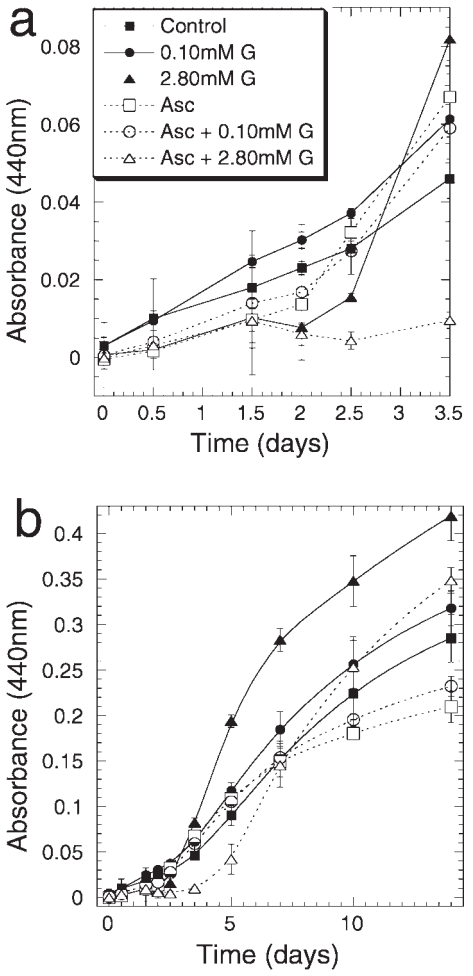


Figure 1. Change in sample absorbance (440 nm) during the oxidation of the model wine system over the first (a) 3.5 days and (b) 14 days.

samples. When (+)-catechin concentration was monitored in the early stages of the reaction, there did not seem to be any relationship between (+)-catechin loss and color formation. The increased color in the 0.10 mM glutathione sample compared to control (Figure 1a) is consistent with the greater loss of (+)-catechin in the former. However, the difference in color between the control and the 2.80 mM glutathione (Figure 1) cannot be explained simply in terms of loss of (+)-catechin.

After day 2, the control and 0.10 mM glutathione samples had similar rates of (+)-catechin loss that coincided with their similar rates of coloration. In the 2.80 mM glutathione sample the (+)-catechin concentration decreased significantly more quickly compared to the control and 0.10 mM samples, reaching <10% of the initial (+)-catechin concentration by the end of the experiment (Figure 2). This accelerated (+)-catechin decrease (Figure 2) preceded the rapid increase in 440 nm absorbance (Figure 1a), which is consistent with the involvement of flavanols in pigment production (Scheme 1).

The measurement of the caffeic acid concentration in the samples was not possible by UPLC due to coeluting compounds (discussed further below). However, the formation of products from caffeic acid during the course of the experiment is discussed in the next section.

Production of Glutathione Phenolic Adducts and Reported Pigment Precursors. The products generated in the first 2 days

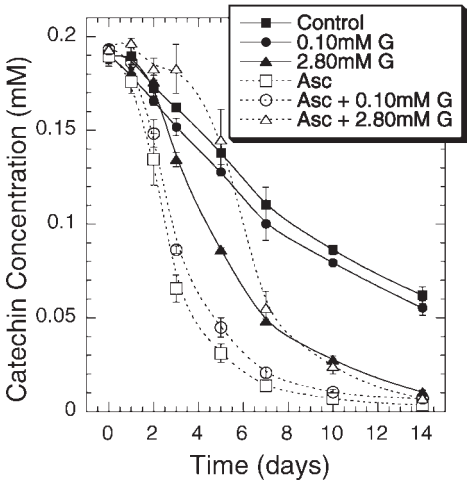


Figure 2. Change in (+)-catechin concentration during the induced oxidation of the model wine system.

were initially investigated. Given the range of products evident in the chromatograms (Figure 3), the focus here was on previously reported glutathione−phenolic compound addition products[13,27] (Scheme 2) and pigment precursors,[9,29] including those in Scheme 1.

