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Investigation of Ethyl Radical Quenching by Phenolics and Thiols in Model Wine

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ABSTRACT: In the present study, the reaction between 1-hydroxyethyl radicals (1-HER) and various wine-related phenolics and thiols, including gallic acid, caffeic acid, ferulic acid, 3-mercaptohexan-1-ol (3MH), cysteine (Cys), and glutathione (GSH), was studied using competitive spin trapping with electron paramagnetic resonance (EPR) and mass spectrometry. Previous studies have reported several important reactions occurring between quinones and other wine components, but the fate of 1-HER within the context of wine oxidation is less understood. Furthermore, the ability of these compounds to prevent formation of acetaldehyde, a known nonenzymatic oxidation product of ethanol, was measured. The hydroxycinnamic acids and thiol compounds tested at 5 mM concentrations significantly inhibited spin adduct formation, indicating their reactivity toward 1-HER. In addition, we confirm that loss of 3MH under model wine conditions is due to quinone trapping as well as 1-HER-induced oxidation.

KEYWORDS: *wine oxidation, 3-mercaptohexan-1-ol, thiols, 1-hydroxyethyl radicals, electron paramagnetic resonance, spin trapping*

INTRODUCTION

Nonenzymatic oxidation greatly affects the stability and, thus, economic value of wines. This is particularly true in the case of white wines, in which oxidation results in browning and loss of important aroma-active compounds that contribute to desirable sensory attributes.[1,2] Varietal thiols are produced during fermentation from enzymatic cleavage of glutathione and cysteine conjugates by yeast activity.[3−5] These thiols are present at exceedingly low concentrations but are critically important to the sensory attributes of wine by contributing pleasant aromas (e.g., grapefruit, passionfruit, and black-currant).[6] However, these same compounds are labile to oxidation and can be rapidly lost in wines,[7] especially if bottled under closures with high oxygen transmission rates or if stored under improper conditions.[8,9] In recent studies, it has been shown that these thiols, which are strong nucleophiles, are particularly susceptible to loss by reacting with electrophilic quinones by Michael-type addition reactions resulting from enzymatic[10] and nonenzymatic[7,11−13] oxidation reactions.

Nonenzymatic wine oxidation is thought to be initiated by metal-catalyzed reduction of dioxygen by transition metals, particularly iron and copper.[14−16] Oxygen is reduced to hydroperoxyl radicals in the presence of reduced transition metals (Scheme 1), and these hydroperoxyl radicals are thought to react quickly with phenolics bearing a catechol or gallate group to yield hydrogen peroxide and semiquionone radicals.[14,17] In the presence of Fe(II) or perhaps Cu(I), hydrogen peroxide is then further reduced to yield highly reactive hydroxyl radicals ($E_{3.6}$ = 2.5 V for $HO^{\bullet}/H_2O$ couple) via the Fenton reaction. These hydroxyl radicals react at diffusion-limited rates and are thought to react nonselectively with wine components.[14,15] As the major organic component in wine, ethanol is known to be a major target for these radicals. Reaction between hydroxyl radicals and ethanol yield ethyl radicals (1-hydroxyethyl radicals and, to a lesser extent, 2-hydroxyethyl radicals), which have been shown to be the major radical species in oxidizing wine.[18,19] While the fate of 1-HER is not fully understood in wine, it is clear that significant amounts of this radical are subsequently oxidized to acetaldehyde (Scheme 2, Reaction 1).

In recent years, there has been great interest in the stability and fate of thiols in wines, especially 3MH.[10,13,20] While thiol loss resulting from Michael-type addition reactions with benzoquinones has been the focus of nearly all of these studies, the present study considers the possibility that thiol loss in wine is also a result of ethyl radical (e.g., 1-HER) oxidation (Scheme 2, Reaction 2). While the hydroxyl radical is certainly capable of directly oxidizing thiols,[21] this reaction is predicted to be of lesser importance in wine given the nonselectivity of the radical and the relative abundance of ethanol (i.e., a more likely substrate for hydroxyl radical oxidation). 1-HER is reported to have a lower reduction potential ($E_{3.6}$ = 1.2 V for the $CH_3CH^{\bullet}OH/CH_3CH_2OH$ couple[22]) relative to the hydroxyl radical ($E_{3.6}$ = 2.5 V for $HO^{\bullet}/H_2O$ couple) and, as such, should react more selectively with wine components compared to hydroxyl radicals. Gislason et al. recently showed that the α,β-unsaturated side chains of hydroxycinnamic acids can react directly with 1-HER to form allylic alcohols (Scheme 2, Reaction 3).[23] Similarly, de Almeida et al. reported that 1-HER is reactive toward certain isohumulones in beer, resulting in decreased bitterness.[24,25] In biological systems, it has been shown that 1-HER is reactive toward GSH, which is oxidized to produce glutathione disulfide.[26] In light of recent studies

