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A Novel Glutathione-Hydroxycinnamic Acid Product Generated in Oxidative Wine Conditions

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Supporting Information

ABSTRACT: This study characterizes a novel glutathione-substituted dihydroxyphenyl compound formed during the oxidation of white wine and model wine solutions, which may contribute to the synergistic role of glutathione and hydroxycinnamic acids in delaying oxidative coloration. The critical components for the formation of the compound were found to be hydroxycinnamic acids and glutathione, while ascorbic acid enabled the product to accumulate to higher concentrations. The presence of the wine components important in other wine oxidation mechanisms, (+)-catechin, ethanol and/or tartaric acid, was not essential for the formation of this new compound. Via LC-MS/MS, HR-MS and ^{1}H NMR (1D and 2D NMR) analyses, the major isomer of the compound formed from glutathione and caffeic acid was found to be 4-[(*E*)-2′-(*S*)-glutathionyl ethenyl]-catechol (GEC). Equivalent products were also confirmed via LC-MS/MS for other hydroxycinnamic acids (i.e., ferulic and coumaric acids). Only trace amounts of GEC were formed with the quinic ester of caffeic acid (i.e., chlorogenic acid), and no equivalent product was found for cinnamic acid. GEC was detected in a variety of white wines supplemented with glutathione and caffeic acid. A radical mechanism for the formation of the styrene-glutathione derivatives is proposed.

KEYWORDS: *ascorbic acid, glutathione, wine, oxidation, 4-[(E)-2′-(S)-glutathionyl ethenyl]-catechol, hydroxycinnamic acid, GEC*

INTRODUCTION

During wine production and storage, the control of wine oxidation is achieved mainly through the use of inert gases and sulfur dioxide additions. The latter has been well studied[1] and is known to protect wine by scavenging the hydrogen peroxide and/or reducing *o*-quinone compounds that are produced during wine oxidation. Furthermore, sulfur dioxide has the ability to form addition products with carbonyl compounds that would otherwise react to form detrimental pigments or aromas in wine.[2] However, sulfur dioxide has been shown to be allergenic for a small group of consumers,[3,4] and hence a market for wines of substantially lower sulfur dioxide concentrations is of interest to winemakers.

In this vein, and for general wine quality improvement, research has been conducted to investigate agents able to aid sulfur dioxide in its role to preserve wine against the effects of oxygen.[5−10] Roussis et al.[8] showed that caffeic acid and/or glutathione were able to limit the loss of several volatile aroma compounds in wines supplemented with lower concentrations of free sulfur dioxide (i.e., 35 mg/L vs 55 mg/L). Other studies have shown that ascorbic acid (vitamin C, 30−100 mg/L) and its epimer erythorbic acid (30−100 mg/L) are superior oxygen scavengers utilized in wine, compared to sulfur dioxide, but they are only effective provided that 'normal' levels of sulfur dioxide (i.e., ∼30 mg/L) are also present.[5−7,9] For these oxygen scavengers, sulfur dioxide is required to remove the hydrogen peroxide generated by their oxidation.

The combination of ascorbic acid and glutathione would appear to be chemically well placed to act as a beneficial oxygen scavenging system in wine. This is due to the ability of ascorbic acid to effectively scavenge oxygen and/or the oxidized form of the metal ion catalysts,[6] and glutathione being able to perform some of the roles of sulfur dioxide. That is, glutathione is well-known to react with oxidized phenolic compounds[11,12] and can form addition products with carbonyl compounds, including glyoxylic acid.[13] Additionally, in acidic aqueous systems, when compared to other thiol compounds, glutathione has been shown to slow the reaction of molecular oxygen with copper(I).[14−16]

Our recent research[17] has also demonstrated that in model wine solutions without sulfur dioxide present, the ascorbic acid/glutathione combination delayed the oxidative coloration of a model wine system until the ascorbic acid became depleted. Furthermore, this combination was found to be more efficient than either glutathione or ascorbic acid alone, with glutathione slowing the degradation of ascorbic acid. During this protective phase, an unknown product accumulated, but once ascorbic acid was depleted it rapidly decreased in concentration, at which point rapid coloration occurred. The formation of the unknown compound was shown to critically depend on the presence of both model wine components glutathione and caffeic acid, and its concentration was enhanced if ascorbic acid was present.

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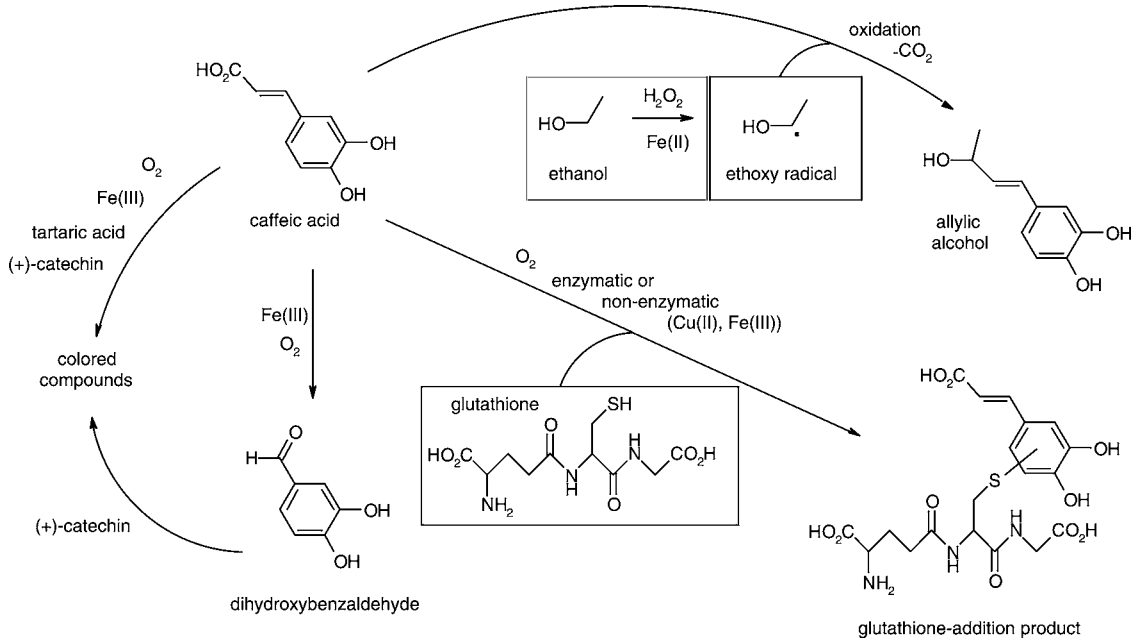

Figure 1. Oxidative mechanisms relevant to caffeic acid under wine or grape conditions.