Glutathione addition products (Scheme 2) of (+)-catechin (peaks 3 and 5, Figure 3) and caffeic acid (peak 7, Figure 3) were detected in all samples containing glutathione. For the 0.10 mM glutathione sample the addition products increased in intensity to day 1, plateaued, and then rapidly decayed after day 2.5. This is consistent with the depletion of glutathione by day 2 in this sample, as these addition products are known to undergo further oxidation processes (i.e., including further *o*-quinone production) upon depletion of glutathione.[13] It has also been reported[30] that the glutathione portion of these products can also undergo hydrolysis and/or esterification during wine storage; however, such products were not detected by LC-MS in this experiment. The monitoring of glutathionylcaffeic acid by UPLC in the 2.80 mM glutathione sample was complicated by coeluting species. However, LC-MS data were used to monitor the relative amounts of adduction products formed with the different concentrations of glutathione. Adducts of glutathionyl-(+)-catechin and glutathionylcaffeic acid were higher in the 0.10 mM glutathione sample by factors of 8.5 and 4.7, respectively. In other words, at 2.80 mM glutathione, adduct formation was inhibited. This phenomenon will be discussed further, below (see Pigment Formation).

The UV−vis spectra, extracted from the UPLC chromatograms (data not shown), for the glutathione-substituted (+)-catechin and caffeic acid products tailed further into the visible region of the spectrum compared to their respective parent phenolic compounds. In fact, at day 2, it was the contribution of these glutathionyl species to the 440 nm chromatograms of the 0.10 mM glutathione sample that was the main point of difference from the equivalent chromatogram of the control sample. This now explains one of the observations above concerning (+)-catechin depletion and color formation. In low glutathione samples, the formed adducts contribute to increased absorbance in the visible region of the spectrum, resulting in the increased color, observed in Figure 1a, relative to control. However, the effect of high glutathione in suppressing color formation, relative to control, cannot be explained at this stage.

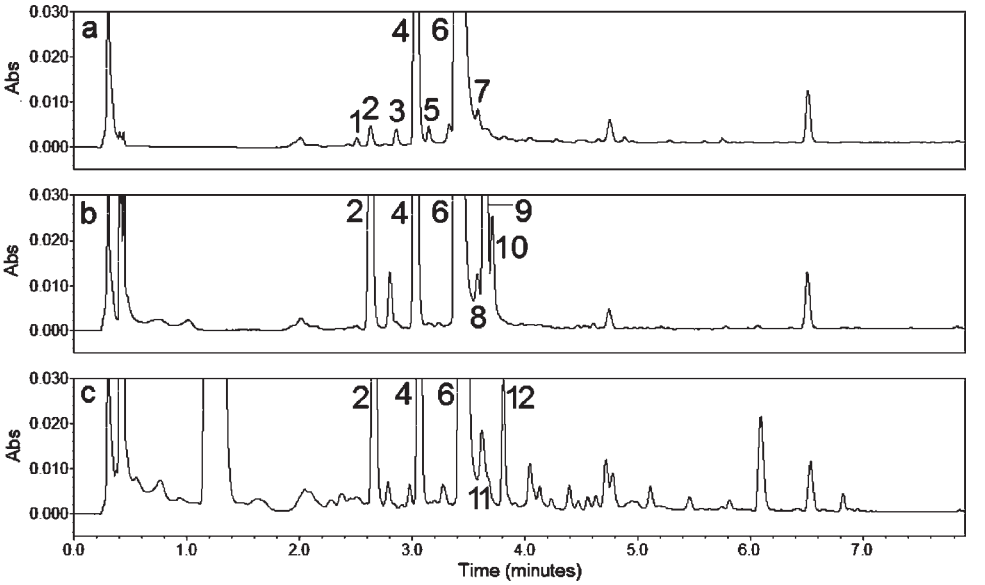


Figure 3. 280 nm chromatograms of the (a) 0.10 mM glutathione, (b) 2.80 mM glutathione, and (c) ascorbic acid samples. Peaks: 1, carboxymethine-linked (+)-catechin dimer; 2, dihydroxybenzaldehyde; 3, glutathionyl-(+)-catechin isomer; 4, (+)-catechin; 5, glutathionyl-(+)-catechin isomer; 6, caffeic acid; 7, glutathionylcaffeic acid; 8−10, unknown compounds; 11 and 12, dihydrofuran-(+)-catechin addition products.