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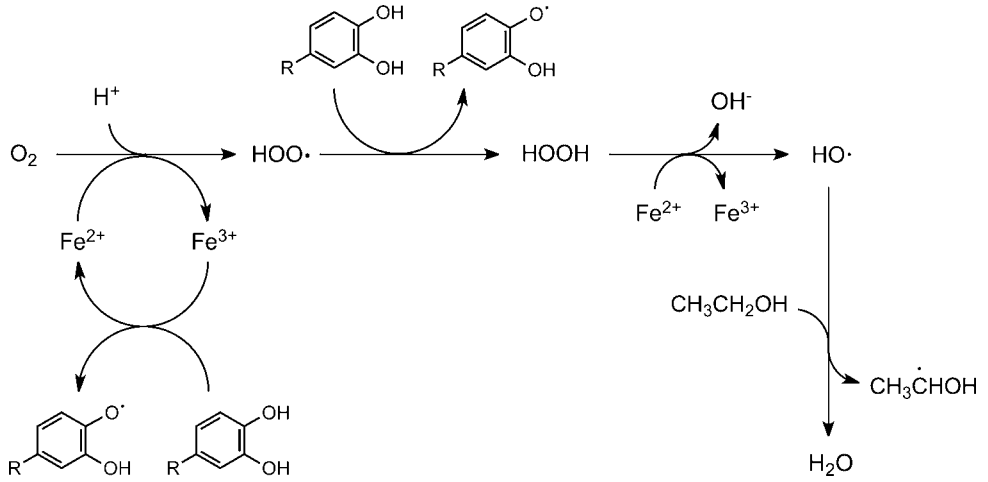
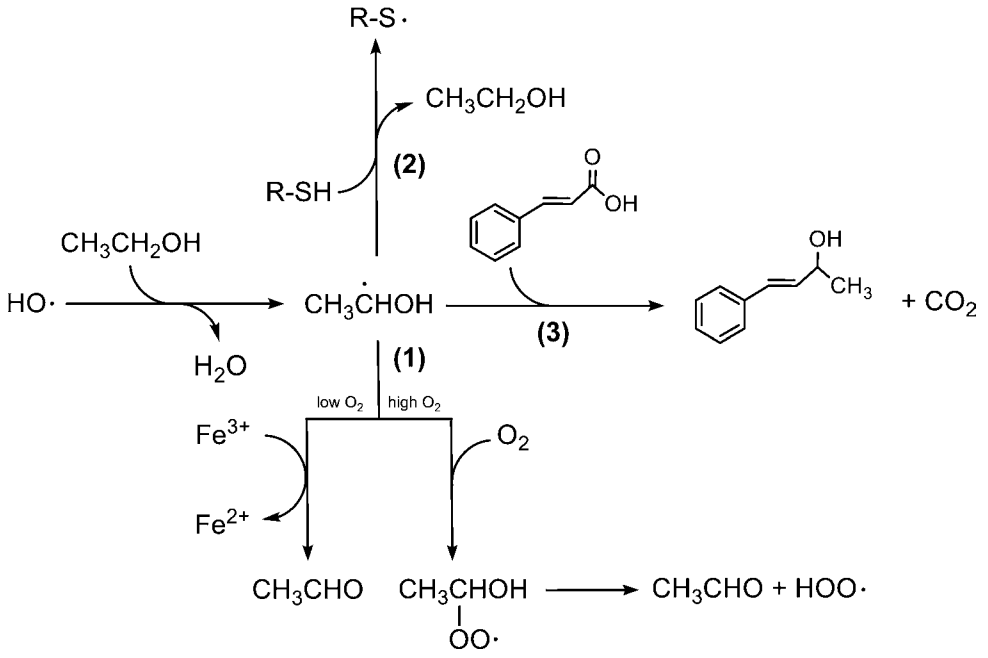
Scheme 1. Proposed Metal-Catalyzed Reduction of Oxygen to Hydrogen Peroxide and Hydroxyl Radicals and Subsequent Oxidation of Ethanol to 1-Hydroxyethyl Radical



Scheme 2. Proposed Reaction Mechanisms of 1-HER with (1) Ferric Ions or Oxygen, (2) Thiols, and (3) Hydroxycinnamic Acids



demonstrating the prevalence and relative stability of 1-HER in wine, our objective was to directly assess the reactivity of 1-HER toward several wine-relevant hydroxycinnamic acids and thiols, including Cys, GSH, and 3MH.

MATERIALS AND METHODS

Materials. Iron(II) sulfate heptahydrate, 4-methylcatechol (4-MeC), ferulic acid (FA), D-mannitol, L-cysteine (Cys), acetaldehyde–DNPH analytical standard, and catalase from bovine liver were obtained from Sigma-Aldrich (St. Louis, MO). 2,4-Dinitrophenylhydrazine (DNPH) was purchased from MCB laboratory chemicals (Norwood, OH). L-Tartaric acid, 3MH, reduced L-glutathione (GSH), 3,4,5-trihydroxybenzoic acid (gallic acid; GA), 3,4-dihydroxycinnamic acid (caffeic acid; CA), and 5,5′-dithiobis(2-nitrobenzoic acid) (DTNB, 99%+) were obtained from Alfa Aesar (Ward Hill, MA). Hydrogen peroxide (30% w/w; H_2O_2) was obtained from EMD Chemicals (Gibbstown, NJ). The spin trap phenyl-*N*-*tert*-butyl nitrone (PBN) was purchased from GeroNova Research Inc. (Garson City, NV). Water was purified through a Millipore Q-Plus system (Milipore Corp., Bedford, MA). All other chemicals and solvents were of analytical or HPLC grade.

Preparation of Model Wine. Model wine was prepared according to previous methods[20] by dissolving 8.0 g of tartaric acid in approximately 700 mL of purified water in a 1 L volumetric flask. Absolute ethanol (120 mL) was added, and the pH was adjusted to 3.6 using 5 N sodium hydroxide. Sufficient water was added to bring the solution to a final volume of 1 L.

