JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY

ARTICLE

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Novel Antioxidant Reactions of Cinnamates in Wine

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Supporting Information

ABSTRACT: Plant-derived polyphenolic compounds have received much attention for their ability to sequester high -energy free radicals in a great variety of food-related and biological systems, protecting those systems from oxidative change. The ability of these compounds to scavenge free radicals has always been attributed to their phenolic functionality, from which a hydrogen atom can be easily abstracted. In this study, the cinnamates and the ubiquitous hydroxycinnamates were found to equally suppress the formation of oxidation products in wine exposed to the Fenton reaction (catalytic Fe(II) with hydrogen peroxide). Mechanistic investigations led to the unexpected discovery that the α,β-unsaturated side chain of cinnamic acids could efficiently trap 1-hydroxyethyl radicals, representing a newly discovered mode of antioxidant radical scavenging activity for these broadly occurring compounds in a food system. The proposed pathway is supported by prior fundamental studies with radiolytically generated radicals.

KEYWORDS: hydroxycinnamate, cinnamate, caffeic acid, ferulic acid, α,β-unsaturated side chain, 1-hydroxyethyl radical, allylic alcohol, radical scavenging, antioxidant, phenolic, wine, oxidation

INTRODUCTION

Hydroxycinnamates represent a broadly distributed class of polyphenolic compounds found in fruits, vegetables, coffee, tea, and wine and are estimated to be the single most abundantly consumed group of dietary polyphenols.[1] The antioxidant properties of hydroxycinnamates have been investigated in systems ranging from food preservation to biological and pharmacological applications, although they have not yet received the same degree of literature attention as flavonoids or stilbenes.[1−6] To date, the observed antioxidant activities of hydroxycinnamates (and all polyphenolic compounds) in foods and biological systems have exclusively been attributed to their phenolic hydroxyl functionalities. It has been widely believed that other parts of the polyphenolic molecules contribute to antioxidant activity only insofar as they effect the stability of phenoxy radicals.[2,7−9] However, drawing on the well-known mechanism of styrene radical polymerization, it is not unreasonable to suspect that the α,β-unsaturated side chains of hydroxycinnamates could themselves be reactive toward radicals found in foods or biological systems. Furthermore, the radical reactivity of these unsaturated side chains has been demonstrated in theoretical systems involving neat ethanol, methanol, or aqueous solvents and pulse radiolysis.[10−13] Here we report for the first time that this side-chain reactivity is important in arresting the oxidation of a model wine, raising the question of how broadly applicable such antioxidant activity could be.

MATERIALS AND METHODS

Reagents. All chromatography was performed using HPLC grade organic solvents, whereas aqueous mobile phases were produced from Milli-Q filtered water and ACS grade or better reagents. All cinnamic acids were obtained from Sigma-Aldrich, and their purity was verified to be >95% using HPLC-DAD detection. Skin and seed tannin extracts were prepared as described by Kennedy et al. from *Vitis vinifera* cv. Cabernet Sauvignon grapes.[14] Hydrogen peroxide was obtained as a stabilized 30 wt % solution from Fisher Scientific and was diluted within 1 week of use, all solutions being stored in the dark at 5 °C. Acetaldehyde standards were prepared from 99.5% Acros Organics stock. For NMR spectra, Sigma-Aldrich methanol-d_4, acetone-d_6, *d*-trifluoroacetic acid, and D_2O were used. Other reagents were obtained from Fisher Scientific.

Model base wine was prepared as previously described,[15] and briefly, (+)-tartaric acid (8.0 g) was dissolved in ∼800 mL of water in a 1.000 L volumetric flask. Ethanol was added to give a 12% by volume solution. This was then titrated to pH 3.60 using 2.5 M sodium hydroxide, also with water added progressively as the total volume approached 1.0 L. The final model wine had a titratable acidity of approximately 4 g/L.