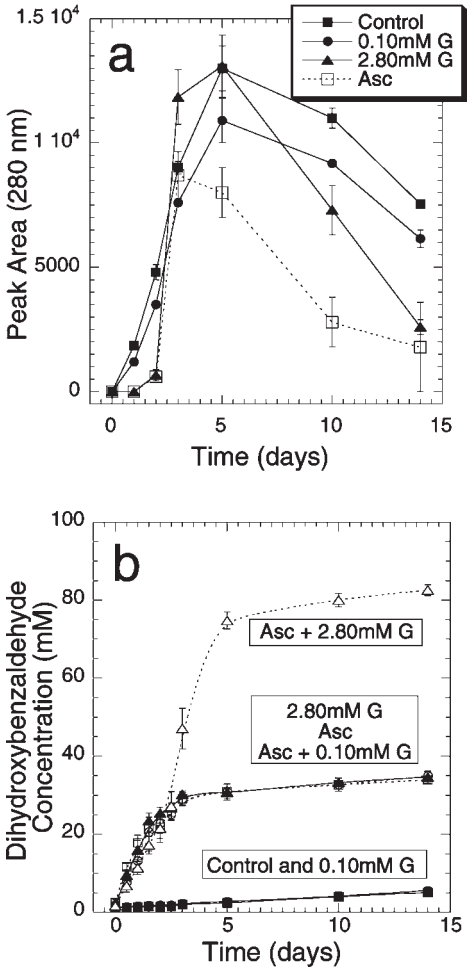


Figure 4. Change in (a) peak area of the carboxymethine-linked (+)-catechin dimer (peak 1 from Figure 3) and (b) concentration of dihydroxybenzaldehyde (peak 2 from Figure 3) during the experiment.

The carboxymethine-linked (+)-catechin dimers (peak 1, Figure 3a), which are precursors to yellow xanthylium cation pigments (Scheme 1), were also monitored during the experiment. Figure 4a shows the peak areas from the major isomer (as depicted in Scheme 1) of these products. From this it is evident that, compared to the control sample, the presence of 0.10 mM glutathione led to slightly lower concentrations of the dimer throughout the experiment. For the 2.80 mM glutathione sample, dimer production was delayed to day 2 followed by an accelerated increase from days 2 to 3 and then a rapid decrease in concentration after day 5. The initial delay in production may be due to the ability of higher concentrations of glutathione to form an addition product with glyoxylic acid, as has been reported in physiological conditions,[16] thereby preventing dimer formation. After day 2, the diminishing levels of free glutathione probably led to dissociation of any glyoxylic acid−glutathione adduct, and therefore the released glyoxylic acid induced dimer formation. The final phase of accelerated loss of the dimer could be due to the lower levels of (+)-catechin available for dimer formation in this sample after day 5 (Figure 2). The trend toward overall lower levels of the dimer with glutathione is consistent with the lower levels of xanthylium cation pigments with increasing glutathione concentration that will be described below.

Dihydroxybenzaldehyde is known to induce pigment formation after reaction with (+)-catechin, although the reaction is relatively slow compared to the glyoxylic acid/(+)-catechin reaction, and its main products are colorless.[31] Figure 4b shows that glutathione, at 2.80 mM, accelerates the production of dihydroxybenzaldehyde to day 3. The dihydroxybenzaldehyde production rate then becomes similar to that in the control and 0.10 mM glutathione samples from days 2 to 14. The UV−vis spectrum of dihydroxybenzaldehyde has no significant absorbance in the visible region, thereby providing some explanation for the production of caffeic acid degradation products in the 2.80 mM glutathione sample but without color formation in the first 2 days. The inability to detect the main colorless products from the reaction between dihydroxybenzaldehyde and

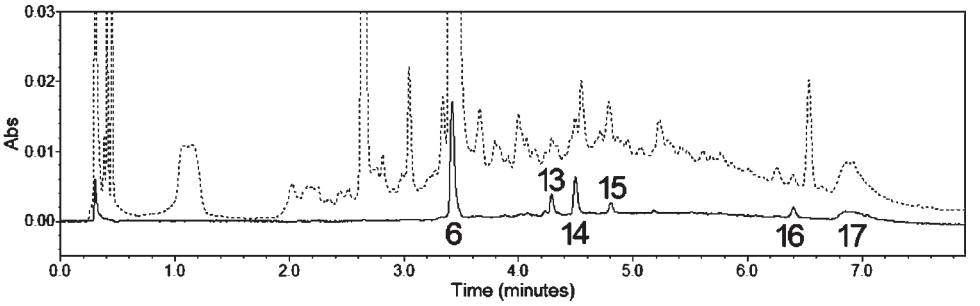


Figure 5. UPLC 440 nm (solid line) and 280 nm (dotted line) chromatograms of the sample with 2.80 mM glutathione at day 14 of the experiment. Peaks: 6, caffeic acid; 13−15, xanthylium cations (acid form); 16, xanthylium cations (ethyl ester form); 17, unknown.