Fenton Reaction Conditions in Model Wine. The PBN spin trap was dissolved directly into model wine solutions to achieve a final concentration of 5 mM. GA, CA, FA, Cys, GSH, and 3MH were also added directly to the model wine solution to achieve final concentrations of 5 mM; these concentrations were chosen in order to establish competitive kinetic conditions between the spin trap and test compounds. A control consisting of model wine containing only 5 mM PBN was used. All reactions were carried out under nitrogen gas. Model wine solutions (1 mL) containing 5 mM of either the phenolic- or the thiol-containing compounds and PBN were transferred to 5 mL

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test tubes and gently sparged with nitrogen gas via glass dispersion tube at low flow rate for 2 min to achieve deoxygenated conditions. Following deoxygenation, Fenton reagents ([H_2O_2] = 1 mM, [Fe(II)] = 100 μM) were added to initiate oxidation reactions according to the method described below. Stock solutions of Fe(II) (10 mM), 4-methylcatechol (4-MeC) (100 mM), and H_2O_2 (100 mM) were prepared daily in water acidified with HCl (pH 2) and mixed by vortex. 4-MeC (10 μL; 1 mM final concentration) was added to approximate the phenolic fraction of a white wine, and Fe(II) (10 μL; 100 μM final concentration) was added to the model wine under nitrogen. H_2O_2 (10 μL, 1 mM final concentration) was then added to initiate the Fenton reaction, which was allowed to proceed at ambient temperature for 1 min under nitrogen. Sample aliquots were withdrawn and analyzed without delay by EPR and LC/MS (methods described below). For treatments requiring acetaldehyde analysis, experiments were performed as described above except in the absence of PBN. Samples used for acetaldehyde analysis were incubated at ambient temperature for 5 min prior to derivatization (described below).

EPR Analysis of PBN/1-HER Spin Adducts. EPR was used to detect PBN/1-HER spin adducts in model wine solution, as described previously.[27] Sample aliquots (50 μL) were loaded into borosilicate micropipets (VWR, Radnor, PA), and EPR spectra were immediately recorded at room temperature on a Bruker eScan R spectrometer (Bruker BioSpin, Rheinstetten, Germany) operating in X-band. Sweep width was set to 50 G, and microwave power was set to 37.86 mW. Modulation frequency and modulation amplitude were set to 86.00 kHz and 2.45 G, respectively. Receiver gain was set to 4.48 × 10^3. Conversion time and sweep time were set to 20.48 ms and 10.49 s, respectively. Total number of scans per sample was 10. The reaction reached a maximum EPR absorbance within 1 min, and the signal remained constant for 30 min before starting to decay (data not shown). Intensity was quantified by adding the maximum and minimum values of the central doublet.

HPLC-MS Analysis of PBN/1-HER Spin Adducts. Samples were diluted 1:20 in Millipore water and filtered over polytetrafluoroethylene (PTFE) syringe tip filters (0.45 μm, 13 mm; AcrodiscTM, Ann Arbor, MI). The HPLC system consisted of a binary pumping system (Shimadzu LC-10ADvp) with high-pressure mixing and sample introduction by means of a Shimadzu SIL 10ADvp autosampler. PBN/1-HER spin adducts were separated on a ZORBAX Eclipse Plus C18 column (4.6 × 150 mm, 5 μm; Agilent Technologies, Santa Clara, CA). The mobile phase consisted of 0.1% v/v formic acid (A) and 0.1% v/v formic acid in methanol (B). PBN/1-HER adducts were eluted by gradient according to the following program: 0 min, 0% B; 0–5 min, 80% B; 5–18 min, 90% B; 18–22 min, 90% B; 22–23 min, 0% B; 23–26 min, 0% B.

Detection and quantification of spin adducts were achieved using a Waters Quattro micro triple quadrupole mass spectrometer (Waters Laboratory Informatics, Milford, MA) coupled to the HPLC. Mass spectra were collected in negative-ion mode using electrospray ionization (ESI). ESI capillary spray was held at 0.50 kV. Cone source voltage was set to 60 V, and source temperature was set to 120 °C. Desolvation gas flow was 250 L/h. Selected ion monitoring mode was set to monitor ions with m/z of 178, 222, 223, 224, and 268, which correspond to unreacted PBN, oxidized PBN/1-HER adduct, radical PBN/1-HER adduct, reduced PBN/1-HER adduct, and PBN-HER biadduct, respectively (Figure 1). Due to the complexity of having pure standards for all of these adducts, their quantification was performed based on raw areas.

Acetaldehyde Analysis. Acetaldehyde was measured in model wine solutions as its DNPH derivative by HPLC according to previous methods.[23,28] Following the Fenton reaction, as described above (in the absence of PBN), 40 μL of sulfuric acid (25%) and 240 μL of DNPH reagent were added to a 100 μL aliquot of model wine in a 1.8 mL capacity microcentrifuge tube. The derivatization reaction was carried out at ambient temperature for 3 h, after which 480 μL of 60:40 acetonitrile:water was added to the sample. The sample was then filtered over a PTFE syringe tip filter (0.45 μm; 13 mm). Chromatographic separation was achieved isocratically using a

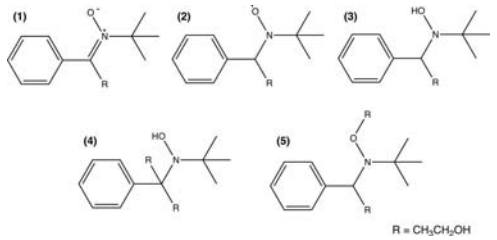


Figure 1. Possible PBN/1-HER adducts formed: (**1**) oxidized (222 m/z), (**2**) radical (223 m/z), (**3**) reduced (224 m/z), and (**4** and **5**) biadducts (268 m/z).