In wine conditions where caffeic acid is present, various oxidation/condensation products have been observed. In the presence of glutathione, the oxidized form of caffeic acid (i.e., the *o*-quinone of caffeic acid) reacts with glutathione to regenerate caffeic acid with glutathione substituted on the catechol-moiety[17] (Figure 1). Such a reaction was first observed for the tartrate ester of caffeic acid (i.e., caftaric acid), which is the main hydroxycinnamate present in grapes.[18] Related products have also been shown to occur, albeit in acetonitrile solvent, after the attack of a cysteinyl-derivative radical on the semiquinone of methyl caffeate, without the need for *o*-quinone production.[19] In the absence of glutathione, two other wine oxidation mechanisms have been reported, which involve the degradation of caffeic acid as mediated by iron(II)/iron(III). For example, Gislason et al.[20] showed that ethoxy radicals could be generated by iron(II) and hydrogen peroxide, which subsequently attack caffeic acid to generate an allylic alcohol (Figure 1). Gislason et al.[20] proposed a carbon centered radical and a carbocation as intermediates in this mechanism and showed that the allylic alcohol could undergo acid-catalyzed dehydration and subsequent attack by nucleophiles. Alternatively, Lutter et al.[21] demonstrated the production of dihydroxybenzaldehyde from caffeic acid via iron(II)/iron(III) and oxygen, and showed that in the presence of (+)-catechin, a grape skin-derived phenolic compound, the benzaldehyde could react to generate red/pink pigments (Figure 1). When such pigments were formed in oxidizing tartrate-buffered model wine systems, which also produced yellow pigments from degradation of tartaric acid and reaction with (+)-catechin, the resulting wine systems appeared brown in color (Figure 1).[22] Other studies have shown the production of dimers and trimers of caffeic acid after oxidation but in matrices without ethanol present.[23−26]

The present study was conducted to identify the unknown compound stemming from the reaction involving glutathione and caffeic acid, and to investigate which of the wine components previously linked to oxidative mechanisms impacted the formation of the unknown compound. Furthermore, the relevance of this compound to wine hydroxycinnamic acids in general was examined and a mechanism for the production of the products is proposed. Finally, the production of these compounds in white wines was assessed.

MATERIALS AND METHODS

General. Water purified through a Milli-Q (Millipore) water system (ISO 9001) was used for all solution preparations and dilutions. L-Ascorbic acid (99%), L-(+)-tartaric acid (>99.5%), (+)-catechin hydrate (98%), caffeic acid (≥98%), chlorogenic acid (≥95%), cinnamic acid (≥95%), coumaric acid (≥98%), ferulic acid (≥99%) (all hydroxycinnamic acids in the *E*-form) and potassium bitartrate (99%) were purchased from Sigma-Aldrich. Deuterated dimethylsulfoxide (DMSO-d_6) (100%, 99.96 atom %D) and deuterium oxide (D_2O) (99.9 atom %D) NMR solvents were also purchased from Sigma-Aldrich. Iron(II) sulfate heptahydrate (99%) was purchased from LabServ and copper(II) sulfate pentahydrate (99−100.5%) from AnalaR. Ethanol (AR grade, >99.5%, Ajax Fine Chemicals), methanol (AR grade, >99.9%, Mallinckrodt Chemicals), glacial acetic acid (AR grade, >99.7%, APS Ajax Fine Chemicals), and formic acid (98%, Fluka) were used without further purification. Sulfuric acid (95−98%) was obtained from Univar.

Analytical liquid chromatography analyses were conducted on an ultra high performance liquid chromatography (UHPLC) system consisting of a Waters Acquity binary solvent manager connected to a sample manager and a diode array 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 operating conditions as per Sonni et al.[17] Liquid chromatography−mass spectrometry (LC-MS) studies were conducted on an Agilent 1200 series Triple Quadrapole (6410) HPLCMS with electrospray ionization (ESI) and run by Mass Hunter Workstation software. The column and LC elution gradient was as described for the UHPLC (above), except for an injection volume of 5 μL and 0.2% (v/v) formic acid replacing acetic acid as the solvent buffer. The MS was operated at 350 °C, gas flow of 9 L/min, nebulizer at 275.8 kPa, and capillary at 4 kV. LC-MS analyses for the samples were carried out in both negative and positive ionization modes with the fragmentor at 80 V and scanning performed between *m*/*z* 100−800. For LC-MS/MS analyses the fragmentor was at 80 V, the

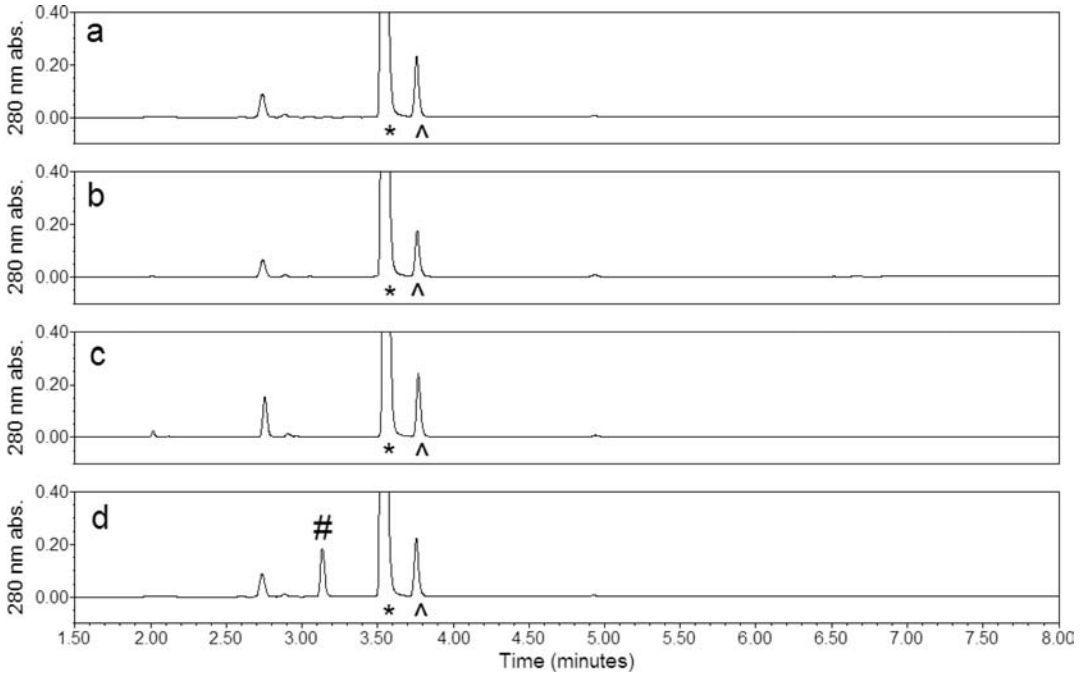

Figure 2. UHPLC chromatograms (280 nm) for model wine systems containing 2.8 mM glutathione, 1.1 mM caffeic acid and 2.8 mM ascorbic acid after 2 days at 45 °C. The model wine systems were all at pH 3.2 and contained (a) tartaric acid/ethanol, (b) formic acid/ethanol, (c) formic acid, or (d) tartaric acid/ethanol/(+)-catechin as outlined in the methods section. The concentrations were 0.02 M formic acid, 0.02 M tartaric acid, 2.0 M ethanol (12% (v/v)) and 0.17 mM (+)-catchin. '*' highlights caffeic acid, '#' highlights (+)-catechin, while '^' highlights the unknown compound (GEC).

collision energy at either 10 or 20 V, and product ion scans performed from m/z 100 to m/z 20 above the ion of interest.