(+)-catechin (i.e., catecholmethine-linked (+)-catechin dimers) was consistent with previous work showing that the reaction rate was slow in model wine conditions.[10,31]

Pigment Formation. The production of compounds responsible for the A_{440} absorbance of the samples (Figure 1) was also assessed. In of all the samples, peaks from (+)-catechin and caffeic acid were present in the 440 nm chromatograms due to tailing of their absorbances into the visible. As already mentioned, the glutathione adducts with (+)-catechin and caffeic acid were the only additional pigments detected in the 0.10 mM glutathione sample at day 2 that were not in the control sample. Apart from these peaks, those corresponding to xanthylium cation pigments (peaks 13−16, Figure 5) were detected in both the control and 0.10 mM glutathione samples, but were not detected in the 2.80 mM glutathione sample. This was expected given that the high glutathione concentration inhibited the precursors to these pigments (Figure 4a). It also accounts for the low color in the 2.80 mM glutathione sample compared to the control. By day 14, the impact of glutathione on xanthylium cation pigments continued, with the total peak areas ($\times 10^4$) attributed to xanthylium cations at 9.1 ± 0.8, 7.5 ± 0.1 and 2.1 ± 0.2 for the 0, 0.10, and 2.80 mM glutathione samples, respectively. Therefore, the production of the xanthylium cations was inhibited permanently by glutathione, even after the depletion of the thiol.

The increased color observed in the glutathione samples at day 14 (Figure 1) can be attributed only to a broad increase in baseline intensity (i.e., from 3 to 8 min) and peak 17 (Figure 5) in the 440 nm chromatograms. These predominant features in the 440 nm chromatogram are consistent with the polymeric nature of pigments generated. A similar 440 nm chromatographic profile, with broad sloping baselines and peaks equivalent to that of peak 17 (Figure 5), was evident in previous model wine studies containing caffeic acid/(+)-catechin and added Fe(II).[32] However, the polymeric pigments contributing to the color in the glutathione samples (Figure 1) are also likely to contain at least some glutathione addition products or derived forms of the glutathione moiety.

As well as the products already discussed, there appeared to be three major peaks (peaks 8−10, Figure 3) in the chromatograms of the 2.80 mM glutathione samples that are worthy of further comment. Peaks 8 and 10 both had an m/z of 745 (negative ion mode) and UV−vis spectra with maxima at 290, 320, and 455 nm. Peak 9 had an m/z of 440 and a UV−vis spectrum with maxima at 290, 315, and 500 nm, with a shoulder at 395 nm. On assessment of the extracted m/z 440 mass chromatogram in this sample, an additional peak that coeluted with caffeic acid was evident and possibly a connective isomer of peak 9. These peaks could only be detected in model wine systems containing both

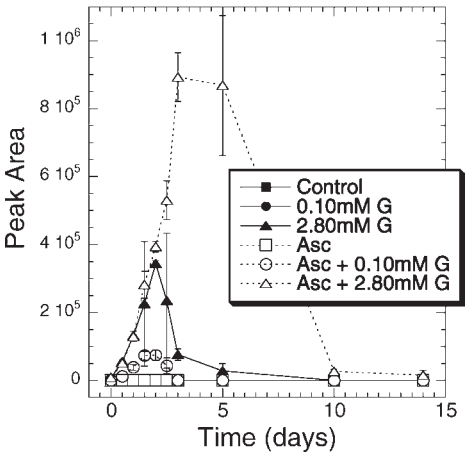


Figure 6. Change in area of peak 9 (from Figure 3) throughout the experiment.