ZORBAX Eclipse Plus C18 column (4.6 × 150 mm, 5 μm; Agilent Technologies) with a mobile phase consisting of 70:30 methanol:-water. The acetaldehyde–DNPH derivative was detected using a diode array detector at 365 nm and quantified based on an external standard curve prepared with an authentic acetaldehyde–DNPH analytical standard.

Quantification of 3MH Loss. In order to study the loss of 3MH by 1-HER, an alternative model wine solution, in which ethanol was replaced by mannitol, was also prepared alongside a standard model wine (i.e., with ethanol). Mannitol, unlike ethanol, does not produce stable radicals that could react with 3MH. To prepare this solution, mannitol (22 g) was added to a 100 mL solution containing 8 g/L tartaric acid at pH 3.6 to achieve a final mannitol concentration of 1.2 M. To either deoxygenated mannitol or deoxygenated ethanol model wine solutions, a final concentration of 100 μM 3MH was established. Fenton reagents and 4-MeC solutions were prepared as described above. Reagents were added to achieve Fenton conditions with or without 4-MeC; in addition, controls which included H_2O_2 only or 4-MeC and Fe(II) were added to verify that the reaction is attributed to radical and quinone addition-type reactions. Reaction vessels were capped and held at ambient temperature for 10 min. 3MH was measured in model wine solutions using Ellman's assay.[29] Following the Fenton reaction, 25 μL of catalase (100 IU) was added directly to model wine solutions followed by 100 μL of phosphate/tris buffer (1 M; pH 8.1) to achieve a final pH of 7.0. Catalase was added to prevent further reaction by hydrogen peroxide formed at this pH, as previously documented.[28] DTNB (375 μL; 2 mM) in phosphate buffer (100 mM; pH 7.0) was then added, and the reaction was allowed to proceed for 10 min. The DTNB derivative was then quantified using a Genesys 10S UV–vis spectrophotometer (ThermoScientific, Waltham, MA) at 412 nm. Quantification of 3MH was based on an external standard curve.

Statistical Analysis. Data were analyzed using one-way ANOVA and Dunnett's post-test or Student's t-test to determine differences from control (Minitab 16 Statistical Software, State College, PA). Treatments were significant when $p < 0.05$. All experiments were performed in triplicate.

RESULTS AND DISCUSSION

Analysis of PBN/1-HER Adducts by EPR. GA, CA, FA, Cys, GSH, and 3MH (Figure 2) were investigated for their ability to quench 1-HER in model wine solutions in which oxidation was initiated by exogenous Fenton reagents (i.e., Fe(II) and H_2O_2). The hydroxyl radicals formed under these conditions are extremely reactive and thought to react with the organic fraction of wine in a concentration-dependent manner. Ethanol (~2 M in wine) is present in molar excess compared to other wine components and thus predicted to be the major target of hydroxyl radicals, resulting in generation of hydroxyethyl radicals.[19,27] It has been previously shown that the accelerated oxidation conditions (i.e., Fenton reaction) employed in the present study yield the same radicals that result from the unforced oxidation of wine.[19,27] Treatment (GA, CA, FA, Cys, GSH, and 3MH) concentrations (5 mM)

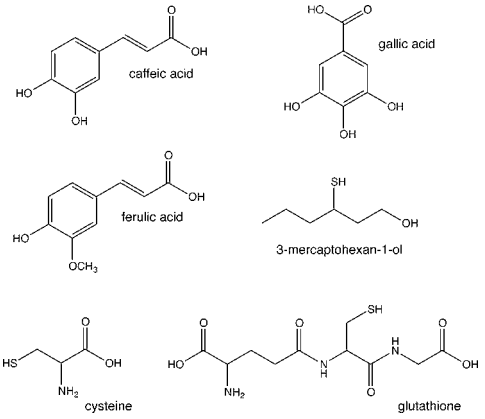


Figure 2. Chemical structures of caffeic acid (CA), gallic acid (GA), ferulic acid (FA), 3-mercaptohexan-1-ol (3MH), cysteine (Cys), and glutathione (GSH).

used in this study are higher than what is typically present in wine but were chosen to establish equimolar concentrations with PBN in order to create competitive kinetic conditions.

A triplet of doublets was observed in the EPR spectrum of oxidized model wine samples (Figure 3), with hyperfine

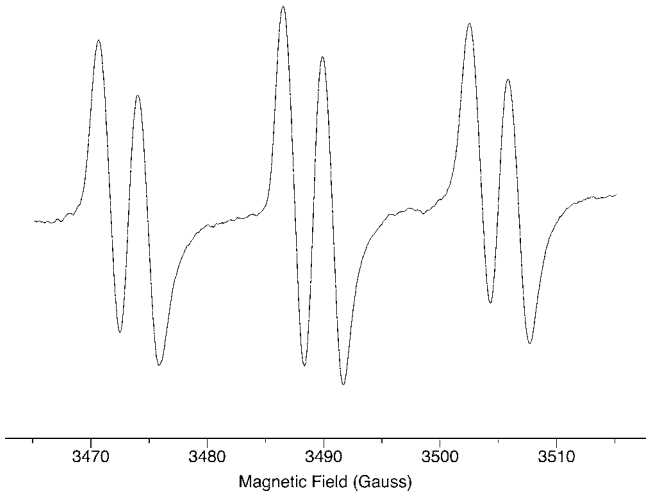


Figure 3. Representative experimental EPR spectrum corresponding to the PBN/1-HER spin adduct in model wine conditions.