Preparative LC separations were conducted on a Perkin-Elmer 250 binary LC-pump connected to a Varian 320 Pro Star UV−visible detector (280 nm) controlled by Varian Star (v 6.41) chromatography workstation. The column was a semipreparative 4 μm Phenomenex Synergy Hydro-RP C18 column (250 × 10 mm) held at room temperature. Injection volume was 2 mL, the flow rate 2 mL/min and the elution gradient consisted of solvent A: 2% (v/v) formic acid in water and B: 2% (v/v) formic acid in methanol, as follows (expressed in solvent A followed by cumulative time): 80% 0 min, 60% 10 min, 48% 12 min, 0% 15 min, 80% 18 min, 80% 28 min. Lyopholization was performed on a Christ-Alpha 2-4D freeze-dryer (Biotech International).

High resolution MS (HR-MS) data were obtained on a Waters LCT PremierXE ESI TOF mass spectrometer run by Mass Lynx software (4.1). The instrument was calibrated in the negative ionization mode with sodium formate in acetonitrile over 50−1000 amu. A Lockmass Solution (leucine enkephalin peptide $[M - H^+]^- = m/z$ 554.27) was infused as well. The samples were dissolved in methanol and infused at a flow rate of 150 μL/min in negative ionization mode and the mass range scanned was 50−1000 amu. Capillary exit voltage was 80 V, cone voltage 30 V, desolvation temperature was 150 °C and the source temperature 100 °C. Elemental Analysis was performed using Mass Lynx 4.1 Elemental Program.

^{1}H (800 MHz) and ^{13}C (200 MHz) NMR spectra were acquired in a (75:25) mixture of DMSO-d_6/D_2O on a Bruker Avance II 800 MHz spectrometer equipped with a TXI Cryoprobe with referencing to solvent signals as follows, DMSO-d_6 (δ 2.49 and 39.5). 1D and 2D NMR experiments included gradient-selected correlation spectroscopy (gCOSY), gradient-selected heteronuclear single quantum coherence (gHSQC), and gradient-selected heteronuclear multiple bond correlation (gHMBC).

Reactions in Model Wines and White Wines. Model wine systems were prepared with either tartrate or formate buffer in 12% (v/v) aqueous ethanol. The tartrate buffer consisted of 0.011 M potassium hydrogen tartrate and 0.008 M tartaric acid (pH 3.2), while the formate buffer consisted of 0.019 M formic acid, 0.011 M potassium hydroxide and acidified to pH 3.2 with 10% (v/v) sulfuric acid. An additional formic acid system was prepared as above but without any ethanol present. The concentration of ascorbic acid, hydroxycinnamic acids and glutathione were based on the study of Sonni et al.[17] in which the caffeic acid/glutathione product was first reported. Ascorbic acid was added at 2.8 mM, hydroxycinnamic acids at 1.1 mM and glutathione at 2.8 mM. The hydroxycinnamic acids utilized were caffeic acid, ferulic acid, coumaric acid, cinnamic acid, and an ester of caffeic acid was also utilized (i.e., quinic acid ester otherwise known as chlorogenic acid) to model the hydroxycinnamic esters found in wine (i.e., caftaric acid). Where indicated sulfur dioxide was added at a 1:1:1 ratio with ascorbic acid and glutathione, such that its concentration was 2.8 mM (180 mg/L). Where utilized, (+)-catechin was added at 50 mg/L as per Sonni et al.[17] Reactions were also conducted in two commercial Australian Riesling wines (pH 3.1, 11.8% (v/v) ethanol), one of which was aged (1992 vintage, 5 mg/L free sulfur dioxide), and another that was young (2011 vintage, 32 mg/L free sulfur dioxide). Both wines were supplemented with ascorbic acid (2.8 mM), caffeic acid (1.1 mM) and glutathione (2.8 mM). Experiments with the wines were conducted in both the presence and absence of added metal ions (i.e., 0.2 mg/L copper(II) and 5.0 mg/L iron(II)).

Oxidation reactions were performed in 250 mL reagent bottles with 100 mL of sample (head space volume 220 mL). The bottles were placed in darkness at 45 °C and aerated twice daily. All samples were prepared in triplicate.

Isolation of Reaction Products. Trial experiments showed that the amounts of the reaction product could be increased by higher caffeic acid concentrations. Consequently, ascorbic acid (2.8 mM), caffeic acid (2.2 mM) and glutathione (2.8 mM) were added to 1 L of the formate buffer (i.e., without ethanol, described above), which was then maintained in darkness at 45 °C, with daily aeration, until the compound of interest reached a maximum concentration (2 days as determined by UHPLC). At this time, further ascorbic acid (1.0 g) was added and the 1 L sample stored at 4 °C to slow any degradation of the accumulated product.

Table 1. LC-MS/MS and UHPLC Data for Targeted Products in Various Reaction Systems

reaction system[a]	parent ion[b] $[M - H^+]^-$ (LC-MS) m/z	fragment ions $[M - H^+]^-$ (LC-MS/MS) m/z	UV λ_{max} (UHPLC) (nm)	retention time (UHPLC) (min)	relative 280 nm peak area[c] (UHPLC) (%)	relative SIM ion current[c] (LC-MS) (%)
caffeic acid (mw)	440 (large)	422, 306, 272, 254, 210, 179, 167, 143, 128	289 (310)	3.67	100	100
	440 (small)	422, 336, 272, 254, 143, 128	not resolved	3.42	not resolved	N/A
ferulic acid (mw)	454	436, 325, 272, 254, 210, 179, 167, 143, 128, 107	289 (310)	4.49	93	110
coumaric acid (mw)	424 (large)	406, 295, 272, 254, 210, 179, 167, 143, 128	289 (310)	4.25	not resolved	49
	424 (small)	406, 272, 254, 153, 143, 128, 115	289 (310)	4.17	not resolved	N/A
cinnamic acid (mw)	408 – not detected		N/A	N/A	N/A	N/A
chlorogenic acid (mw)	440		280	3.64	0.3	0.3
2011 wine (no metal added)	440		not resolved		not resolved	2
2011 wine	440		290 (307)	3.65	1[d]	1
1992 wine (no metal added)	440		289 (310)	3.67	7[d]	6
1992 wine	440		289 (310)	3.67	38[d]	25

[a]mw = model wine with added hydroxycinnamic acid, ascorbic acid, glutathione and metal ions, wine = wine with added caffeic acid, ascorbic acid, glutathione and metal ions (unless otherwise indicated). [b]Large = high intensity parent ion peak, small = low intensity parent ion peak. [c]The values are relative to chromatographic peak intensity for GEC in the caffeic acid sample. The SIM ion current is the total current from all isomers (i.e., *Z*- and *E*-). The average standard deviation for this data is 2% unless otherwise indicated (see below). [d]Larger uncertainty associated with these values (standard deviation ~5%) due to high background in the UV–visible detector for these samples.