Model Wine Fenton Reactions for the Determination of Acetaldehyde Production. To determine the ability of individual phenolic species to suppress the formation of oxidized end-products in oxidizing model wine, the formation of acetaldehyde (the dominant end-product of chemical oxidation in wine) in response to oxidation by the Fenton reaction was quantified. Model wine (preparation described above) was then dosed with concentrations of phenol and Fe^{II} that are typical of those encountered in real wines (300 and 90 μM, respectively).[16,17] Argon was bubbled through the model wine and phenol mixture (without Fe^{II} or H_2O_2) for 30 min to reduce dissolved oxygen to below 0.15 ppm, verified with a PreSense Fibox 3 − trace v3 Oxymeter (Presens − Precision Sensing GmbH). After the argon sparge, Fe(II)SO_4 in 0.1 M HCl was added using a syringe to achieve 90 μM Fe^{II}, followed 15 min later by a H_2O_2 spike that yielded 300 μM H_2O_2 in the model wine, thereby initiating the Fenton reaction.

To assess the kinetics of acetaldehyde evolution, 1 mL samples of the model wine reaction were removed at timed intervals with a syringe and were added immediately to a 30 μL aliquot of aqueous 0.15 M potassium metabisulfite to rapidly react with any remaining H_2O_2 and quench further acetaldehyde production. These bisulfite-quenched samples were immediately derivatized with 2,4-dinitrophenylhydrazine

Received: January 10, 2011
Revised: April 20, 2011
Accepted: April 29, 2011
Published: April 30, 2011

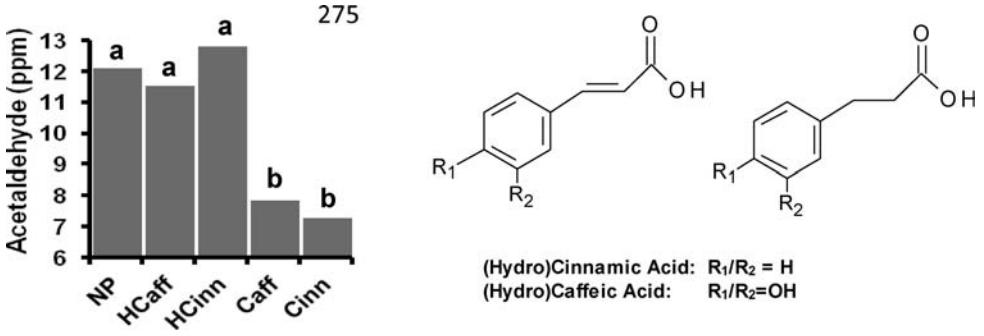


Figure 1. Selected cinnamates demonstrate involvement of the α,β-double bond in acetaldehyde depression in oxidizing model wine. Cinnamate analogues were used to establish the significance of the α,β-unsaturated side chain in the suppression of oxidized end products in wine (right) and acetaldehyde production for 300 μM cinnamic and caffeic acids (Caff/Cinn) and their hydro analogues (HCaff/HCinn), compared to the no-phenol control (NP) in deaerated model wine, oxidized by 300 μM H_2O_2/90 μM Fe(II) (left). Significant differences among the acetaldehyde means are denoted by lower case letters on the plot, where the same letter indicates that the means are indistinguishable (N = 6, α = 0.01, LSD = 2.9 ppm).

(DNPH) to quantify the acetaldehyde present. To assess the stoichiometry of acetaldehyde production produced by the Fenton reaction in the presence of various phenols, the model wine reactions were allowed to complete over the course of 1.5 h before a sample was removed, quenched, and derivatized with DNPH.