coupling constants (a_N = 15.5 G, a_H = 3.3 G) indicative of PBN/1-HER adducts.[27] As expected, the intensity of PBN/1-HER spin adduct formation for GA was not significantly different from the control (Figure 4). GA was, in essence, used as a negative control as it plays a similar role to 4-MeC which was present in all other treatments. GA was used at 5 mM to verify that the gallate group does not react with either 1-HER or hydroxyl radicals to a significant extent at the concentrations used. As these reactions were run in the absence of oxygen, Fe(II) was not considered to be limiting in the Fenton reaction, as 1-HER quickly reduced Fe(III) under low oxygen conditions (Scheme 2, Reaction 1).[27] If the experiment was conducted in the presence of oxygen, 1-HER would be more likely to react with oxygen to form peroxyl radicals rather than reduce Fe(III) to Fe(II) ions. In the absence of a reducing agent capable of cycling Fe(III) back to its Fe(II) state, it would be expected that the PBN/1-HER spin adduct intensity observed in the GA

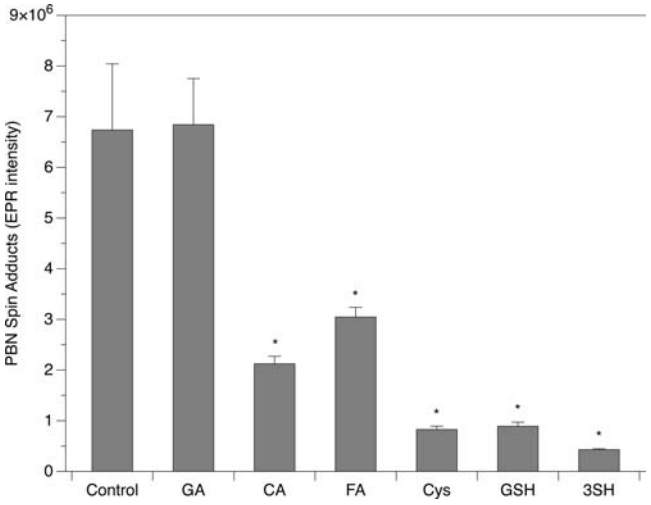


Figure 4. EPR spin adduct intensities of PBN/1-HER in the presence of 4-MeC, PBN, and selected treatments in model wine in the absence of oxygen. Spectra were obtained at room temperature after 1 min following addition of Fe(II) and H_2O_2. Asterisk (*) indicateds statistically significant difference ($P < 0.05$) from control by one-way ANOVA with Dunnett's post-test.

treatment would be higher than the control, as gallate groups facilitate Fe(III)/Fe(II) redox cycling.[13,14]

Significantly lower PBN/1-HER spin adducts were observed in solutions containing hydroxycinnamic acids (FA and CA) and thiol-containing compounds (Cys, GSH, 3MH) compared to the control model wine (Figure 4). On the basis of the results seen from the GA treatment, it is unlikely that the concentrations used were sufficiently high to compete with ethanol for hydroxyl radicals, as ethanol is present at ca. 400 molar excess. Therefore, these results suggest evidence of direct competition between the treatments and PBN for 1-HER.

CA inhibited production of PBN/1-HER spin adducts by ca. 69% compared to the control, despite the presence of a catechol group and a similar reduction potential to that of GA.[14] A decrease in observed spin adducts can be attributed to the high reactivity of CA's α,β-unsaturated side-chain group, which has recently been shown by Gislason et al. to efficiently scavenge 1-HER at the α position.[23] The resulting complex is a stable benzyl radical and in the presence of Fe(III) rearranges to its carbocation form and, eventually, into an allylic alcohol.[23] The ability of FA to compete with PBN for 1-HER was also investigated, as it contains an α,β-unsaturated side chain yet, unlike CA, does not contain a catechol group and therefore does not form an *o*-benzoquinone upon oxidation. As was the case with CA, FA was observed to scavenge 1-HER radicals, thereby suppressing PBN/1-HER adduct formation by ca. 55% compared to the control.

The thiol-containing treatments inhibited spin adduct formation to the greatest extent, indicating their reactivity toward 1-HER under wine conditions. Cys, GSH, and 3MH suppressed formation of PBN/1-HER spin adducts by 88%, 87%, and 96%, respectively (Figure 4). It is possible that these compounds showed high reduction in spin adduct formation due to the fact that thiol groups have a relatively low reduction potential and can effectively reduce 1-HER. However, reduction potentials do not necessarily predict the reaction rates. For the 2-electron reduction potential, RSH/RSSR couple is a stronger reducing agent than polypenols;[30] however, a sulfhydryl group has a reduction potential of approximately −1.16 V for the

