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Impact of Glutathione on the Formation of Methylmethine- and Carboxymethine-Bridged (+)-Catechin Dimers in a Model Wine System

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ABSTRACT: This study was performed to assess the impact of glutathione on the reaction between (+)-catechin and carbonyl compounds in wine-related conditions. (+)-Catechin (0.50 mM) and either glyoxylic acid (0.25 mM) or acetaldehyde (0.25 mM) were added to a model wine system with 0.0, 0.25, and 2.5 mM of glutathione added. UPLC-DAD and LC-MS analysis showed that the formation of carbonyl-bridged (+)-catechin dimers was inhibited in the samples with a glutathione to carbonyl ratio of 10:1 compared to the samples without glutathione. At a ratio of 1:1, glutathione inhibited the acetaldehyde-bridged dimers but only had a minor impact on the glyoxylic acid-bridged dimers. Further investigations showed that this trend of inhibition by glutathione on the glyoxylic acid-derived dimer was independent of temperatures, 20 °C vs 45 °C, or the presence of metal ions, 0.2 mg/L copper(II) and 5 mg/L iron(II). ^{1}H NMR analysis and LC-MS analysis provided evidence that glutathione inhibited dimer formation via different mechanisms depending on the carbonyl compound. For acetaldehyde-derived dimers, the main mode of inhibition was the ability of glutathione to form a (methyl-glutathionyl-methine)-(+)-catechin complex. Alternatively, the formation of a glutathione-glyoxylic acid addition product impeded the reaction between glyoxylic acid with (+)-catechin. These results demonstrate that glutathione, at sufficient concentration, can have a substantial impact on carbonyl-derived polymerization reactions in wine-like conditions.

KEYWORDS: Browning, wine, (+)-catechin, xanthylium cation, glutathione, glyoxylic acid

INTRODUCTION

In wines, and/or model wine systems, the carbonyl compounds acetaldehyde and glyoxylic acid can induce color changes by initiating the polymerization of grape skin or seed-derived flavonoid compounds.[1−5] Acetaldehyde may be present in wine after primary fermentation as a residual yeast metabolite, while both acetaldehyde and glyoxylic acid can be formed if wine encounters sufficient oxygen. In this latter case, acetaldehyde is formed from the oxidation of ethanol,[6] while glyoxylic acid is formed from the oxidative cleavage of tartaric acid, a common organic acid found in wine.[7] The rate of glyoxylic acid production from tartaric acid can be accelerated if the wine has both exposure to sunlight and oxygen.[7]

The carbonyl compounds have marked reactivity toward flavonoid compounds, such as the flavanol (+)-catechin, due to the nucleophilic character of the phloroglucinol-type moiety in flavanol compounds (Figure 1) and the electrophilic character of the aldehyde group, especially at wine-related pH values. In the case of (+)-catechin, the resulting reaction produces either a methylmethine-bridged (+)-catechin dimer from acetaldehyde[4] or a carboxymethine-bridged (+)-catechin dimer from glyoxylic acid[2] (Figure 1). The site of attachment between each (+)-catechin unit can be at either carbon-6 or carbon-8, which results in four possible bridged isomers (i.e., 8−8, 6−8, 8−6, or 6−6), of which the 8−8 dimer has the highest yield.[8,9]

Provided sufficient acetaldehyde and (+)-catechin are present, the methylmethine-bridged (+)-catechin dimer can undergo further reaction to generate larger polymers (Figure 1), which can form colloids and precipitate if they are of sufficient size.[10] They can also undergo depolymerisation with wine aging[11] and have been proposed to result in the formation of reactive vinylflavanol products[11,12] (Figure 1). In red wine, the participation of anthocyanins in the bridging reactions of acetaldehyde leads to the production of anthocyanin−flavanol copolymers that provide different colors to the parent anthocyanin (i.e., violet vs red).[5] Furthermore, the polymeric pigments are more resistant to the loss of their chromophore by reaction with water or hydrogen sulfite.[13]

The carboxymethine-bridged (+)-catechin dimer (Figure 1) can also form larger polymers if sufficient glyoxylic acid is present[9] (Figure 1), but lower concentrations of glyoxylic acid favor dehydration of the dimer to form a xanthene, before a final oxidation step to generate yellow xanthylium cation pigments[14] (Figure 1). These xanthylium cations have been identified in oxidized red and white wines[3,15] and can react further with nonflavonoid phenolic compounds, derived from the pulp of the

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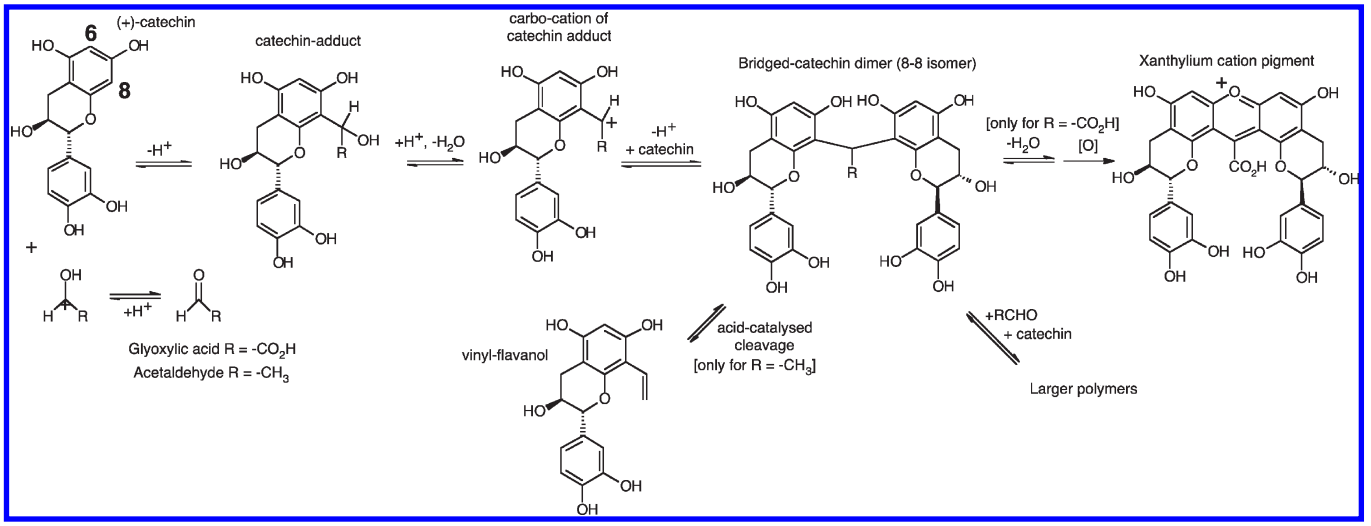

Figure 1. Reaction of acetaldehyde or glyoxylic acid with catechin.