Acetaldehyde Determination. A DNPH derivatization procedure based on a previously reported method for acetaldehyde determination in wine and model wine systems was developed for this study.[18] First, the DNPH reagent was prepared by dissolving 200 mg of recrystallized DNPH in 100 mL of acetonitrile, followed by acidification with 4 mL of 70 wt % perchloric acid as described by Elias et al. Then, to derivatize acetaldehyde, a 100 μL aliquot of the model wine was mixed with 240 μL of DNPH reagent and 40 μL of 25 wt % H_2SO_4 in a 2 mL glass chromatography vial. The tightly capped vial was heated to 60 °C for 2 h in a water bath to accelerate formation of the acetaldehyde-DNPH derivative. After cooling to room temperature, the contents of the vial were mixed with 480 μL of 60:40 acetonitrile/water. After the samples had been filtered using a 0.45 μm PTFE membrane filter, the acetaldehyde−DNPH derivative was directly quantified using HPLC-DAD (A_{365}) by comparison against a standard curve. Chromatographic conditions were 0.5 mL/min isocratic elution with 70:30 acetonitrile/water on a 50 mm × 4.6 mm × 1.8 μm Agilent XDB-C18 column (t_R = 2.35 min for the acetaldehyde−DNPH derivative). Separation of means and least significant difference (LSD) were calculated using SAS statistical software and a nested ANOVA model.

Cinnamate Oxidation Product Separation/Identification. To characterize the oxidation products of caffeic, ferulic, *o*-coumaric, and cinnamic acids when these compounds were exposed to the Fenton reaction in model wine under the conditions described for acetaldehyde production (above), HPLC-DAD/MS was utilized (Agilent Series 1100/1100MSD). Separation of cinnamates and their oxidation products was achieved with 0.5 mL/min isocratic elution of 50:50 methanol/1% by wt aqueous formic acid on a 150 mm × 4.6 mm × 5 μm Phenomenex Hypersil C18 column. Chromatograms are expressed as UV−vis absorbance at 254 nm. Products were characterized by collecting full UV−vis spectra during HPLC-DAD, as well as mass spectra with m/z 100−300 or 300−600 scan ranges and electrospray ionization with 30 or 50 V fragmentation voltages in positive or negative mode with 3000 V capillary voltage.

To further characterize the oxidation products of ferulic acid (exposed to a Fenton reaction in model wine), these species were isolated using preparatory scale liquid chromatography. Separation was achieved using an Agilent 1100 series Prep-LC and a LiChrospher 100 RP-18 end-capped 10 μm column with 4.0 mL/min gradient elution of 1% aqueous acetic acid and methanol, beginning at 40% methanol and ending at 60%. Collected fractions were concentrated using a Buchi Rotavapor and then freeze-dried, before reconstitution in acetone-d_6 for one-dimensional ^1H and ^{13}C experiments in an Avance 500 NMR spectrometer.

To examine the equilibrium of allylic methoxy and ethoxy exchanged products, the isolated allylic products were reconstituted in acetone-d_6 to which either 10% ethanol, methanol, or water and 0.2% trifluoroacetic acid were added (v/v), and this forced the equilibrium to complete within a matter of seconds or minutes (to either the −OEt, −OMe, or −OH allylic product, respectively). When the same concentrations of product, ethanol, or methanol were added to a 1% acetic acid in acetone solution, the equilibration of the allylic alcohol took place over hours or days (and was thus not a problem for on-column conversion).

Identification of Cinnamate Oxidation Products in White Wine. Aliquots of 2009 UC-Davis French Colombard white wine (TSO_2 < 5 ppm, pH 3.16, TA = 7.5 g/L as tartaric acid) were spiked with 300 μM caffeic or ferulic acid and 90 μM Fe^{II} before oxidation of the wines with 12 mM H_2O_2 was performed, exactly as for the acetaldehyde assessments described above. After the in-wine Fenton reactions were complete, the mixtures were assessed using HPLC-MS under conditions identical to those used to identify the cinnamate oxidation products in model wine.