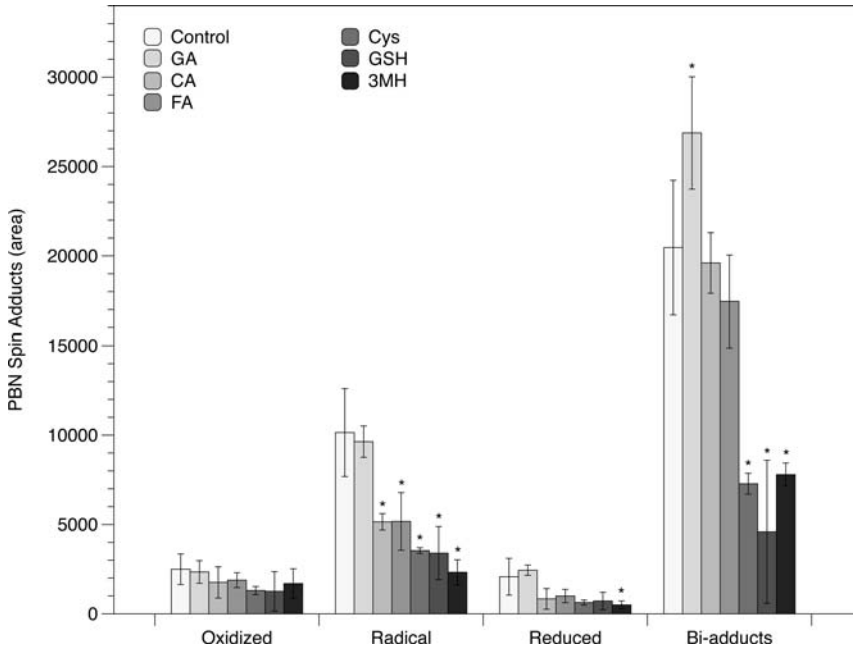


Figure 5. Total MS spin adduct intensities of all possible PBN/1-HER adducts (oxidized, 222 m/z; radical, 223 m/z; reduced, 224 m/z; biadducts, 268 m/z) in the presence of 4-MeC, PBN, and selected treatments in model wine in the absence of oxygen. Spectra were obtained at room temperature after 1 min following addition of Fe(II) and H_2O_2. Asterisk (*) indicates statistically significant difference ($P < 0.05$) from control by one-way ANOVA with Dunnett's post-test.

RSH/RS$^\bullet$ couple at wine pH;[13] this is lower than the catechol system ($E_{3.6} = -1.0$ V for catechol/semiquinone couple). This indicates that upon formation of a thiyl radical, it is likely quickly scavenged by a catechol, which is present in excess compared to the thiol in wine. We observed that the dimerized form of 3MH was formed during the Fenton reaction in the absence of 4-MeC, but this was not significant in the presence of 4-MeC (data not shown). However, it is possible for the disulfide to form, as it has been observed in botrytized and aged wines.[31] We propose that the formation of this disulfide can also occur by reducing 1-HER back to ethanol while simultaneously forming a thiyl radical followed by dimerization (Scheme 2, Reaction 2).

Analysis of PBN/1-HER Adducts by LC-MS. In order to confirm the EPR spin-trapping results reported above, PBN/1-HER adducts were also measured using LC-MS. This was important due to the potential instability of the EPR-active form of the adduct under the conditions employed in the study. Both hydroxylamine and nitrone forms of the spin adducts (i.e., reduced and oxidized radical adducts, respectively) are EPR-silent species due to loss of their unpaired electrons. Previous studies have demonstrated that under some conditions (e.g., biological systems) reducing agents such as Cys and GSH can result in reduction of paramagnetic radical spin adducts to their EPR-silent hydroxylamine forms.[32] It has also been shown that under beer conditions α-(4-pyridyl-1-oxide)-*N*-*tert*-butylnitrone (POBN)/1-HER adducts, which are structurally analogous to PBN/1-HER, can be oxidized to their nitrone forms or react with a second 1-HER to yield a biadduct, both of which are undetectable by EPR.[24] Therefore, the nitrone, nitroxide radical, hydroxylamine, and biadduct forms of the PBN/1-HER adducts were measured by LC-MS (m/z = 222, 223, 224, and 268, respectively) to account for any losses of the EPR-active nitroxide adducts.

No significant differences between the control and treatments with respect to formation of the nitrone (oxidized form of the adduct, 222 m/z) were observed (Figure 5). This was expected as samples were analyzed immediately following induction of the Fenton reaction and there was limited opportunity for oxidation of the nitroxide radical adduct. Samples that were allowed to stand for extended periods prior to LC-MS analysis were found to contain a higher peak area of biadducts and nitrone forms and lower peak area of hydroxylamine and nitroxide radical forms (data not shown). The peak area for the EPR-active PBN/1-HER adduct (nitroxide radical; 223 m/z) detected in model wine solutions containing the GA treatment was not significantly different from the control; however, the total nitroxide radical peak area was significantly lower for the hydroxycinnamic acid and thiol treatments compared to the control, thus corroborating our EPR spin-trapping results. Interestingly, the total peak area value for the reduced (hydroxylamine) PBN/1-HER adduct (224 m/z) for 3MH was significantly lower than those observed in the control. Previous studies have suggested that addition of a thiol (e.g., GSH) to a preformed PBN/1-HER adduct does not, in fact, affect EPR signal intensity but would effectively inhibit spin adduct formation if the thiol were present prior to reaction,[26] thereby suggesting competition with PBN for 1-HER. With respect to biadduct (268 m/z) formation, the peak area for the GA treatment was significantly higher than the control, yet for the hydroxycinnamic acids (CA, FA) there was no difference from control. Biadduct formation was significantly lower in the thiol-containing treatment compared to control.