caffeic acid and 2.80 mM glutathione, demonstrating that both of these components were critical for their formation. The assignment of these peaks to specific compounds still requires further investigation; however, the m/z 440 and 745 mass data are consistent with vinylcatechol substituted with either one or two glutathione units, respectively. Vinylcatechol has been implicated as a possible degradation product of caffeic acid capable of producing pyranoanthocyanin pigments in red wine[33] and can be formed from lactic acid bacterial action (i.e., *Lactobacullus plantarum*) on caffeic acid.[34]

Figure 6 shows that peak 9 reached a maximum area at day 2 in the 2.80 mM glutathione sample followed by a rapid decay (i.e., trace levels at day 3), which coincided with the production of color in this sample, further implicating it as a possible participant in the rapid oxidative coloration process. The initial production of peaks 8 and 10 occurred half a day later than for peak 9, and they also reached their maximum peak area at day 2.5 when peak 9 was already in decline, before themselves undergoing a rapid decrease from day 2.5 to 3 (data not shown). These kinetics data suggest that peaks 8 and 10 are most likely formed from peak 9, consistent with their tentative assignment as disubstituted glutathione compounds. They also suggest an oxidative step is required for the rapid depletion of peaks 8, 9, and 10 that is inhibited while glutathione remains present. Due to their absorbance in the visible region, these peaks were the largest product peaks in the 440 nm chromatogram of the 2.80 mM glutathione sample at day 2. However, despite their absorbance in the chromatographic system (i.e., ~40% aqueous methanol) at 440 nm, their molar absorptivity at this wavelength in the model

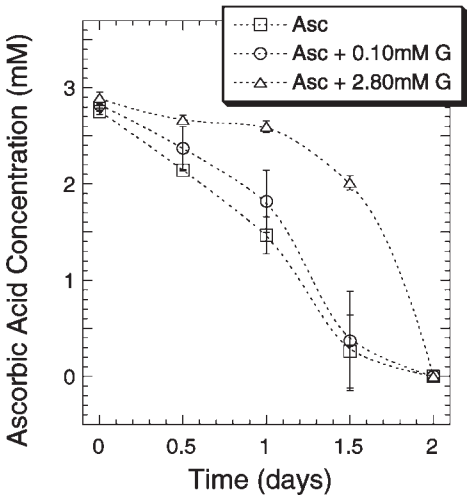


Figure 7. Change in ascorbic acid concentration throughout the experiment.

wine system must have been sufficiently low to provide only a low A_{440} measurement at day 2 in the 2.80 mM glutathione sample (Figure 1).

The outcome of this study demonstrates that glutathione has the ability to prevent the formation of oxidative pigments and certain precursors when at sufficient concentrations but leads to the formation of a new range of polymeric pigments upon its depletion. While glutathione is present and protecting against coloration, (+)-catechin is conserved, but degradation products of caffeic acid accumulate. During the second stage of oxidative color production, (+)-catechin and several caffeic acid degradation products are rapidly consumed.

Coloration of Model Wine Samples with Added Ascorbic Acid. *Color.* Figure 1 shows that there is a trend to lower color in samples with ascorbic acid, compared to the respective samples without ascorbic acid, throughout the entire experiment. In contrast to past studies,[7,10] at the end of the experiment, the coloration is less in the sample with ascorbic acid compared to the control. This is because of the lower levels of (+)-catechin in this model wine compared to past studies,[7,10] leading to eventual exhaustion of the pigment precursor (+)-catechin (Figure 2) and thereby limiting the conversion of ascorbic acid-derived pigment precursors to their final pigments (Scheme 1).

As for the impact of glutathione on the oxidative color formation in samples with ascorbic acid, there was little difference evident between the samples with 0 and 0.10 mM glutathione. The most dramatic color effect was evident in the sample with both ascorbic acid and 2.80 mM glutathione. This sample had the least color of any up to day 7, and then it underwent accelerated coloration to be the most intensely colored of the ascorbic acid samples by day 14. This sample having the longest period before accelerated coloration compared to any other sample in Figure 1 demonstrates that the glutathione/ascorbic acid pair has an additive protective effect against coloration.