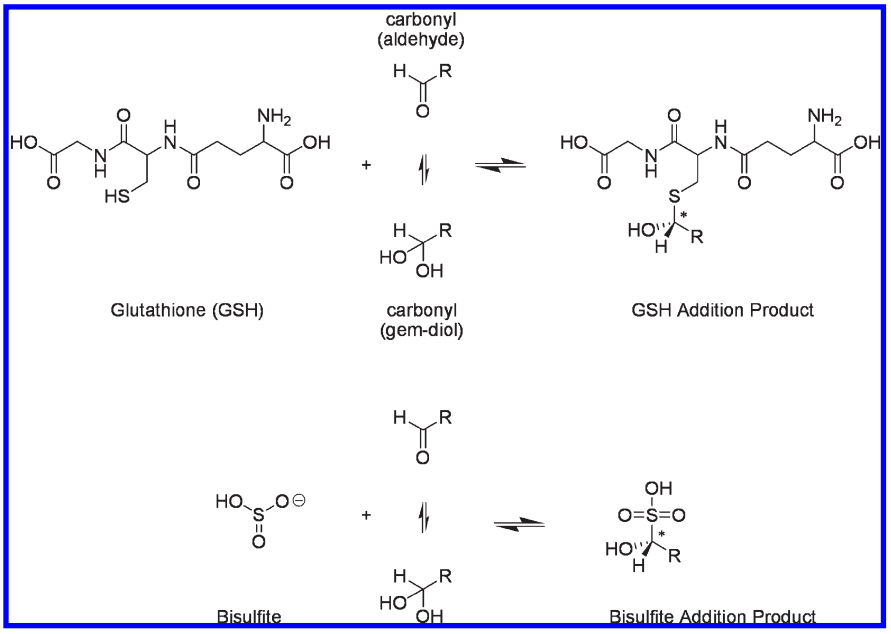

Figure 2. Addition products between bisulfite and glutathione with carbonyl compounds. For acetaldehyde, R = $-CH_3$ and for glyoxylic acid, R = $-CO_2H$ or $-CO_2^-$.

grape, to form other brown pigments.[16] An equivalent xanthylium cation derived from acetaldehyde has not been reported.

Sulfur dioxide (SO_2) is utilized in oenology to limit the detrimental impact of any oxygen ingress into the wine. Its main function in this role is to efficiently scavenge hydrogen peroxide and *ortho*-quinone compounds,[17] but it also forms addition products with carbonyl compounds, particularly acetaldehyde (Figure 2).[18] Indeed, virtually no free acetaldehyde will remain in solution provided some free sulfur dioxide (i.e., not bound) is present. However, as sulfur dioxide can induce allergic reactions in certain consumers there are obvious incentives to lower the concentrations of this preservative in wine.[19,20]

As a consequence, recent studies have shown the ability of glutathione, in combination with caffeic acid and/or with sulfur dioxide at lower levels than normally adopted, to inhibit the loss of desirable aroma compounds in white wines and model wines.[21] Similarly, glutathione was observed by Ugliano et al.[22] to protect 3-mercaptohexanol, which contributes beneficial tropical aromas, during the bottle aging of Sauvignon blanc wine. However, not all aspects of glutathione usage in wine were found to be particularly beneficial. Ugliano et al.[22] also highlighted that in particularly low oxygen conditions and/or with elevated copper(II) concentrations, glutathione also induced hydrogen sulfide production (i.e., off-odors) during bottle aging of Sauvignon Blanc. Patel et al.[23] showed that prefermentation additions of glutathione resulted in lower concentrations of varietal related thiols in finished Sauvignon blanc wines. Finally, the glutathione moiety of glutathionyl-caftaric acid has been shown to undergo gradual hydrolysis during the aging of wine.[24] Therefore, it is reasonable to assume that similar hydrolysis may occur to the free form of glutathione during wine aging.

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In comparing the known antioxidant action between glutathione and sulfur dioxide, it is evident that glutathione can certainly undergo addition reactions with oxidized *ortho*-quinone compounds, converting them back to their reduced and less reactive phenolic forms, albeit with a substituted glutathionyl group attached.[25] This reaction is known to occur during the enzymatic oxidation of phenolic compounds, particularly caftaric acid, when grapes are first crushed,[25] and also during the nonenzymatic oxidation, which is more prevalent after fermentation.[26,27] Not surprisingly, it is in wines whose must or juice has been protected from oxygen during processing, thereby limiting the production of *ortho*-quinone compounds, that the highest levels of glutathione are available in their corresponding wines.[28]

Less certain is the ability of glutathione to fulfill the remaining antioxidant roles of sulfur dioxide in wine conditions: to bind to aldehyde compounds (Figure 2) and its efficiency in scavenging hydrogen peroxide. In physiological conditions, it is known that acetaldehyde and glutathione do not readily react,[29] while glyoxylic acid and glutathione do form an addition product.[30] Our past work[27] in highly oxidizing model wine systems, demonstrated that glutathione, while present at sufficient concentrations, could delay the formation of carboxymethine-bridged (+)-catechin dimers formed in the model wine system. However, it was not certain whether this delay was due to a glutathione−glyoxylic acid interaction or rather some other antioxdant action of glutathione (e.g., radical or hydrogen peroxide scavenging) that prevented glyoxylic acid formation.