The 1 L solution was then passed through a solid-phase extraction cartridge (Strata C18-E, 70 g/150 mL, Phenomenex) to concentrate and provide a crude purification of the unknown compound. The cartridge was first conditioned with 0.2% (v/v) formic acid in methanol (150 mL), followed by 0.2% (v/v) formic acid in water (300 mL). The sample was then absorbed and washed with 0.2% (v/v) formic acid in water (150 mL) and eluted with 100 mL solutions of increasing methanol concentration (i.e., increasing in 10% (v/v) steps from 10 to 100% (v/v)). Collected 100 mL fractions were analyzed by UHPLC and those containing the compound of interest were combined and concentrated via a vacuum rotary evaporator. The concentrate was then injected directly onto preparative scale LC.

RESULTS AND DISCUSSION

In the model wine system, based on that described by Sonni et al.,[17] the concentration of the hydroxycinnamic acid (i.e., caffeic acid, 1.1 mM) modeled the total non-flavonoid concentrations commonly reported in white wines.[27,28] The metal ions utilized were copper(II) (3.1×10^{-3} mM) and iron(II) (9.0×10^{-2} mM) also typical of the concentrations found in white wines.[29] (+)-Catechin was utilized at a concentration (0.17 mM) that was akin to the total concentration of flavan-3-ols in a white wine produced from heavily pressed grapes.[28] The ascorbic acid concentration (2.8 mM), although around 5-fold higher than normally added to wines by winemakers,[2,27] allowed enhanced accumulation of the unknown compound under study. The glutathione concentration was matched to that of ascorbic acid (2.8 mM) because when sulfur dioxide is utilized with ascorbic acid, the active form (i.e., free sulfur dioxide) is at a molar equivalent or above to that of ascorbic acid.[5] It must be noted that the normal levels of glutathione in finished white wines are considerably lower than the concentration used in this study, with maximum levels of 0.1 mM quoted for wines produced from grapes processed in 'reduced' conditions (i.e., covered with inert gases).[30,31] However, recently there has been the development of yeast strains that express elevated concentrations of glutathione in finished wines,[32] as well as discussions by the International Organisation of Vine and Wine (OIV) to allow it as a legal additive (not allowed to date).[33] That is, significantly higher concentrations of glutathione in finished wines may be possible in future winemaking regimes.

Impacts of Ethanol, Organic Acid Buffers and (+)-Catechin. Previous work[17] demonstrated that both caffeic acid and glutathione were critical to the formation of the unknown compound under study here. Given the importance of wine components such as ethanol, tartaric acid and (+)-catechin[20,22,34] in many oxidation mechanisms (Figure 1), the impact of these components on the unknown compound was first ascertained.

Figure 2a shows the 280 nm chromatogram of a 12% (v/v) ethanol solution, buffered to pH 3.2 with tartaric acid, containing ascorbic acid (2.8 mM), caffeic acid (1.1 mM), and glutathione (2.8 mM) that was incubated for two days at 45 °C under saturated aerobic oxygen conditions. The unknown compound was detected (peak area $(4.4 \pm 0.3) \times 10^5$, $n = 3$, stdev.) and its UV spectra (λ_{max}; 289 nm and shoulder at 308 nm in a solvent consisting of 0.5/24.8/74.7% (v/v) acetic acid/methanol/water) was consistent with that previously reported,[17] as was the presence of low-intensity absorption bands at 378 and 495 nm in the visible region of the spectrum. The previous study[17] also proposed, based on LC-MS data, that a less concentrated isomer to the unknown compound, was coeluting with caffeic acid. This was also the case for the sample shown in Figure 2a (see LC-MS/MS data in Table 1). The peak eluting at ~2.6 min in Figure 2a was identified as dihydroxybenzaldehyde (Figure 1), by LC-MS and coelution with a standard, and has been previously reported as an oxidation product of caffeic acid in model wine systems.[21]

In equivalent solutions buffered with formic acid (i.e., instead of tartaric acid), and with or without ethanol, the unknown compound was detected with peak areas of $(3.4 \pm 0.2) \times 10^5$ and $(4.5 \pm 0.2) \times 10^5$, respectively (Figure 2, panels b and c, respectively). Finally, under identical conditions to the caffeic acid sample in Figure 2a, but with the addition of (+)-catechin (0.17 mM), the unknown compound was again detected (i.e., peak area = $(4.2 \pm 0.3) \times 10^5$, Figure 2d). These results confirm that glutathione and caffeic acid were the critical components of the model wine matrix responsible for the production of the unknown compound, while tartaric acid, (+)-catechin and ethanol were not critical to its formation. Sonni et al.[17] had already shown previously that ascorbic acid

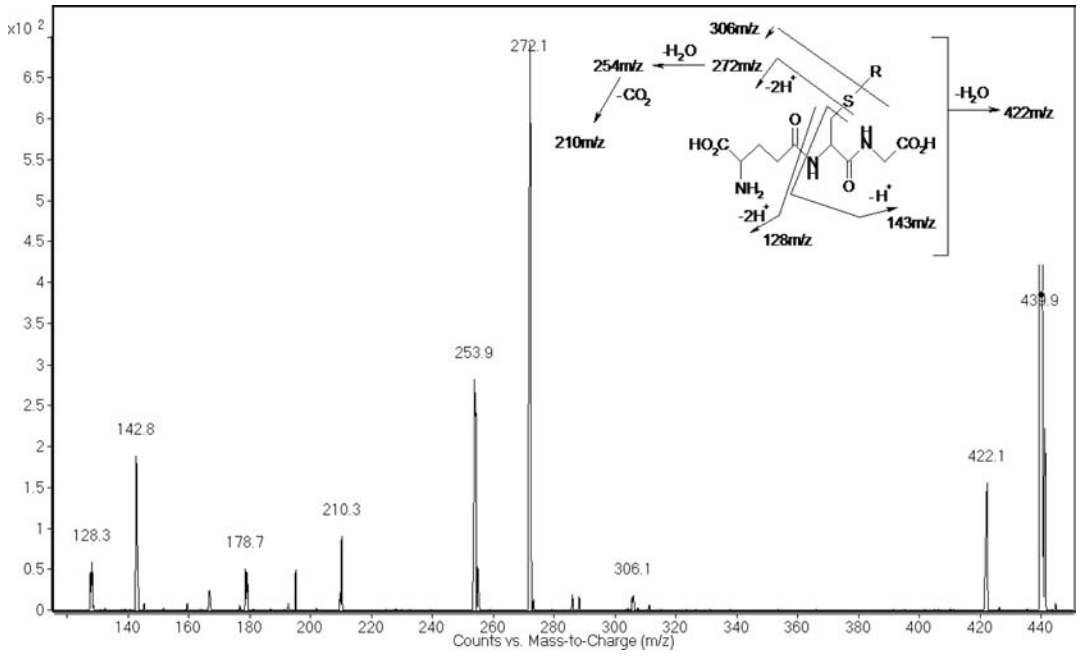

Figure 3. LC-MS/MS product ion scan of m/z 440 ion from a caffeic acid-glutathione model wine sample and proposed fragmentation.

had a significant impact on the extent of accumulation of the unknown compound rather than being critical for its formation.