Model Wine Components. The LC-MS analysis of glutathione and its disulfide in the samples with ascorbic acid showed results similar to those described in the samples without ascorbic acid. At day 2, no residual glutathione was detected in the 0.10 mM glutathione/ascorbic acid sample, whereas it was still present in the 2.80 mM glutathione/ascorbic acid sample, and at day 14, no glutathione was detected in either sample. The oxidized form of glutathione (i.e., glutathione disulfide) was detected in both samples containing glutathione at both days 2 and 14, again demonstrating that oxidation of glutathione was also occurring in samples with ascorbic acid.

The change in ascorbic acid concentration is shown in Figure 7. In the sample with ascorbic acid alone, its concentration decreased to near depletion by day 1.5. A similar result is observed in the presence of 0.10 mM glutathione, although there is a trend toward higher ascorbic acid concentrations than without glutathione. Alternatively, the 2.80 mM glutathione sample provides a lowered ascorbic acid decay rate, whereby ascorbic acid is only 30% depleted by day 1.5. After day 1.5, the ascorbic acid concentration in this sample then decreases rapidly to be depleted by day 2. The ability of glutathione to delay loss of ascorbic acid in the first 1.5 days suggests that at sufficient concentrations, glutathione could either scavenge oxygen or hydrogen peroxide or reduce dehydroascorbic acid back to ascorbic acid. However, the latter is less likely given the lower redox potential for the dehydroascorbic acid/ascorbic acid couple than the glutathione disulfide/glutathione couple in wine conditions.[12] In any case, delaying the loss of ascorbic acid is one mechanism that could account for the glutathione/ascorbic acid combination retarding the onset of accelerated oxidative coloration (Figure 1).

The decrease in (+)-catechin concentration (Figure 2) was reassessed for the ascorbic acid/glutathione samples. In the ascorbic acid sample, there was a rapid loss of (+)-catechin initially and then a diminished loss at day 5 once the (+)-catechin was nearly depleted. In the added presence of 0.10 mM glutathione, the result was similar, although there was marginally slower, albeit significant ($p = 0.05$), loss. Both of these samples demonstrated much faster rates of (+)-catechin consumption compared to the equivalent samples without ascorbic acid, which was expected given that xylosone is an oxidative degradation product of ascorbic acid that can react with (+)-catechin[9] (Scheme 1). In contrast, the sample with both ascorbic acid and 2.80 mM glutathione had the slowest loss of (+)-catechin of any sample in the first 3 days. The protective effect of the high glutathione concentration on (+)-catechin was presumably related to its ability to delay ascorbic acid loss and hence affect the production of xylosone for subsequent reaction with (+)-catechin (Scheme 1). After day 3, the rate of (+)-catechin loss in the ascorbic acid/2.80 mM glutathione sample accelerated such that it was similar to the initial rate of loss in (+)-catechin concentration (i.e., days 0.5−5) observed in the other ascorbic acid samples. The onset of the accelerated (+)-catechin loss in this sample (Figure 2) was consistent with the onset of rapid color formation (Figure 1).

Production of Glutathione Phenolic Adducts and Reported Pigment Precursors. The glutathionylcaffeic acid or glutathionyl-(+)-catechin products (Scheme 2) were not detected in any of the ascorbic acid samples at day 2 but were present at low concentrations in the 2.80 mM glutathione/ascorbic acid sample at day 14. The absence of these gluthionyl compounds at day 2 was consistent with the ability of ascorbic acid to scavenge oxygen (Scheme 1). In doing so the oxidation of phenolic compounds to *o*-quinone compounds would be inhibited and, thereby, limit the formation of the glutathione-substituted species (Scheme 2). It appeared that ascorbic acid could achieve this more efficiently than 2.80 mM glutathione alone, as both glutathionylcaffeic acid and glutathionyl-(+)-catechin were detected in the latter, as described above.

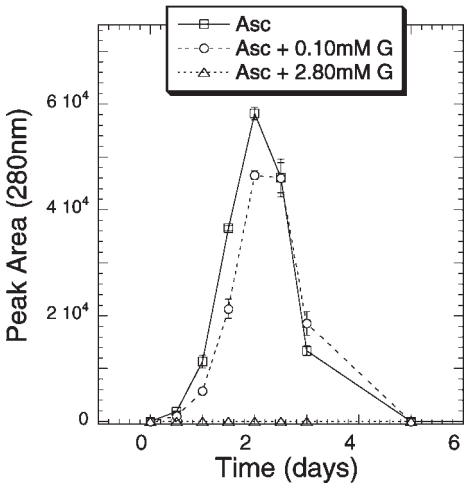


Figure 8. Change in the dihydrofuran-substituted (+)-catechin peak area (280 nm) throughout the experiment. The full name for this compound is (+)-1-methylene-6-hydroxy-2*H*-furan-5-one-8-catechin, and its structure is shown in Scheme 1. The data are from the combined peak areas for peaks 11 and 12 from Figure 3c.