RESULTS AND DISCUSSION

During a study on the effects of various hydroxycinnamates, tannins, flavonoids, hydroxybenzoic acids, and anthocyanins on the Fenton reaction as it occurs during wine aging and oxidation,[19] it was observed that even low (300 μM) concentrations of hydroxycinnamates in a model wine were significantly suppressing the formation of acetaldehyde, a primary oxidative end-product in wine (N = 6, α = 0.01). None of the other phenols tested resulted in less acetaldehyde production than the no-phenol control. This unexpected outcome prompted a repeat of the model wine oxidations using the series of cinnamate structural analogues shown in Figure 1. As seen in this figure, acetaldehyde formation was suppressed only by the analogues containing the α,β- unsaturated side chain, clearly implicating this structural feature with the observed acetaldehyde reduction; in fact, even cinnamic acid with no phenol group at all still exhibited this behavior, suggesting an unexpected direct reaction of the cinnamate side chain during radical oxidation of a food system.

To further investigate the mechanism by which the cinnamate side chain was scavenging free radicals, HPLC-DAD/MS

Table 1. Hydroxycinnamates and Proposed Oxidation Products[a]

compound	t_R (min)	UV−vis λ_{max} (nm)	predominant fragment (m/z)	
			−	+
caffeic acid	8.448	324, 298, 240	179	181
caffeic allylic OH	8.816	298, 262, 229	179	163
caffeic allylic OEt	12.657	298, 264, 225	207	163
ferulic acid	9.888	323, 297, 237	193	195
ferulic allylic OH	10.277	294, 264, 227	193	177
ferulic allylic OEt	14.788	295, 265, 222	NA	177
o-coumaric acid	10.745	326, 276, 230	163	165
o-coumaric allylic OH	11.008	300, 250, 225	163	147
o-coumaric allylic OEt	15.135	302, 250, 216	NA	147
cinnamic acid	12.592	276	147	149
cinnamic allylic OH	13.410	250	NA	131
ferulic allylic OH formed in propanol model wine	11.617	296, 264, 226	NA	191
ferulic allylic OPr formed in propanol model wine	18.486	296, 266, 215	NA	191

[a] Model wines containing a single hydroxycinnamate each (300 μM) were oxidized by 3 mM H_2O_2/90 μM Fe(II), then characterized using HPLC-DAD/MS. Mass spectrometer was operated at 30 V fragmentor voltage. Values are reported for the unoxidized cinnamic acids, their primary allylic alcohol oxidation products, and the minor allylic exchange (−OH for $-OCH_2CH_3$) products thereof.

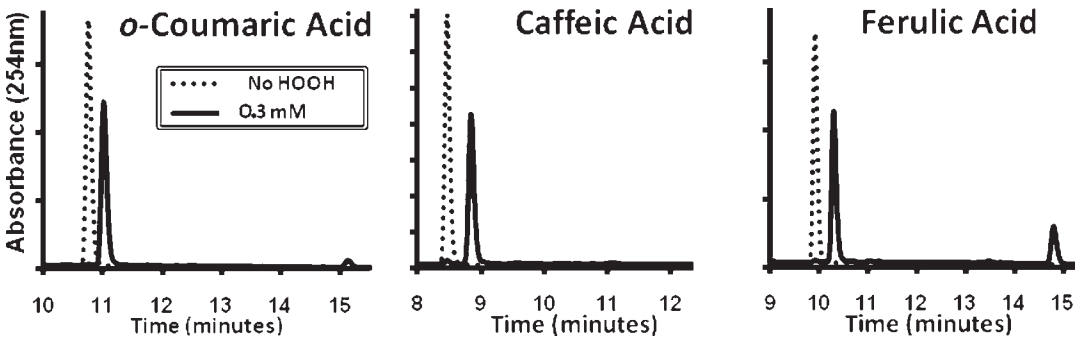


Figure 2. LC chromatograms of model wines that contain hydroxycinnamates at 300 μM, before and after oxidation with 300 μM H_2O_2/90 μM Fe(II).