Acetaldehyde Analysis. With respect to acetaldehyde formation (i.e., an alternative way to asses ethanol oxidation), the results were consistent with the above-reported EPR and LC-MS analysis of 1-HER. GA did not significantly prevent acetaldehyde formation compared to the control (Figure 6). Significantly less acetaldehyde was observed in samples

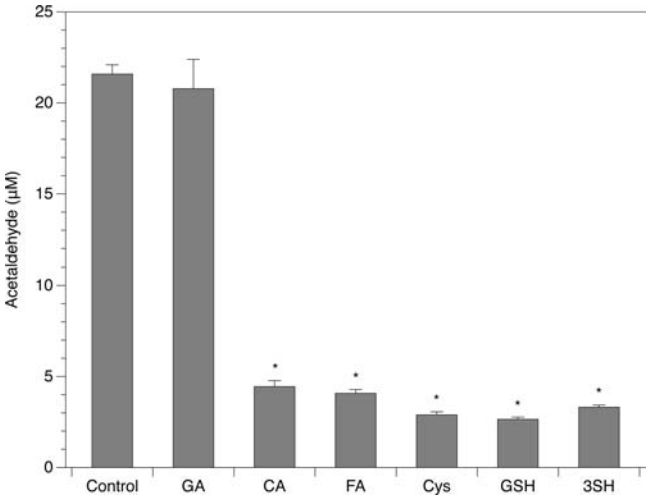


Figure 6. Acetaldehyde formation in the presence of 4-MeC and phenolic and thiol treatments in model wine in the absence of oxygen. Asterisk (*) indicates statistically significant difference ($P < 0.05$) from control by one-way ANOVA with Dunnett's post-test.

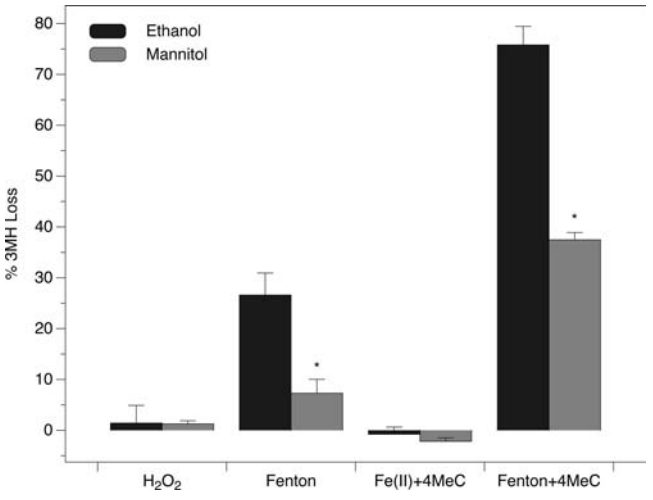


Figure 7. 3MH loss resulting from the following treatments: H_2O_2 only; Fenton reaction (Fe(II) + H_2O_2); Fe(II) + 4MeC; Fenton reaction (Fe(II) + H_2O_2) + 4MeC. Reactions was run in either model wine (pH 3.6) or mannitol solution (pH 3.6) in the absence of oxygen. Asterisk (*) indicates statistically significant difference ($P < 0.05$) from model wine by Student's *t*-test.

containing the hydroxycinnamic acid and thiol treatments compared to the control. The overall trend for acetaldehyde formation was similar to 1-HER formation, as measured by both EPR and LC-MS, thus supporting our proposed mechanism.

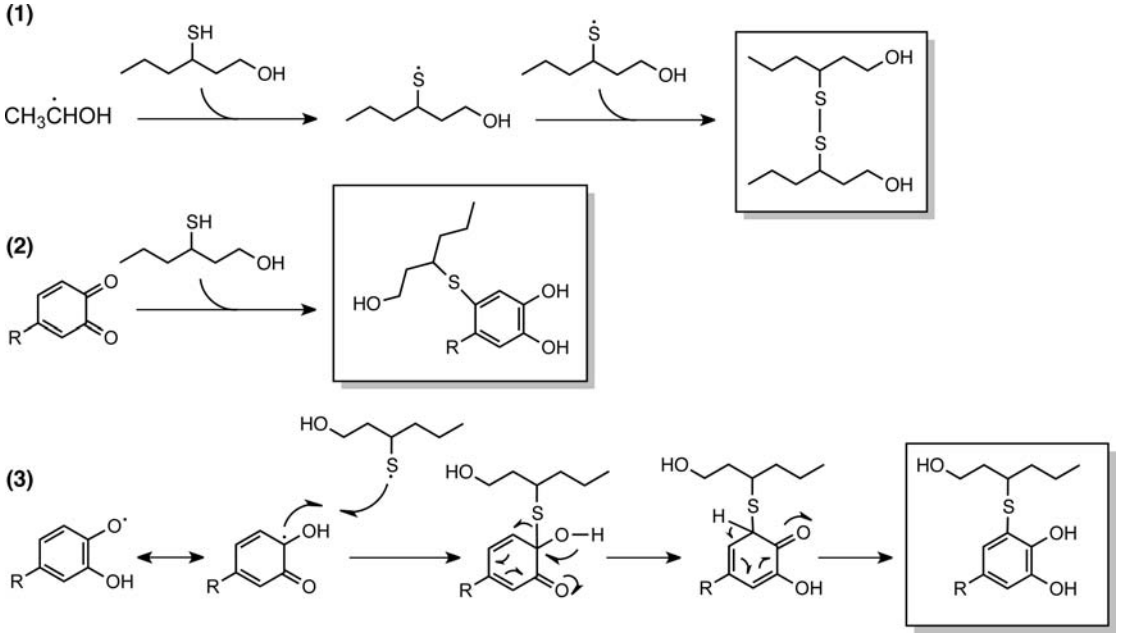
Contribution of 1-HER to 3MH Loss. In order to directly assess the extent to which a test thiol (3MH in this case) is oxidized by 1-HER, wine samples were oxizided in either the presence or the absence of 4-MeC. This was done to determine the relative contribution of quinone reactions (via 1,4-Michael addition reactions) versus 1-HER-induced oxidation reactions to the overall loss of the model thiol under wine conditions. Mannitol, a sugar alcohol that is a known substrate for hydroxyl radicals,[33] was substituted for ethanol in some experiments. Unlike ethanol, mannitol has been shown to be rapidly degraded following its reaction with hydroxyl radicals and, thus, would not be expected to promote thiol (e.g., 3MH) oxidation as 1-HER would.