The carboxymethine-linked (+)-catechin dimers could be detected by UPLC only in the 0 mM glutathione/ascorbic acid sample (Figure 4a), as the remaining samples with ascorbic acid had a coeluting species. In the 0 mM glutathione/ascorbic acid sample there was a delay in dimer production in the first 2 days, while ascorbic acid was still in solution, and then rapid production from days 2 to 3, followed by a decrease from days 5 to 14. This initial delay in dimer production was similar to that observed in the 2.80 mM glutathione sample (Figure 4a). Previous studies had demonstrated the ability of ascorbic acid to delay the onset of xanthylium cation pigment formation,[10] but the results in Figure 4a are the first to show that it also delays the onset of a precursor to these pigments. To gain some insight into the dimer production in the presence of both ascorbic acid and glutathione, LC-MS analysis showed that at day 2, the dimer was present in the ascorbic acid and ascorbic acid/0.10 mM glutathione sample but not in the ascorbic acid/2.80 mM glutathione sample. By day 14, all of the ascorbic acid samples had dimer present but in the order 0.10 mM glutathione > 0 mM glutathione > 2.80 mM glutathione. This suggests that the 2.80 mM glutathione/ascorbic acid combination can delay production of this pigment precursor for longer than any other sample, but after depletion of glutathione and ascorbic acid, the pigment precursor is still formed.

The dihydrofuran−(+)-catechin addition product (Scheme 1) was also monitored as it is another precursor to the xanthylium cation pigments that requires oxidation of ascorbic acid for its formation. Two connective isomers are possible for this addition product, as outlined by Barril et al.,[9] and the chromatographic peaks for both are shown in Figure 3 (peaks 11 and 12). Figure 8 shows the expected kinetics for these peaks in the ascorbic acid sample without glutathione present, whereby they increase as ascorbic acid decreases in concentration, and then the peaks rapidly decrease once ascorbic acid has been depleted from solution. The 0.10 mM glutathione sample with ascorbic acid showed a similar trend, whereas in the 2.80 mM glutathione/ascorbic acid sample the addition product was not detected throughout the entire experiment. Therefore, not only was glutathione able to delay the degradation of ascorbic acid but at high concentrations it could also prevent the accumulation of an ascorbic acid-derived pigment precursor.

Figure 4b demonstrates the ability of ascorbic acid to induce production of dihydroxybenzaldehyde from caffeic acid. This result has been previously reported[10] and is attributed to efficient production of hydrogen peroxide by ascorbic acid after reaction with oxygen (Scheme 1). Akin to the ability of glutathione, ascorbic acid has the ability to enhance oxygen consumption in the model wine system, and consequently hydrogen peroxide production, that in turn leads to dihydroxybenzaldehyde formation from caffeic acid. The similar rate of production of dihydroxybenzaldehyde for all of the samples with ascorbic acid and/or 2.80 mM glutathione, from days 0 to 2, suggests that the rate-limiting factor is most likely the rate at which molecular oxygen dissolves in the 45 °C samples from the headspace. The highest concentration of dihydroxybenzaldehyde is reached in the sample with both ascorbic acid and 2.80 mM glutathione, consistent with an additive potential for oxygen consumption when these model wine components are both present.

Pigments. In the samples with ascorbic acid, there were no xanthylium cation pigments detected at day 2. However, by day 14, xanthylium cations were present and there were significantly lower concentrations of the xanthylium cations in the 2.80 mM glutathione/ascorbic acid sample compared to any other samples, with peak areas ($\times 10^4$) of 0.9 ± 0.1, 1.2 ± 0.1, and 0.05 ± 0.01 for the 0, 0.10, and 2.80 mM glutathione/ascorbic acid samples, respectively. This outcome was consistent with the ability of 2.80 mM glutathione, in combination with ascorbic acid, to limit production of both precursors (i.e., carboxymethine-linked catechin dimers and dihydrofuran−(+)-catechin addition product, Scheme 1) to the xanthylium cation pigments. The increased 440 nm absorbance for the 2.80 mM glutathione/ascorbic acid pair was again a consequence of a broad increase in the baseline absorbance from 2 to 8 min in the chromatogram (i.e., polymers) and peak 17.