This study was undertaken to investigate the potential for glutathione to inhibit the production of bridged-(+)-catechin dimers, formed from the reaction of (+)-catechin with acetaldehyde or glyoxylic acid in a model wine system. The impact of glutathione concentration on the production of the dimers was monitored by UV/visible spectroscopy, UPLC-PDA, and LC-MS, while the interaction of glutathione with the carbonyl compounds (Figure 2) was determined by ^{1}H NMR and LC-MS.

MATERIALS AND METHODS

Reagents and Apparatus. All glassware and plasticware were soaked for at least 16 h in 10% nitric acid (BDH, AnalaR) and then rinsed with copious amounts of grade 1 water (ISO 3696). Solutions and dilutions were prepared using grade 1 water. (+)-Catechin monohydrate (98%), potassium hydrogen tartrate (>99%), L-(+)-tartaric acid (>99.5%), glutathione (>98%), glyoxylic acid (98%), sodium metabisulfite (99%), and copper(II) sulfate pentahydrate (98%) were purchased from Sigma-Aldrich (USA). Iron(II) sulfate heptahydrate (>98%) was purchased from Ajax Fine Chemicals (Australia). Ethanol (AR grade, >99.5%) and methanol (AR grade, >99.9%) were purchased from Ajax Fine Chemicals (Australia) and Mallinckrodt (USA), respectively. A stock solution of acetaldehyde was prepared by diluting the concentrated acetaldehyde (Sigma-Aldrich, Switzerland) 100-fold with a 12% aqueous ethanol solution buffered to pH 3.20 with tartaric acid (as described below). The stock solution was standardized via a second 100-fold dilution followed by the addition of excess sulfur dioxide (2.5 mM) and a resulting determination of the bound sulfur dioxide using the FIAstar 5000 FSO_2 and TSO_2 analyzer (FOSS, Sweden). The stock solution was found to be 0.1442 ± 0.0008 M acetaldehyde (n = 4, 95% confidence limit).

Absorbance measurements and spectra were recorded on a μQuant Universal Microplate Spectrophotometer (Biotek Instruments, New York, USA) with the software KC4 v3.0 (Biotek Instruments), using the wine-like solution as the blank solution.

Liquid chromatography for samples with (+)-catechin were conducted with an Ultra Performance Liquid Chromatography (UPLC) system consisting of a Waters Acquity binary solvent manager connected to a sample manager and a PDA detector all run by Empower^2 chromatography manager software. The column was a Waters Acquity BEH C18 (2.1 × 50 mm) with 1.7 μm particle diameter. Injection volume was 7.5 μL, and the elution gradient consisted of solvent A, 0.5% acetic acid in water, and B, 0.5% acetic acid in methanol, as follows (expressed in solvent A): 100% at 0 min, 100% at 1 min, 95% at 1.31 min, 62% at 5.25 min, 56% at 6.27 min, 48% at 6.34 min, 45% at 7.22 min, 0% at 8.85 min, 0% at 9.85 min, 100% at 10.19 min, and 100% at 11.20 min. The flow rate was 0.45 mL/min. Chromatograms and UV/visible spectra were recorded over the range from 200 to 500 nm.

Liquid chromatography−mass spectrometry (LC-MS) studies were conducted on an Agilent 1200 series Triple Quadrupole (6410) HPLC-MS. The column and LC conditions were as described for the UPLC (above), except for an injection volume of 20 μL. The MS was operated with the drying gas temperature at 350 °C, gas flow of 9 L/min, nebulizer pressure at 40 psi, and capillary voltage at 4 kV. MS analyses were carried out in the positive and negative ion modes with the fragmentor at both 80 and 150 V, the former providing parent ion signals and the latter inducing fragmentation.

^{1}H NMR spectra were acquired using a Varian INOVA spectrometer operating at 399.75 MHz, using buffered D_2O containing 11 mM potassium hydrogen tartrate and 8 mM L(+)-tartaric acid as the solvent.

Reactions. The wine-like solution was prepared by adding 0.011 M potassium hydrogen tartrate and 0.008 M tartaric acid to aqueous ethanol (12% v/v, 2 L) and stirring overnight at room temperature. The pH of the wine-like solution was 3.2 ± 0.1. To this solution was added 0.5 mM (+)-catechin and either acetaldehyde (0.25 mM) or glyoxylic acid (0.25 mM). Glutathione was added at concentrations of 0, 0.25, and 2.5 mM to afford ratios of the glutathione to carbonyl compounds of 0:1, 1:1, and 10:1. All samples were prepared in triplicate. The samples (65 mL) were placed in 50 mL Schott Duran reagent bottles, with an actual full capacity of 69 mL, so as to minimize the headspace volume (~4 mL) and thereby limit the amount of volatile acetaldehyde that may reside in the headspace. The samples were stored at 20 °C in darkness for 20 days and only opened for sampling.

Further experiments were conducted to induce the formation of xanthylium cation pigments from the glyoxylic acid/(+)-catechin reaction system, which involved addition of either metal ions to the reaction system or storage at elevated temperature. All these samples (100 mL) were placed in 250 mL Schott Duran reagent bottles with a headspace of 220 mL to ensure sufficient oxygen availability to enhance production of the xanthylium cation pigments. In the case of the glyoxylic acid/(+)-catechin samples stored at 45 °C, they were prepared as described above, and during storage (in darkness), they were aerated twice a day by rapid stirring while uncovered for 5 min. In the case of the samples with added metal ions, the glyoxylic acid/(+)-catechin samples were prepared as described above, but 0.2 mg/L copper(II) and 5 mg/L iron(II) was added to all samples. These metal concentrations are within the respective ranges that have been reported in wine.[31] The samples were stored at 20 °C in darkness and aerated twice a day by rapid stirring while uncovered for 5 min.