Identification of the Unknown Compound. The unknown compound, corresponding to the major peak eluting after caffeic acid in Figure 2a (i.e., labeled "^"), was isolated as a faintly yellow colored solid, and subjected to LC-MS/MS, HR-MS and 1D and 2D NMR analyses. The UV−vis spectrum for the isolated compound was as already described above (i.e., λ_{max} = 289 (308) nm with additional low-intensity absorption bands in the visible region). The HR-MS analysis provided a $[M - H^+]^-$ parent ion signal of m/z 440.1123 (negative ionization mode), establishing a molecular formula of $C_{18}H_{23}N_3O_8S$ (±1.1 ppm) and indicating 9 degrees of unsaturation. Figure 3 shows the mass spectrum generated from fragmentation of the m/z 440 ion and provides the proposed fragmentation mechanisms. Except for a dehydration fragment of the parent ion at m/z 422, the majority of the fragmentation peaks were consistent with the presence of a glutathionyl moiety in the compound, as these fragments had previously been observed in studies on derivatives of glutathione.[35] Furthermore, the peak at m/z 306 corresponded to the $[M - H^+]^-$ ion for glutathione itself. The remaining non-glutathione fragment of the compound (i.e., $[R]^-$ = m/z 135 = $C_8H_7O_2$) was not detected.

Analysis of the NMR spectra (Supplementary Table 1, Supporting Information) indicated the presence of four methylene groups [(δ_H 3.51, 3.49, *m*, 2H; δ_C 42.9, C10), (δ_H 3.15, 2.88, *m*, 2H; δ_C 34.2, C12), (δ_H 2.31, *m*, 2H; δC 30.9, C4), (δ_H 1.92, *br s*, 2H; δ_C 26.6, C3)] and seven methine groups [(δ_H 6.76, *br s*, 1H; δ_C 112.2, C17), (δ_H 6.66, *d*, J = 8.0 Hz, 1H; δ_C 115.8, C20), (δ_H 6.65, *d*, J = 8.0 Hz, 1H; δ_C 118.1, C21), (δ_H 6.50, *d*, J = 16.0 Hz, 1H; δ_C 129.9, C14), (δ_H 6.38, *d*, J = 16.0 Hz, 1H; δ_C 120.4, C15), (δ_H 4.41, *m*, 1H; δ_C 53.2, C7) and (δ_H 3.44, *m*, 1H; δ_C 54.2, C2)]. HSQC NMR experiments supported the presence of four methylenes and seven methine carbons. Seven quaternary carbons were identified on the basis of gHMBC correlations (Supplementary Table 1). The exchangeable protons were not detected in this solvent composition. The presence of an aromatic methine at H21 (δ_H 6.65) showed a correlation *ortho* to the methine at H20 (δ_H 6.66) in the COSY spectrum. Both H20 and H21 showed $^3J_{CH}$ coupling to C18 (δ_C 144.7) and C19 (δ_C 144.2) respectively (Supplementary Table 1). The ^{13}C chemical shifts at C18 (δ_C 144.7) and C19 (δ_C 144.2) were indicative of hydroxyl, aromatic bearing carbons. Furthermore, the two methine groups at H17 (δ_H 6.76) and H21 (δ_H 6.65) showed key $^2J_{CH}$ correlations to the quaternary carbon at C16 (δ_C 128.6) in the gHMBC spectrum (Supplementary Table 1), consistent with a trisubstituted six membered catechol moiety. A combination of the described gCOSY and gHMBC NMR experiments as well as comparison to the published NMR data readily confirmed the presence of a catechol moiety for the first structural fragment.[20]

The olefinic methine groups at H14 (δ_H 6.50) and H15 (δ_H 6.38) were consistent with a *trans* (J = 16.0 Hz) geometry based on the gCOSY spectrum (Supplementary Table 1). H15 showed a key $^3J_{CH}$ coupling to C21 (δ_C 118.1) and C17 (δ_C 112.2) to the catechol fragment. A $^3J_{CH}$ from H14 (δ_H 6.50) ultimately positioned the *trans*-olefinic moiety to the quaternary carbon at C16 (δ_C 128.6).

Finally key $^2J_{CH}$ HMBC correlations (Figure 4) were observed from the methine at H2 (δ_H 3.44) to the quaternary carbon at C1 (δ_C 172.5) and to the methylene at C3 (δ_C 26.6). The methylene protons at H3 (δ_H 1.92) which were coupled to H4 (Supplementary Table 1), showed a $^2J_{CH}$ HMBC correlation to the amine bearing carbon at C2 (δ_C 54.2) and to the adjacent methylene at C4 (δ_C 30.9) (Figure 4). Furthermore, a key $^2J_{CH}$ HMBC correlation was observed from H4 (δ_H 2.31) to the amide carbon at C5 (δ_C 173.8).The methylene at H10 (δ_H 3.49 and 3.51) showed a $^2J_{CH}$ HMBC correlation to the carboxylic acid at C11 (δ_C 174.2) and a $^3J_{CH}$ HMBC correlation to the amide bearing carbon at C8 (δ_C 170.7) (Figure 4). The final methine at H7 (δ_H 4.41) showed COSY correlations to the unaccounted methylene at H12 (δ_H 3.15 and 2.88) and a $^2J_{CH}$ HMBC correlation to C12 (δ_C 34.2). Both H12 (δ_H 3.15 and 2.88) exhibited a $^3J_{CH}$ HMBC correlation to the amide bearing carbon at C8 (δ_C 170.7) and a

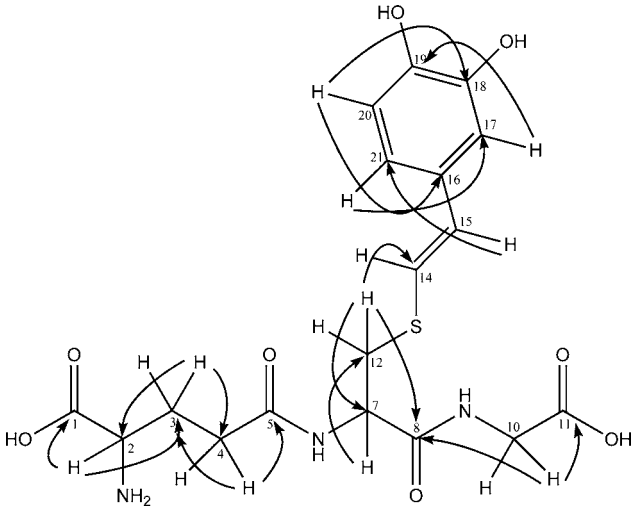

Figure 4. Key gHMBC correlations for GEC.