The unidentified compound responsible for peak 9 (m/z 440, Figure 3) was not detected in the ascorbic acid alone sample, but was in the combined glutathione/ascorbic acid samples. Of the glutathione/ascorbic acid samples, the area of peak 9 reached a maximum >10-fold higher in the 2.80 mM than in 0.10 mM glutathione sample (Figure 6) and also diminished later during the time course of the experiment in 2.80 mM glutathione sample. The rapid decrease in peak 9 coincided with the production of color in both of these samples (Figure 1). The presence of ascorbic acid also led to over double the maximum intensity of peak 9 in the 2.80 mM glutathione samples (i.e., (8.9 vs 3.4) × 10^5, Figure 6). Intriguingly, during days 0−2, the rate of increase of peak 9 was identical in the 2.80 mM glutathione samples regardless of the presence of ascorbic acid. This matching rate of production between the different samples was similar to the behavior of the dihydroxybenzaldehyde production (Figure 4b), and therefore the production of peak 9 was also most likely linked to the rate at which oxygen could dissolve into the sample. These results show that the presence of ascorbic acid with glutathione can increase the concentrations for the compound associated with peak 9, and like high glutathione concentrations, it can afford stability to the compound. The former outcome is most likely a consequence of the higher amounts of oxygen consumed, and hydrogen peroxide produced, in the ascorbic acid/2.80 mM glutathione sample that would induce caffeic acid decay via Fenton chemistry. The latter was probably due to the ability of ascorbic acid to inhibit an oxidation step that would consume the compound responsible for peak 9.

Peaks 8 and 10 were not detected in the samples with ascorbic acid, and their tentative assignment as being vinylcatechol

disubstituted with glutathione suggests that ascorbic acid is preventing substitution of a glutathione unit. This ability of ascorbic acid had been demonstrated in its inhibition of glutathionyl-(+)-catechin and glutathionylcaffeic acid (discussed earlier). It also suggests that if peak 9 is vinylcatechol with a monosubstituted glutathione unit, then its formation is probably not via the *o*-quinone form of the vinylcatechol.

The results demonstrate that, under the conditions adopted, the ascorbic acid and glutathione combination offers greater protection against oxidative coloration when present together rather than in isolation. However, the extent of protection was favored by the higher glutathione to ascorbic acid ratio (i.e., 1:1). Glutathione was able to delay ascorbic acid degradation and also inhibit the formation of a known pigment precursor formed from an ascorbic acid degradation product. Alternative caffeic acid degradation products were formed from the glutathione/ascorbic acid combination that upon decay led to a diverse range of polymeric pigments that were undefined by UPLC.

These results provide mechanistic insights into the role of glutathione, and the glutathione/ascorbic acid combination, during the storage of a model wine system in oxidizing conditions. They demonstrate the protective effect possible with the antioxidants and their ability to change the range of pigments generated along with the corresponding pigment precursors. Further studies may now be built upon these results to assess these antioxidants in combination with sulfur dioxide and also under reduced oxygen supply and lower temperatures, whereby the conditions are more relevant to typical bottled wine storage conditions. Finally, although this work has concentrated on coloration and the associated phenolic compound reactions, the potential volatile degradation products of glutathione and their impact on wine quality also need to be considered.

■ ASSOCIATED CONTENT

Supporting Information. Supplementary Figure 1. This material is available free of charge via the Internet at http://pubs.acs.org.

■ AUTHOR INFORMATION

Corresponding Author

*Phone: +612-6933-4181. Fax: +612-6933-2107. E-mail: aclark@csu.edu.au.

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■ ABBREVIATIONS USED

LC-MS, high-performance liquid chromatography with mass spectrometry detection; UPLC, ultraperformance liquid chromatography with UV−vis detection; UV−vis, ultraviolet−visible.

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