Identification of Products. The methylmethine- and carboxymethine-bridged (+)-catechin dimers were identified on the basis of a comparison of their UV/vis spectra and LC-MS data to that reported previously.[32] This included UV/vis absorbance maxima at 279 nm for both dimers, and signals at 605 m/z (negative ion mode) and 607 m/z (positive ion mode) for the methylmethine-bridged (+)-catechin dimer, and 635 m/z (negative ion mode) and 637 m/z (postive ion mode) for the carboxymethine-bridged (+)-catechin dimer. The xanthylium cation pigments (617 m/z) were identified by matching their retention

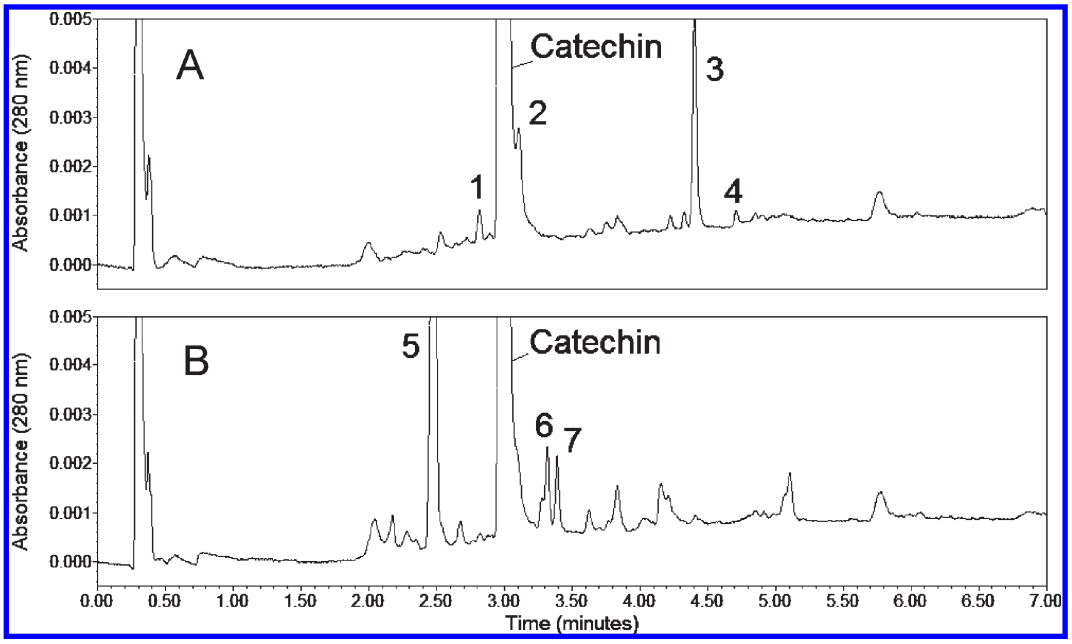

Figure 3. Two hundred eighty nanometer chromatograms at day-20 for the 0.50 mM (+)-catechin and 0.25 mM glutathione samples with either 0.25 mM acetaldehyde (A) or 0.25 mM glyoxylic acid (B). The peak assignments are peaks 1 and 2, glutathionyl-(+)-catechin isomers; peak 3, (methyl-glutathionyl-methine)-(+)-catechin; peak 4, methylmethine-bridged (+)-catechin dimer; and peak 5−7, carboxymethine-bridged (+)-catechin dimers.

time, UV/vis spectra, and mass data (positive ion mode) with the values quoted in ref 33.

Assignment of the glutathionyl-(+)-catechin products was achieved by a comparison of their retention time, UV/vis spectra, and LC-MS data to the equivalent addition products formed enzymatically. The enzymatic preparation was based on the method of Moridani et al.[34] The glutathionyl-(+)-catechin addition products showed two isomers with parent ion signals at 594 *m/z*, both with fragment ions indicative of cleavage around sulfur (i.e., 321 *m/z* for (+)-catechin sulfide, and 272 *m/z* for [(glutathione-HS)-2H$^+$]$^-$) and absorbance maxima at 290 nm.

Determination of Apparent Equilibrium Constants by ^{1}H NMR. A stock solution of buffered D_2O (20 mL) containing 0.011 M potassium hydrogen tartrate and 0.008 M L-(+)-tartaric acid was prepared by the addition of 41.4 mg and 24.0 mg of the respective solids, which was sealed and stirred overnight at room temperature to dissolve. The resulting pH of this wine-like solution measured using a pH meter was 3.0 ± 0.1. A portion (ca. 1 mL) of this solution was used to prepare a 20 mM solution of gluthathione as a reference to assist with assignment of the ^{1}H NMR signals, and the remainder was used in the preparation of samples for equilibrium constants determinations.

For glyoxylic acid, 11.22 mg of glyoxylic acid monohydrate was dissolved in 5.5 mL of buffered D_2O solvent to yield a concentration of 22 mM, and this solution was then split into 4 × 1 mL samples, to each of which was titrated a portion of concentrated glutathione (GSH) solution (~1.2 M) in the same solvent. These samples were diluted to a constant volume of 1.1 mL yielding final concentrations of 20 mM glyoxylic acid and GSH concentrations of 0, 10, 20, and 100 mM. For acetaldehyde, an initial solution at 1.797 M was prepared by pipetting 112 μL of acetaldehyde into 1 mL of buffered D_2O solvent. Then 67.4 μL of this solution was subsequently diluted into 5.5 mL of buffered D_2O solvent to yield a concentration of 22 mM, and this solution was split into 4 × 1 mL samples as before, to each of which was titrated a portion of concentrated glutathione (GSH) solution (~1.2 M) in the same solvent. These samples were similarly diluted to a constant volume of 1.1 mL yielding final concentrations of 20 mM acetaldehyde and GSH concentrations of 0, 10, 20, and 100 mM. All samples were sealed immediately after preparation, and equilibrated overnight at room temperature, then transferred to standard 5 mm NMR tubes immediately prior to measurements. Post-acquisition data processing and integration of the relevant ^{1}H NMR peaks was performed using the MestReC 4.8.6.0 software package.

For glyoxylic acid, the integral of the free glyoxylic acid methine resonance which appeared as a sharp singlet at ca. 5.12 ppm compared to the total integral region between 5.05 to 5.25 ppm upon titration with GSH was used to calculate the equilibrium concentrations of free glyoxylic acid and glyoxylic acid bound to GSH. For acetaldehyde, the integral of a new methyl resonance at ca. 1.34 ppm which appeared upon titration with GSH compared to the free acetaldehyde methyl resonances (doublets at ca. 1.2 ppm and ca. 2.10 ppm) was used to calculate the equilibrium concentrations.