was utilized to examine the oxidation products of cinnamic, *o*-coumaric, ferulic, and caffeic acids when subjected to a Fe^{II}/H_2O_2 Fenton reaction[19] in model wine having pH 3.6 and ethanol = 12% v/v.[15] All of the cinnamates appeared to be analogously oxidized, each cleanly yielding a major and minor product that both had a dominant MS fragment of m/z $[P^+ - 17]$ (where P is the mass of the parent cinnamate), whereas the unsaturated side-chain UV−vis absorbances of the products were all blue-shifted by ∼26 nm when compared to the unoxidized cinnamates, as seen in Table 1 and Figure 2.[20] Because all of the cinnamates appeared to be oxidizing in an analogous fashion, the two products of ferulic acid were selected for isolation and structural elucidation by preparative HPLC and $^1H/^{13}C$ NMR, respectively, as a representative compound for all of the cinnamates, due to the ease of separation and workup.

The ^1H and ^{13}C NMR spectra of the major ferulic acid oxidation product in acetone-d_6 were consistent with an allylic alcohol that maintained the parent ferulic acid ring structure and

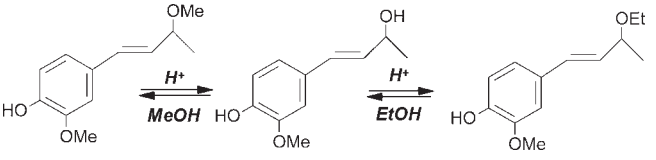
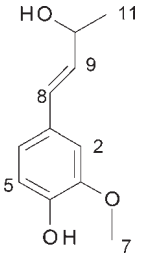


Figure 3. Equilibrium of ferulic acid derived allylic alcohol with methyl and ethyl ether forms in acidic media.

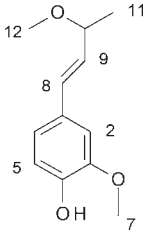
the *trans* unsaturated side chain, whereas the minor products were the methyl or ethyl ether of this same base alcohol (reconstituted in acidic methanol or ethanol respectively) as shown in Figure 3 and Tables 1−3. Due to the allylic nature of the alcohol, it was exchangeable for either methanol or ethanol in acidic media. Under highly acidic conditions (0.2% formic acid) exchange of the allylic alcohol with the methoxy or ethoxy forms took place within a matter of seconds or minutes, whereas in 1% acetic acid it was many hours. Although only the ferulic acid products were fully characterized with NMR, it is highly likely

Table 2. NMR of Ferulic Acid Allylic Alcohol (4-[(*E*)-3-Hydroxybut-1-enyl]-2-methoxyphenol) Oxidation Product in a Mixture of Acetone-d_6, D_2O, and TFA Using a 500 MHz Avance NMR



				connectivities	
	δ ^{13}C	δ ^{1}H (J_{HH} in Hz)	$\int^1$H	COSY	HSQC
1	129.9				
2	110.1	7.04 d (2.0)	1		C2−H2
3	148.3				
4	146.7				
5	115.7	6.76 d (8.0)	1		C5−H5
6	120.4	6.84 dd (8.0; 2.0)	1		C6−H6
7	56.0	3.86 s	3		C7−H7
8	129.1	6.45 d (15.5)	1	H_8-H_9	C9−H9
9	132.1	6.14 dd (15.5; 6.0)	1	H_8-H_9; H_9-H_{10}	C9−H9
10	68.5	4.37 m	1	H_9-H_{10}; $H_{10}-H_{11}$	C10−H10
11	23.3	1.26 d (6.5)	3	$H_{10}-H_{11}$	C11−H11