$^{2}J_{CH}$ HMBC correlation to the methine C7 (δ_C 53.2) consistent with a glutathione moiety. Furthermore, HR-MS had previously established a molecular formula of $C_{18}H_{23}N_3O_8S$, indicating 9 degrees of unsaturation, and it was clear that sulfur bridged the caffeic acid moiety at C14 and the glutathionyl functionality at C12. Finally, linking the *trans*-caffeic acid moiety to the glutathionyl functionality was confirmed through the observation of $^{3}J_{CH}$ HMBC coupling from the methylene protons at H12 (δ_H 2.88 and 3.15) to the vicinal methine on the caffeic acid moiety at C14 (δ_C 129.9) (Figure 4) confirming the gross structure identified as 4-[(*E*)-2′-(*S*)-glutathionyl ethenyl]-benzene-1,2-diol, more simply: 4-[(*E*)-2′-(*S*)-glutathionyl ethenyl]-catechol (GEC). It is important to mention that pure standards of both glutathione and caffeic acid were also analyzed by ^{1}H NMR, in the same deuterated solvent mixture which confirmed the chemical shifts relevant to the caffeic acid and glutathionyl fragments for this compound. A similar structural analogue, differing only by the addition of a methylene group α to the olefinic moiety, has previously been reported by Bolton et al.[36] which discuss the hepatocarcinogenicity of safrole. However, our work is the first report for the (glutathionyl ethenyl)-catechol derivative in a wine-like media.

Given that the LC-MS/MS fragmentation data was similar for the high and low intensity m/z 440 ion peaks (Table 1), it is likely that the minor m/z 440 peak is the *Z*-isomer of GEC. As previously observed for the elution pattern of *Z*- and *E*-hydroxycinnamic acids,[37] the proposed *Z*-form of CEG eluted before the *E*-form. Single ion monitoring LC-MS data showed that the ratio of the *E*:*Z* isomers was around 14:1.

As mentioned earlier, GEC provided a low intensity absorbance band at 495 nm (and shoulder at 378 nm) in the data extracted from the UHPLC, which has been reported for complexes of related phenol vinyl sulfide compounds.[38] Inoue and Otsu[38] reported absorbances at these wavelengths due to charge transfer bands observed for phenyl vinyl sulfide complexing to π-electron acceptors. Similar complexing may be occurring for GEC with itself at the high concentrations encountered during the UHPLC separation in acidic methanolic conditions.

Given the attachment of glutathione to the 'olefinic' unit of caffeic acid, the formation of GEC would appear to have some commonality with the formation of allylic alcohol[20] (Figure 1). However, the production of GEC in the absence of ethanol demonstrates an ethanol/ethoxy radical-independent mechanism for GEC formation. In our case, it was most likely that a glutathiyl radical, rather than the ethoxy radical, attacks the caffeic acid double bond (Figure 5) to generate a carbon-centered radical.

The production of radicals from glutathione has been confirmed by EPR studies, but their detection requires trapping agents due to their high instability.[39−41] Thiyl radicals can be formed by radical reactions, enzymes, light (with and without the presence of sensitizers), heat and also by metal ions.[40,42−45] On the basis of kinetic studies, the production of glutathiyl radicals was proposed in acidic aqueous solutions containing either copper(II) or iron(III) and glutathione,[15,42,43,46] whereby the metal ions were reduced to copper(I) or iron(II) and glutathione formed a disulfide. Consequently, the presence of metal ions, glutathione and oxygen in the model wine system would appear conducive to glutathiyl radicals, and indeed glutathione disulfides were detected in our previous study.[17] Other studies have shown that during the glutathiyl radical

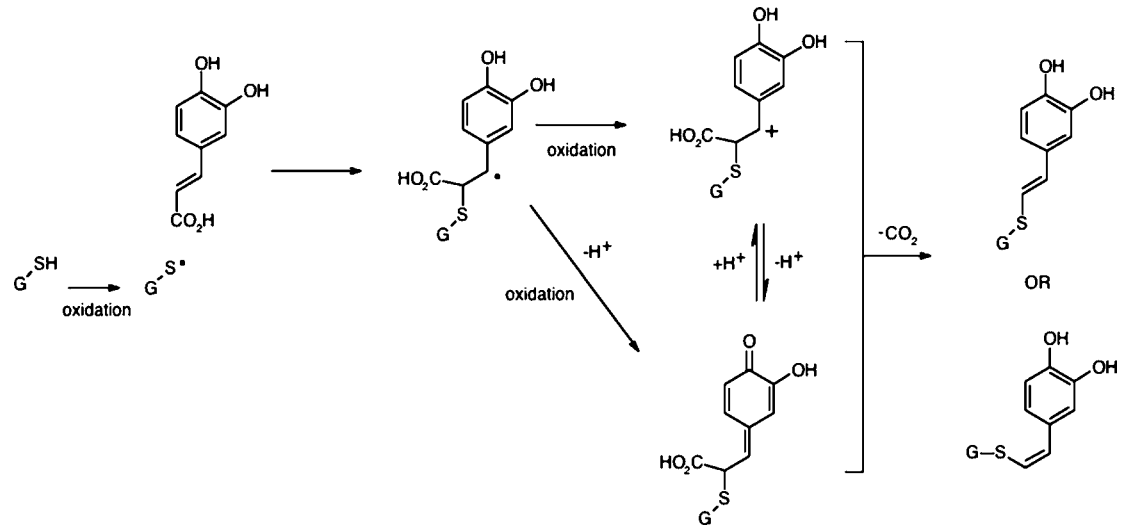

Figure 5. Proposed mechanism for the formation of GEC.

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production by copper(II) ions excess concentrations of glutathione can complex the generated copper(I) ions and thereby slow the oxidation of copper(I) by oxygen and/or hydrogen peroxide.[14,16,47,48]

Thiyl radicals are particularly electrophilic,[49] and addition of the glutathiyl radical to olefinic double bonds has been well documented.[45,49] Stock et al.[45] showed that when enzymatically generated glutathiyl radicals attacked styrene to produce a glutathione/styrene addition product similar to GEC (Figure 5), an intermediate radical was generated with the radical electron situated α to the phenyl group (i.e., similar to that depicted in Figure 5). The radical would be stabilized by resonance of the unpaired electron into the adjacent phenyl ring system.