Apparent equilibrium constants, K_{app}, were then evaluated as the product of the equilibrium concentrations of GSH and the remaining free carbonyl compound divided by the bound form of the relevant carbonyl compound (i.e., K_{app} = [GSH][R-CHO]/[R-CH(OH)-GSH] where R = $-CH_3$ or $-COOH$ for acetaldehyde or glyoxylic acid, respectively). The apparent equilibrium constants were calculated for each carbonyl to glutathione ratio and then averaged.

RESULTS AND DISCUSSION

The reaction of (+)-catechin (0.5 mM) with either acetaldehyde (0.25 mM) or glyoxylic acid (0.25 mM) was conducted at a (+)-catechin to carbonyl ratio of 1:0.5 consistent with the stoichiometry of these reagents in the bridged-(+)-catechin dimers. This ratio would also limit the production of larger polymer chains observed in studies utilizing the carbonyl compound in excess of (+)-catechin.[8] Glutathione was then added at 0.0, 0.25, and 2.50 mM to achieve ratios of 0:1, 1:1, and 10:1 of glutathione to carbonyl compound. The 1:1 ratio was chosen to mimic the lower range concentration of total acetaldehyde (i.e., free and sulfur dioxide bound forms) in red and white wines (4−11 mg/L)[35] compared to the higher range of glutathione

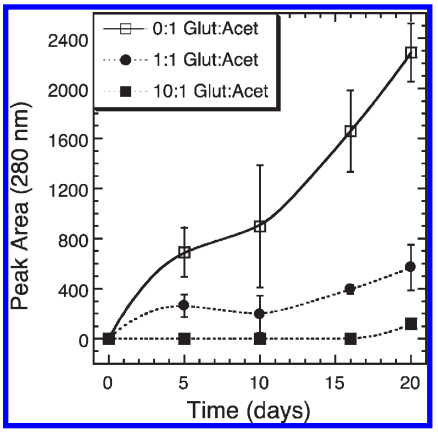

Figure 4. Production of the methylmethine-linked (+)-catechin dimer during the storage of samples at 20 °C.

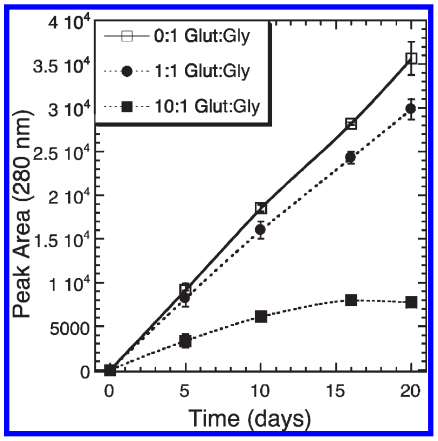

Figure 5. Production of the carboxymethine-linked (+)-catechin dimer during the storage of samples at 20 °C.

concentration found in red and white wines[28,36] (e.g., 30–40 mg/L). Glutathione concentrations of 10 mg/L, or less, would be more typical of most wines at bottling.[22,23] The 10:1 ratio was adopted to assess the glutathione in molar excess of acetaldehyde, a situation that would invariably occur in wines where much of the carbonyl compound was bound by sulfur dioxide. Given the lack of reported data for glyoxylic acid in wine, it was utilized at the same concentration as acetaldehyde. The ratios adopted would provide some insight into the efficiency of glutathione for the inhibition of the bridged-(+)-catechin formation.

Once prepared, all solutions were essentially colorless with absorbance values of less than 0.003 at 440 nm (A440), a wavelength indicative of the formation of yellow color in the model wine samples.[37] After storage at 20 °C for 20 days, no sample exceed an A440 value of 0.005 and consequently had little development of color, as assessed visually, and also had no haze formation.

Figure 3 shows the 280 nm chromatograms of the samples after 20 days. Peak 4 (Figure 3A) corresponded to the methylmethine-bridged (+)-catechin dimer (from acetaldehyde), while peak 5 (Figure 3B) corresponded to the carboxymethine-bridged (+)-catechin dimer (from glyoxylic acid). Other isomers of the dimers were detected, for example, peaks 6 and 7 for the carboxymethine-bridged (+)-catechin dimer, but as expected their intensity was much lower than that of the major isomer. On the basis of previous work,[8,9] the major isomers are known to be the two (+)-catechin units bridged together via carbon-8 on catechin. Also, evident in the chromatograms were the presence of peaks (1 and 2, Figure 3) corresponding to glutathionyl-catechin addition products known to be formed from the oxidation of catechin to its *ortho*-quinone and subsequent reaction of the *ortho*-quinone with glutathione.[34] The production of the glutathionyl-catechin addition products was only minor given that the samples contained no added metal ions required to efficiently catalyze the oxidation of catechin.[31]

For the samples containing acetaldehyde, after the 20 day reaction period the (+)-catechin concentration was 96 ± 1%, 95 ± 1%, and 95 ± 1% the initial concentration (i.e., 0.5 mM) in the 0, 0.25 mM, and 2.50 mM glutathione samples, respectively. For the samples containing glyoxylic acid, after 20 days the (+)-catechin concentration was 92 ± 1%, 91.7 ± 0.3%, and 92.2 ± 0.5% the initial concentration (i.e., 0.5 mM) in the 0, 0.25 mM, and 2.50 mM glutathione samples, respectively. Therefore, there was no significant effect ($p = 0.05$) of glutathione on the loss of (+)-catechin for a given carbonyl compound. This lack of impact of glutathione on catechin loss was most likely a consequence of the relatively minor amounts of catechin consumed throughout the experiment, as well as the presence of competing reactions for catechin (described below). The increased loss of (+)-catechin in the samples with glyoxylic acid compared to acetaldehyde was consistent with glyoxylic acid being more reactive with (+)-catechin than acetaldehyde as determined previously.[8,32]

Figures 4 and 5 show the formation of the major isomers of the bridged-(+)-catechin dimers based upon their detection by UPLC (Figure 3). From the data in Figure 4, it is evident that accumulation of the methylmethine-bridged (+)-catechin dimer is inhibited by the presence of glutathione regardless of the concentration of the thiol. However, there was significantly greater inhibition ($p = 0.05$) at the higher glutathione concentration. In the sample without glutathione, the bridged-(+)-catechin dimer increased in concentration throughout the experiment. The LC-MS data showed no evidence for the presence of bridged-(+)-catechin trimers, oligomers, or vinyl (+)-catechin (Figure 1), indicating that further reaction of the dimer was not occurring under the conditions of this experiment.