Table 3. NMR of Ferulic Allylic Methyl Ether (2-Methoxy-4-[(*E*)-3-Methoxybut-1-enyl]phenol) Oxidation Product in Acetone-d_6, Using a 500 MHz Avance NMR



				connectivities	
	δ ^{13}C	δ ^{1}H (J_{HH} in Hz)	$\int^1$H	COSY	HSQC
1	129.8				
2	110.0	7.09 d (2.0)	1		C2−H2
3	148.5				
4	147.1				
5	115.8	6.78 d (8.0)	1		C5−H5
6	120.9	6.88 dd (8.0; 2.0)	1		C6−H6
7	55.9	3.86 s	3		C7−H7
8	129.6	6.48 d (16.0)	1	H_8-H_9	C9−H9
9	132.1	5.95 dd (16.0; 7.5)	1	H_8-H_9	C9−H9
10	78.5	4.21 m	1	$H_{10}-H_{11}$	
11	24.0	1.23 d (6.5)	3	$H_{10}-H_{11}$	C11−H11
12	55.5	3.22 s	3		C12−H12

that the oxidation products of caffeic and coumaric acid are structured analogously, as indicated by the similar HPLC chromatograms and MS and UV−vis spectra for these compounds (Figure 2; Tables 1−3).

A proposed mechanism for the formation of the allylic alcohol from the parent cinnamic acids is shown in Figure 4. When wine is oxidized by the Fenton reaction, it has been established that 1-hydroxyethyl radicals are the predominant radical species present,[19] and in our model wine these could attack the side-chain double bond, attaching at the α carbon, producing a relatively stable benzyl radical. To test this hypothesis, ferulic acid was oxidized by a Fenton reaction in a model wine made with 1-propanol instead of ethanol. This yielded a dominant product having UV−vis spectra nearly identical to the allylic alcohol product formed in ethanol model wine (λ_{max} = 296, 264, 226 nm), but with a dominant MS fragment of m/z $[M - 17]^+$ that was 14 amu ($-CH_2-$) higher than for the allylic alcohol formed in ethanolic model wine, consistent with the attachment of the 1-propoxyl radical to the α-position, leading to the product R = CH_2CH_3 in Figure 4.

After the 1-hydroxyethyl radical attaches to the α carbon of the unsaturated side chain, a resonance-stabilized benzyl radical is produced. It has been previously established that a variety of Fe^{III} complexes can oxidize benzyl and *tert*-butyl radicals to the carbocation form,[21−24] and because there is Fe^{III} present during oxidation of wine and here in our study,[16] this mechanism is likely. One electron oxidation of the benzyl radical by Fe^{III} would produce a carbocation β to the carboxyl group, an arrangement that has been previously demonstrated to readily decarboxylate

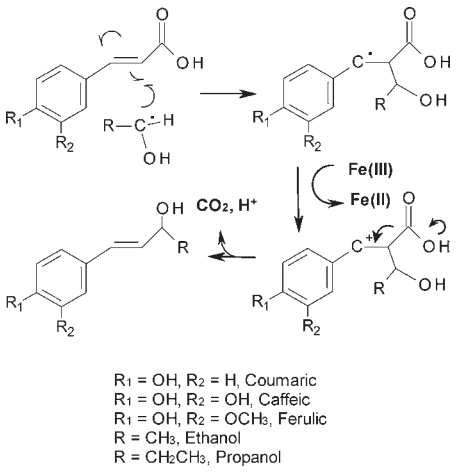


Figure 4. Proposed mechanism for radical oxidation of cinnamic acids to the allylic alcohol via the 1-hydroxyethyl radical.

and re-establish the double bond.[25] In this way, the highly reactive 1-hydroxyethyl radical has been directly sequestered by the unsaturated side chain of a hydroxycinnamate.