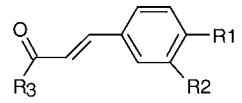
The carbon-centered (glutathionyl ethenyl)-catechol radical is most likely oxidized and decarboxylated to form GEC (Figure 5). This may occur differently depending on the favored resonance form of the radical. Gislason et al.[20] proposed that in the production of allylic alcohols from hydroxycinnamic acids (Figure 1), the oxidation step occurred via metal ions inducing production of the carbocation as shown in Figure 5, followed by subsequent decarboxylation. Alternatively, resonance of the unpaired electron to the oxygen at the *para*-hydroxyphenyl position, and subsequent hydrogen atom loss (i.e., oxidation), would result in a hydroxyquinone methide (Figure 5) capable of decarboxylation[50] and CEG production.

An alternative pathway of oxidation rather than by metal ions may be via interaction of the carbon-centered radical in Figure 5 with molecular oxygen to form the peroxide radical and then loss of the hydroperoxyl radical to form the carbocation, but this pathway would seem to induce other products that were not identified in our model wine system. For example, in the absence of metal ions, Stock et al.[45] showed that molecular oxygen added to the glutathione-styrene radical to generate the radical peroxide, which then underwent hydrogen atom abstraction from free glutathione to form a peroxide, and then reduction to the alcohol (i.e., to generate [(*E*)-2′-(*S*)-glutathionyl-1′-hydroxy-ethyl]-benzene) either enzymatically or chemically. In anaerobic conditions, again without metal ions, Stock et al.[45] showed the production of a range of different products, but were only able to isolate [(*E*)-2′-(*S*)-glutathionyl ethyl]-benzene. This latter compound was proposed to be formed by the intermediate carbon-centered radical directly abstracting a hydrogen atom from free glutathione. None of the equivalent aerobic or anaerobic products identified by Stock et al.[45] were identified in our model wine system, and this suggests the high concentration of metal ions in our reaction system and/or added resonance of vinyl phenol radicals, compared to styrene radicals, may have favored GEC formation.

During the final decarboxylation step of the mechanism (Figure 5), the isomeric forms of GEC are generated, whereby the *E*-form is favored due to steric constraints. While the model styrene compounds in the study of Stock et al.[45] did not have the ability to decarboxylate to regenerate the ethene group, this decarboxylation step was observed in the formation of the allylic alcohol.[20]

An alternative possibility for the formation of GEC is perhaps the reaction of a glutathiyl radical with the caffeic acid semiquinone radical, the latter formed from metal-catalyzed oxidation of caffeic acid in the presence of molecular oxygen.[24] The glutathiyl radical could conceivably attack the unpaired electron in the semiquinone, after delocalization to the carbon α to the carboxyl group, followed by decarboxylation resulting in GEC. However, given the oxidation reactions occurring in the order of days and the low concentration of radicals, the propensity of such a mechanism to produce GEC was considered less likely than the mechanism shown in Figure 5.

Reactions of Other Cinammic Acid Analogues. To examine the relevance of the reaction to other hydroxycinnamic acids, caffeic acid was substituted in the model wine system (i.e., containing ethanol/tartaric acid/ascorbic acid/metal ions) by either ferulic acid or coumaric acid (Figure 6). UHPLC and



Caffeic acid; R1 = OH, R2 = OH, R3 = OH

Coumaric acid; R1 = OH, R2 = H, R3 = OH

Cinnamic acid; R1 = H, R2 = H, R3 = OH

Chlorogenic acid; R1 = OH, R2 = OH, R3 = quinic acid

Ferulic acid; R1 = OH, R2 = OCH_3, R3 = OH

Figure 6. Cinnamic acid and its derivatives utilized for reaction with glutathione in the model wine system.

LC-MS/MS analyses demonstrated that after two days storage at 45 °C, equivalent products were generated in both cases (Figure 7, Table 1), with 4-[(*E*)-2′-(*S*)-glutathionyl ethenyl]-phenol from coumaric acid and 4-[(*E*)-2′-(*S*)-glutathionyl ethenyl]-guaiacol from ferulic acid. On the UHPLC chromatograms, 4-[(*E*)-2′-(*S*)-glutathionyl ethenyl]-phenol coeluted with coumaric acid, but could be resolved on the LC-MS system. The LC-MS/MS data for the ion peaks provided fragmentation data that was similar to that of GEC, with ions corresponding to dehydration of the $[M - H^+]^-$ ion as well as the fragments of the glutathione moiety (Table 1).

In the case of the coumaric acid derivative, two ion peaks at *m*/*z* 424 were evident in the LC-MS ion chromatograms again suggesting two isomers, with the major isomer eluting before the minor isomer. Although the geometrical isomerism could not be ascertained from the LC-MS analysis, it is likely that the major isomer was again the *E*-isomer, and the minor isomer the *Z*-isomer. However, only one ion peak (*m*/*z* 455) was observed for the ferulic acid derivative, suggesting either nonresolution of isomers within the chromatographic conditions utilized or single isomer production due to steric constraints induced by the extra methoxy-group.

On the basis of relative single ion monitoring (SIM) data from the LC-MS (Table 1), and assuming similar efficiencies of ionization by ESI, the amount of the glutathione-hydroxycinnamic analogues after two days were similar for caffeic acid and ferulic acid, suggesting that the structural variations between these compounds did not have any major impact on the yield of the compounds. However, the amount of the coumaric acid product generated was half that of caffeic acid and ferulic acid. This suggested that the extent of electron donating groups attached to the phenyl system was of importance to the yield of the glutathione substituted products.

When the experiment was repeated with cinnamic acid, only a small number of low-intensity peaks were evident in the 280 nm chromatogram and the *m*/*z* ion corresponding to [(*E*)-2′-(*S*)-glutathionyl ethenyl]-benzene could not be detected by LC-MS. This suggested that the hydroxyl-substitution on the