A large peak (peak 3, Figure 3A) was detected in the 280 nm chromatograms of the acetaldehyde samples that contained glutathione. Given that it was possibly related to the inhibitory effect of glutathione on (+)-catechin dimer formation, it was investigated further. LC-MS analysis showed that the parent ion corresponded to m/z values of 622 and 624 in the negative and positive ion modes, and exhibited fragment ions at 315 and 306 m/z in the negative ion modes and 317 and 308 m/z in the positive ion mode. Such data were consistent with the formation of the structure shown in Figure 6A with glutathione bonded to the methylmethine-moiety of a substituted (+)-catechin, and the fragmentation can be explained by cleavage between the sulfur and methine bond. Formation of this compound could occur from glutathione reacting with an intermediate compound in the production of the dimer, and the most likely candidate would be the carbo-cation of the (+)-catechin adduct (Figure 1). An alternative mechanism would be the reaction of glutathione with a degradation product of the methylmethine-bridged (+)-catechin dimer; however, there was no evidence that these were forming under the conditions adopted for this experiment (i.e., no detection of vinyl catechin). In both glutathione samples

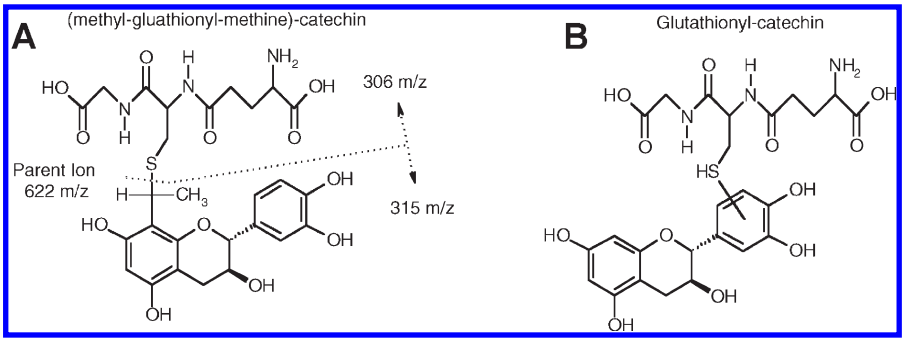

Figure 6. Proposed structure (A) of the compound responsible for peak 3 in Figure 3A and the gluathionyl-(+)-catechin product (B).

Table 1. Peak Areas ($\times 10^3$) for the Carboxymethine-Bridged (+)-Catechin Dimer (at 280 nm) and the Xanthylium Cation Pigments (at 440 nm) in the Glyoxylic Acid and (+)-Catechin Samples[a]

	20 °C[b]	20 °C + metal ions[b]	45 °C[c]
ratio of glyoxylic acid to glutathione	carboxymethine-bridged (+)-catechin dimer		
1:0	36 ± 2	246 ± 4	20 ± 4
1:1	30 ± 1	160 ± 20	15 ± 2
1:10	7.7 ± 0.4	44 ± 3	6 ± 2
ratio of glyoxylic acid to glutathione	xanthylium cation		
1:0	0.4 ± 0.1	33 ± 5	200 ± 20
1:1	0.5 ± 0.2	22.2 ± 0.8	150 ± 40
1:10	not detected	3 ± 2	7 ± 1

[a] The values quoted are the average peak areas with 95% confidence limits. [b] Measured after 20 days. [c] Measured after 12 days.

(i.e., 1:1 and 10:1), peak 3 increased in a linear manner from days 0 to 20 (data not shown), but the sample with higher glutathione had the most intense (280 nm) peak at day 20 (i.e., 14.3 ± 0.7 ($\times 10^3$) vs 17 ± 1 ($\times 10^3$) absorbance units). However, the data indicate that even the lower concentration of glutathione allows efficient production of the compound responsible for peak 3.

The glutathionyl-catechin addition product (peaks 1 and 2, Figure 3 and Figure 6B) was observed in the all samples with added glutathione. It was anticipated, on the basis of the reported formation of this compound from the reaction of glutathione with the (+)-catechin *ortho*-quinone,[34] that by following glutathionyl-(+)-catechin production, some insight into glutathione availability in the samples would be gained. Glutathionyl-(+)-catechin products were detected in both the 0.25 mM and 2.50 mM glutathione samples with peak areas (280 nm) of 2.8 ± 0.3 ($\times 10^3$) and 11.2 ± 0.5 ($\times 10^3$) absorbance units, respectively, at day 20. These products had increased in a linear manner up to day 20 (data not shown). This meant that despite the potential for glutathione to form an addition product with acetaldehyde, there was still sufficient free glutathione in the 0.25 mM and 2.50 mM glutathione samples to scavenge *ortho*-quinone compounds before their reaction with other components of the model wine system. For instance, the (+)-catechin *ortho*-quinone is known to react with the phloroglucinol ring of another (+)-catechin unit to generate a (+)-catechin dimer.[38] No products were detected by LC-MS under the conditions of this experiment that would have been consistent with hydrolysis of the glutathione-moiety in glutathionyl-(+)-catechin.