When model wine samples were oxidized via the Fenton reaction (same conditions as described above) in the absence of 4-MeC, a 26.6 ± 2.2% loss of 3MH was observed (Figure 7). We propose that under these conditions hydroxyl radicals oxidize ethanol to 1-HER, which then proceed to oxidize 3MH. The likely product of this reaction is a 3MH thiyl radical, which is expected to react with a second thiyl radical to yield a disulfide (Scheme 3, Reaction 1). In order to support this hypothesis and verify that there is no direct loss of 3MH to H_2O_2, as suggested previously,[12] a treatment containing H_2O_2 (1 mM) only (i.e., without added iron) was included. No significant loss of 3MH was observed under these conditions, indicating that in the time frame of the reaction H_2O_2 was not capable of directly oxidizing the thiol. In the presence of 4-MeC, which are readily oxidized to quinones under wine conditions,[14,22] total loss of 3MH increased to 75.8 ± 1.8%, indicating that quinones play a major role in the loss of this thiol due to Michael-type addition reactions, as has been shown previously.[8,10−12] However, it is also possible, yet probably less likely, for the thiyl radical to react with a 4-MeC semiquinone radical to form a catechol−thiol adduct[34] (Scheme 3). In addition, the catechol may scavenge the resulting thiyl radical and form a semiquinone radical. Further studies should investigate formation of the disulfide.

In model solutions where mannitol was substituted for ethanol, the Fenton reaction (in the absence of 4-MeC) is expected to yield hydroxyl radicals that quickly react with mannitol, which is present in molar excess. However, unlike ethanol, mannitol is not known to form stable radicals upon its reaction with hydroxyl radicals.[35] Consequently, when ethanol was replaced with mannitol, only a 7.3 ± 0.9% loss of 3MH was observed, compared to a 26.6 ± 2.2% loss of 3MH in the presence of ethanol and, thus, 1-HER radicals. It is unlikely that a significant portion of 3MH reacted directly with hydroxyl radicals, as mannitol is present in large molar excess (ca. 240×) of 3MH; however, it is possible that 3MH is lost to oxidation by Fe(III) in this system slowly, as suggested previously.[13] As was the case with model wine (i.e., in the presence of ethanol), H_2O_2 did not directly oxidize 3MH during the time frame of the experiment. When the Fenton reaction was carried out in the presence of 4-MeC, there was a 37.5 ± 0.7% loss of 3MH in mannitol-containing solutions, compared to a 75.8 ± 1.8% loss of 3MH in the presence of ethanol (and 1-HER radicals). Loss of 3MH is likely attributed to Michael-type addition reactions with quinones, as have been seen in previous studies. While quinones can in theory be formed by direct reaction of catechols with hydroxyl radicals, it is more likely that these quinones are formed by metal-catalyzed reactions. Elias and Waterhouse showed that in the presence of 0.68 mM 4-MeC, 50 μM of Fe(III) is completely reduced to Fe(II) within 200 s.[27] A treatment containing 4-MeC and Fe(II) (i.e., no H_2O_2) was also used as a control, as no reaction would be expected to occur in the absence of oxygen. No significant loss of 3MH was observed over the time frame of this experiment. This also suggests that the conditions of the DTNB derivatization did not result in accelerated oxidation of the catechol, and demonstrates that 3MH is stable in the presence of 4-MeC or Fe(II) alone under our conditions.

This study provides further evidence that selected hydroxycinnamic acids (i.e., CA and FA) react directly with ethyl radicals in wine, thus supporting the mechanism recently

Scheme 3. Proposed Mechanisms for Loss of 3MH by (1) Reaction with 1-HER and Subsequent Disulfide Formation, (2) Michael-Type Addition Reaction to *o*-Quinone, and (3) Reaction with Semiquinone Radicals



proposed by Gislason et al.[23] We also demonstrate, for the first time to our knowledge, direct evidence of the reactivity between selected thiol compounds with 1-HER under wine conditions, which argues for a mechanism other than nonradical quinone adduction as a contributor to thiol loss. However, our results indicate that loss of thiols, such as 3MH, to quinones by two-electron, Michael-type additions reactions is probably the dominant mechanism in wine due to the abundance of phenolics. In addition, there may be an initial radical reaction by formation of thiyl radicals, followed by catechol scavenging and Michael-type addition reaction. Future work should be conducted in real wine to confirm these results.

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Notes

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