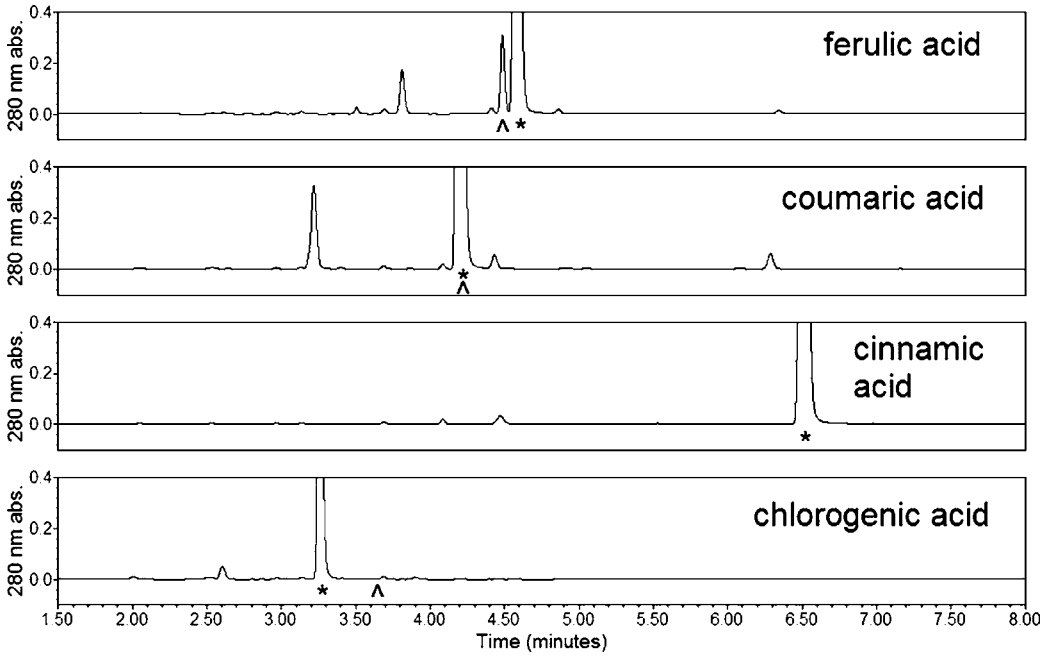


Figure 7. UHPLC chromatograms (280 nm) of cinnamic acid-glutathione-derived systems after 2 days darkness at 45 °C. In all cases the model wine system consisted of a pH 3.2 tartaric acid-buffered 12% (v/v) ethanol solution, with ascorbic acid (2.8 mM) also present. "*" highlights the parent cinnamic acid, while "^" highlights the product of interest.

phenyl moiety, and subsequent increased electron-donating character, was important in allowing production of the (glutathionyl ethenyl)-phenyl adducts. Either cinnamic acid was not sufficiently electron-rich to undergo significant attack by glutathione radicals or different glutathione-adducts were generated with low molar absorptivity at 280 nm (i.e., generating the small peaks in Figure 7).

In grapes, caffeic acid predominantly exists in the form of an ester (i.e., caftaric acid) whereby tartaric acid is attached via an alcohol moiety to the acid group of caffeic acid.[51] After crushing of the grape during wine production, the ester can gradually undergo hydrolysis.[52] To assess the impact of esterification on the production of GEC, the quinic acid ester of caffeic acid (i.e., chlorogenic acid) was utilized. Although not identified in wine, this specific ester has recently been detected as a product of fermentation with *Saccharomyces cerevisiae* in a synthetic must medium.[53] Upon incubation in the model wine system, only minor amounts of GEC were detected by UHPLC and LC-MS/MS (Table 1). Given that the production of GEC requires decarboxylation of the acid moiety in caffeic acid, it is expected that the esterification of caffeic acid will hinder this step. In this case, it is more likely that the carbocation, or hydroxyquinone methide (Figure 1), generated from the ester will undergo intermolecular nucleophilic attack by other wine components, such as glutathione, hydrogen sulfite, and water, rather than undergoing decarboxylation. Investigation of products generated from the esterified version of caffeic acid is the subject of further work.

Detection of CEG in Modified White Wine. Two commercially available Riesling wines were modified with ascorbic acid, glutathione and caffeic acid to the concentrations utilized in the model wine systems (i.e., 2.8, 2.8, and 1.1 mM, respectively) in order to assess if the mechanism highlighted in Figure 5 could occur in a complete white wine matrix. One Riesling wine was young (2011) with 32 mg/L free sulfur dioxide, while the other was older (1992) with 5 mg/L free sulfur dioxide, but both had identical pH (3.1) and ethanol concentrations (11.8% (v/v)). Both wines were split into two groups, one of which was further modified by the addition of metal ions (i.e., 0.2 mg/L copper(II) and 5.0 mg/L iron(II)). The wines were then stored in darkness at 45 °C. Table 1 shows that GEC was detected in all the wines samples, confirming that the mechanism in Figure 5 occurred in white wine. The sample that most favored the formation of the GEC was the old wine supplemented with metal ions, while the young wine without metal addition gave the least amount of GEC. Therefore, the metal ions appeared critical in the production of GEC and are most likely linked to rates of oxidation of both glutathione, to generate the radical, and also of conversion of the carbon-centered radical (Figure 5) to GEC. The impact of the wine age on GEC production was most likely a consequence of the higher concentrations of sulfur dioxide in the young wine. To confirm this, caffeic acid was incubated in the ethanol/tartrate model wine system with glutathione and ascorbic acid, but with and without 180 mg/L of added sulfur dioxide. This concentration afforded molar equivalent ratios of sulfur dioxide, ascorbic acid and glutathione. After two days in darkness at 45 °C, the relative peak area (280 nm) of GEC was 100% and 7% in the samples without and with sulfur dioxide, respectively, suggesting that SO_2 can indeed hinder the production of GEC. The absence of any significant peaks in the 280 nm chromatogram suggested that the inhibition may have been prior to the attack of glutathione on caffeic acid, but further work is required to establish the mode of the inhibition by sulfur dioxide.

This work shows the accumulation of a novel glutathione-caffeic acid addition product during the protective phase of the ascorbic acid/glutathione couple against wine oxidation. This mechanism is most relevant to the oxidation of wines containing ascorbic and hydroxycinnamic acids, low concentrations of sulfur dioxide and high concentrations of glutathione. Given these requirements it would be more likely

to occur in white wines whose glutathione concentrations have either been supplemented (not currently legal winemaking practice) or induced to higher level by specific yeast strains.[32] The production of GEC could occur during fermentation, but the free hydroxycinnamic acids may be limiting components.

The link between the production of GEC during the protective phase of ascorbic acid and glutathione in model wine systems[17] requires further investigation. However, it would appear that different radical species are propagating in the presence of glutathione and caffeic acid (Figure 5) that ultimately enable GEC production. In their absence, the main radicals propagating would be the ethoxy radical[20] and perhaps also tartaric acid derived radicals that are suggested precursors to oxidative pigments[54] (Figure 1). The different radical-propagating mechanisms are supported by the fact that a different range of colored compounds are generated in model wine systems depending on whether glutathione and caffeic acid are present.[17] The production of CEG may also contribute to the synergistic role for glutathione and caffeic acid in the protection of wine volatile compounds.[8]

■ ASSOCIATED CONTENT

Ⓢ Supporting Information

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

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Notes

The authors declare no competing financial interest.

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■ ABBREVIATIONS USED

LC-MS, high performance liquid chromatography mass spectrometry; UHPLC, ultra high performance liquid chromatography; DMSO-d_6, deuterated dimethyl sulfoxide; UV, ultraviolet; HR-MS, high resolution mass spectrometry; FT-ICR-MS, Fourier transformion cyclotron resonance mass spectrometry; ESI, electrospray ionization; SIM, single ion monitoring; NMR, nuclear magnetic resonance; gCOSY, gradient-selected correlation spectroscopy; gHMBC, gradient-selected heteronuclear multiple bond correlation; gHSQC, gradient-selected heteronuclear single quantum coherence

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