In the reaction between glyoxylic acid and catechin, the data in Figure 5 show that accumulation of the carboxymethine-bridged (+)-catechin dimer is inhibited most by the presence of 2.50 mM glutathione and only marginally by 0.25 mM glutathione. The carboxymethine-bridged (+)-catechin dimer increased in a linear manner, from days 0 to 20, in the 0 and 0.25 mM glutathione samples, and there was little production of the xanthylium cation (Table 1) as supported by the lack of color in the samples by the end of the experiment (i.e., A440 < 0.005). Unlike the presence of peak 3 in samples with both glutathione and acetaldehyde (Figure 3A), there appeared to be no dominant product peak/s in the samples with both glutathione and glyoxylic acid (besides the bridged-(+)-catechin dimer in the 0.25 glutathione sample) (peaks 5 and 7, Figure 3B). LC-MS analysis showed no clear evidence for a glyoxylic acid product equivalent to that in Figure 6A besides some small signals in the 363 *m/z* (negative ion mode) ion chromatogram contributing to the peaks just prior to peak 5 (Figure 3B). This suggested that the intermediates in the production of the carboxymethine-linked (+)-catechin dimer were less reactive to glutathione than was the case for the (+)-catechin/acetaldehyde system. Indeed, Drinkine et al.[8] provided kinetic evidence that supports the increased reactivity of the acetaldehyde-derived (+)-catechin adduct (Figure 1) compared to that of the glyoxylic acid-derived (+)-catechin adduct, which is consistent with the results above.

The formation of the glutathionyl-(+)-catechin products (Figure 6B) were also investigated in the (+)-catechin and glyoxylic acid samples. At day 20 the glutathionyl-(+)-catechin isomers were only detected at trace levels in the 0.25 mM glutathione sample (peak area of 300 ± 30) but were much larger in the 2.50 mM glutathione sample (peak area of 3800 ± 80). As these values were much lower than those found in the acetaldehyde/(+)-catechin samples, it suggested that glutathione was less available to scavenge *ortho*-quinone compounds in the glyoxylic acid/(+)-catechin system, and therefore, glutathione was more likely bound to glyoxylic acid in this system.

As metal ions and higher temperatures are known to accelerate the reactions between (+)-catechin and glyoxylic acid,[32] further experiments were conducted to induce the formation of the yellow xanthylium cations and to assess if the inhibitory action of glutathione was still evident under these conditions. The results from Table 1 show that during storage for 20 days at 20 °C and in the presence of metal ions, or during storage for 12 days at 45 °C with no added metal ions, the same trend is observed for inhibition of the carboxymethine-linked (+)-catechin dimer by

Table 2. Apparent Equilibrium Constants for the Reactions Shown in Figure 2[a]

	glutathione		hydrogen sulfite
	measured K_{app}	literature K_{app}	literature K_{app}
acetaldehyde	$(5 \pm 1) \times 10^{-2}$ (pH 3.0)	8.3×10^{-2} (pH 4–5)[39]	1.5×10^{-6} (pH 3.0) [40] 3.7×10^{-6} (pH 4.3) [41]
glyoxylic acid	$(1.1 \pm 0.1) \times 10^{-3}$ (pH 3.0)	1.5×10^{-3} (pH 7)[30]	3.7×10^{-6} (pH 3.2) [42]

[a] The measured values are the average calculated from the data in Figure 9, and the error represents the standard deviation. The pH utilized for measurements/literature values are in parentheses, and the error limits for the measured values are the SD.

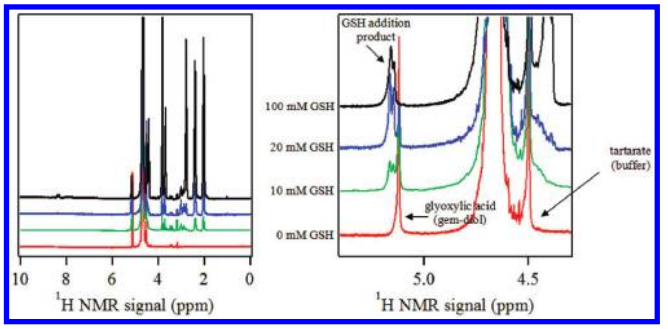

Figure 7. Observed ^{1}H NMR spectra of glyoxylic acid in tartrate buffered D_2O (pH ~3.0) upon increasing additions of GSH (left) and expansion of methine region (right).

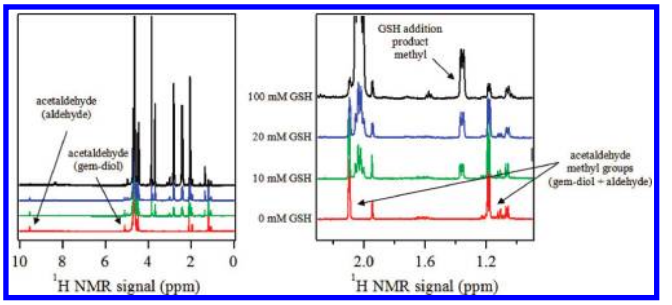

Figure 8. Observed ^{1}H NMR spectra of acetaldehyde in tartrate buffered D_2O (pH ~3.0) upon increasing additions of GSH (left) and expansion of the methyl group region (right).

glutathione, that is, a slight inhibition at 0.25 mM glutathione and a large inhibition at the higher glutathione concentrations. As expected,[32] the xanthylium cation pigments were also generated at much higher concentrations in the experiments with metal ions or stored at 45 °C (Table 1). For the xanthylium cation pigments, again the same trend of glutathione inhibition is evident (Table 1).

To understand further the ability of glutathione to inhibit the formation of the bridged-(+)-catechin dimers, the interaction between glutathione and the carbonyl compounds was investigated. Table 2 shows the apparent equilibrium constants taken from published sources which are relevant to the equilibria shown in Figure 2. The bisulfite binding to the carbonyl compounds was included for comparison. The literature data in Table 2 show that the apparent equilibrium constants are lower for hydrogen sulfite binding to the carbonyl compounds, indicating stronger binding, compared to that for glutathione. It also shows that glutathione appears to bind more strongly with glyoxylic acid than acetaldehyde. However, these literature data utilize pH conditions that are higher than those found in wine, particularly for the glutathione/glyoxylic acid combination at pH 7. Indeed, the comparison of apparent equilibrium constants determined at different pH values is not always appropriate given that different proportions of ionized glyoxylic acid and glutathione may impact the respective equilibria.