To explicitly demonstrate this newly found radical scavenging activity of hydroxycinnamates in a real food system, a white wine was oxidized by 12 mM H_2O_2 after its naturally occurring pools of Fe^{II} and caffeic acid had been supplemented by 90 and 300 μM, respectively (see the Supporting Information). Hydrogen peroxide is widely known to be formed in wine as a part of the

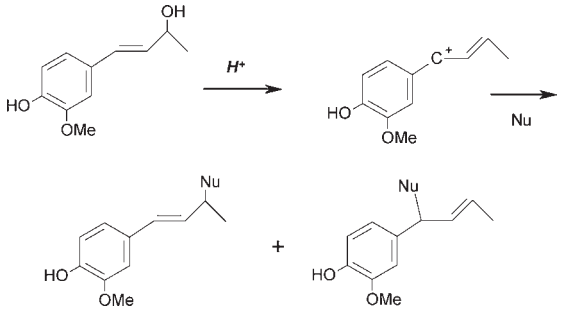


Figure 5. Possible reaction products for observed allylic alcohol under wine conditions. Nu = nucleophiles (i.e., thiols, phloroglucinol moiety, sulfite).

oxidative cascade and plays a key role during the oxidation process.[16] Iron concentrations in wine have been reported to range from 50 to 286 μM and hydroxycinnamate concentrations between 111 and 1700 μM;[16,17] therefore, the additions of Fe^{II} and caffeic acid to the wine do not represent a departure from levels typically encountered in wine. After oxidation by H_2O_2, the white wine clearly contained the allylic alcohol caffeic oxidation product (t_R = 8.738 min, λ_{max} = 301, 262, 228 nm, $[M - H]^-$ = 179), albeit in lower yields (with respect to the hydrogen peroxide added) than in the model wine system. Due to the great variety of nucleophiles in the real white wine compared to the model wine system, it would be expected that oxidation of the caffeic side chain would lead to a variety of exchanged allylic products, causing a decreased apparent yield of the allylic alcohol itself. Under the acidic conditions of wine, the allylic product reacts with acid, releasing water and generating a reactive carbocation that could then react with any number of nucleophiles, leading to numerous trace products (Figure 5). This feature of the allylic product may explain the lack of prior observations of cinnamate side-chain reactivity in wine, because the allylic alcohol is probably quickly converted to many diverse compounds formed in small quantities. Future studies will be needed to see what actually happens to the allylic alcohol. This chemical complexity of real wine, that is, alternate nucleophiles, precludes the use of the allylic alcohol as a quantitative indicator of cinnamate side-chain reactivity but, rather, is useful solely as a qualitative one.

The appearance of the allylic alcohol in wine demonstrates for the first time the reactivity of the unsaturated side chain of cinnamic acids toward free radicals in a real food system, supported by previous theoretical studies that predicted the potential for such reactivity. This represents a fundamentally new mode of hydroxycinnamate antioxidant activity, clearly distinguished from the phenol-centered view that was previously held for these ubiquitous compounds. The results presented here should prompt investigations into the range and characteristics of food systems in which the α,β-unsaturated side-chain functional group can efficiently scavenge radicals, protecting these systems from oxidative damage. As is well understood, the radical scavenging efficacy for any given compound is highly specific to the characteristics of the matrix, so although in our wine system the cinnamate side chain could scavenge 1-hydroxyalkyl radicals, in other food systems, the initial hydroxyl radical is certain to produce secondary radicals that may also be quenched by cinnamates. The ability of the unsaturated side chain to scavenge these or other radicals in other food systems must be investigated on a case-by-case basis. If it is discovered that the unsaturated side chains can usefully scavenge radicals in other systems, then analogous to previous work directed at tuning polyphenol antioxidant efficacy through rationalized substituent selection, the same could be done for the side-chain moiety.[2,9,26] It is hoped that the present discovery will contribute to the understanding and development of useful naturally derived antioxidants for foods, cosmetics, industrial materials, and biological and/or pharmaceutical systems.

■ ASSOCIATED CONTENT

Supporting Information. Additional figure of HPLC separation of 2009 UC-Davis French Colombard white wine. This material is available free of charge via the Internet at http://pubs.acs.org.

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