As such, ^{1}H NMR studies were conducted to assess the apparent equilibrium constants for the binding of glutathione to the carbonyl compounds at a pH relevant to wine. The formation of the glutathione addition products as shown in Figure 2 upon the addition of increasing glutathione concentrations was confirmed by ^{1}H NMR analysis.

Analysis of the ^{1}H NMR spectra showed that the equilibrium position for free glyoxylic acid at ca. pH 3.0 exists exclusively toward the gem-diol form, with a sharp singlet methine CH resonance apparent at 5.12 ppm as shown in Figure 7, and no evidence of a corresponding aldehyde peak. Other than the residual HDO signal at 4.64 ppm, the only other peak, apparent as a sharp singlet at 4.49 ppm, was assigned to nonexchangeable CH protons of the tartrate buffer system by comparison to the spectra for the neat solvent. Upon incremental addition of GSH, the signal for the gem-diol proton of glyoxylic acid at ca. 5.12 ppm is diminished, and the growth of two new peaks at ca. 5.15 and 5.17 ppm are clearly evident in the spectra. We have assigned these two new peaks to the two isomeric addition products, as shown in Figure 2, which differ in the relative stereo configuration at the substituted carbon center. This assignment is corroborated by LC-MS analysis, which showed only a single addition product. Interestingly, the integrals of these two peaks are not equal, suggesting that one epimer is present in a slight excess, likely due to differences in steric interactions with the glutathione backbone. However, the experimental data available do not allow us to identify which isomer is preferred.

For experiments involving acetaldehyde, again, the residual HDO signal of the solvent appeared at ca. 4.64 ppm, with a second sharp singlet at 4.49 ppm due to the tartrate buffer system. In this case, however, the ^{1}H NMR spectrum (Figure 8) showed peaks attributable to both the keto form of acetaldehyde, with a singlet aldehyde proton signal at ca. 9.54 ppm, and the corresponding gem-diol form, which gave a methine proton signal at ca. 5.10 ppm, split into a quartet by the adjacent methyl group. The corresponding methyl resonances appear as doublets at ca. 2.10 and 1.2 ppm, respectively, for the aldehyde and gem-diol. Upon titration with GSH, a new doublet peak centered at ca. 1.36 ppm appeared, which we attribute to the formation of the GSH addition product (Figure 2), and a concomitant reduction in intensities for the free acetaldehyde methyl resonances was noted.

Last, also apparent in both spectra upon incremental addition of GSH are six other signals in the alkyl region (ca. 2.02 ppm, quartet, 2.42 ppm, multiplet, 2.81 ppm, multiplet, 3.68 ppm,

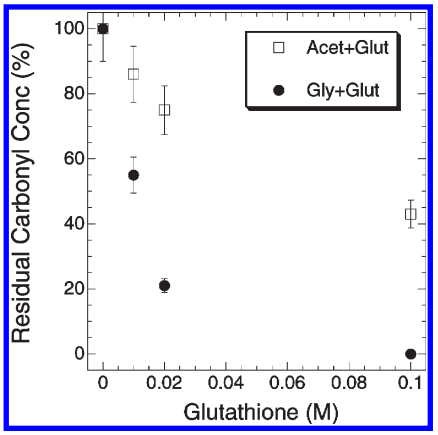

Figure 9. Impact of glutathione on carbonyl compound concentration. The initial carbonyl concentration was 0.02 M.

triplet, 3.83 ppm, singlet, and 4.38 ppm, multiplet). Using the ^{1}H NMR spectrum of GSH under identical conditions as a reference, we can assign these signals to the distal alkyl groups of both the bound and free forms of GSH.

Figure 9 shows the decrease in carbonyl compound signal with increasing glutathione. Consistent with the literature data presented in Table 2, glutathione is much more efficient at binding glyoxylic acid compared to acetaldehyde. The apparent equilibrium constants were calculated for each of the binding systems on the basis of the data in Figure 9 and are shown in Table 2. The fact that the calculated apparent equilibrium constants in Table 2 agree quite well with the literature values perhaps reflects the independence of the glutathione equilibria, presented in Figure 2, on pH.

The data in Table 2 are consistent with the inability of glutathione, at the lower glutathione to acetaldehyde ratio (i.e., 1:1), to prevent the initial reaction between acetaldehyde and catechin and instead support the role of glutathione as scavenging an intermediate in the production of the methylmethine-bridged (+)-catechin dimer. Alternatively, the data in Table 2 are consistent with the ability of glutathione to slow the reaction of glyoxylic acid with catechin, due to the increased stability of the glutathione/glyoxylic acid addition product. However, the ability of glutathione to inhibit the glyoxylic acid reaction with (+)-catechin is greatly enhanced once the ratio of glutathione to glyoxylic acid is large, and hence, the majority of the glyoxylic acid exists in the bound addition product form. This is especially the case with the experimental conditions adopted for the collection of the data in Table 1 as there are other competing reactions for glutathione that can lower its concentration relative to glyoxylic acid. For example, the oxidation product of (+)-catechin can more readily react with glutathione in the acetaldehyde system as it is more freely available than in the glyoxylic acid system.

On the basis of these results, it appears that glutathione is a promising candidate for the inhibition of bridged-(+)-catechin polymers provided that it is present at sufficient concentrations. The results of this research will become more relevant should glutathione become an approved additive to wines, as is currently being proposed in certain countries. It is also of importance to winemakers intending to induce polymerization reactions via microoxygenation to perhaps consider the presence of glutathione in the wine. The ability of glutathione to inhibit the acetaldehyde polymerization reaction and not the glyoxylic acid polymerization reaction at certain concentrations may provide a means of having some regulation over the preferred polymerization mechanism. Further research is required to assess the contribution of glutathione to prevent phenolic polymers when used in combination with sulfur dioxide and to further understand the impact of the acid-catalyzed hydrolysis of glutathione on phenolic polymerization. Finally, the fate and reactivity of glutathione products should be assessed in wine conditions.

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ABBREVIATIONS USED

UPLC-PDA, ultraperformance liquid chromatography with photodiode array detector; LC-MS, liquid chromatography mass spectrometry; UV, ultraviolet; CIE, International Commission on Illumination; ^{1}H NMR, proton nuclear magnetic resonance.

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