JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY

Article

pubs.acs.org/JAFC

Identification of Adducts between an Odoriferous Volatile Thiol and Oxidized Grape Phenolic Compounds: Kinetic Study of Adduct Formation under Chemical and Enzymatic Oxidation Conditions

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Supporting Information

ABSTRACT: HPLC−MS and ^{1}H, ^{13}C, and 2D NMR analyses were used to identify new addition products between 3-sulfanylhexan-1-ol (3SH) and *o*-quinones derived from (+)-catechin, (−)-epicatechin, and caftaric acid. The kinetics of formation of these adducts were monitored in a wine model solution and in a must-like medium by HPLC−UV−MS with the aim of understanding the chemical mechanism involved in reactions between volatile thiols and *o*-quinones. One *o*-quinone-caftaric acid/3SH adduct, three *o*-quinone-(+)-catechin/3SH adducts, and three *o*-quinone-(−)-epicatechin/3SH adducts were characterized. Caftaric acid was oxidized faster than (−)-epicatechin and (+)-catechin when these phenolic compounds were incubated in a one-component mixture with polyphenoloxidase (PPO) in the presence of 3SH. Consequently, *o*-quinone-caftaric acid formed adducts with 3SH more rapidly than *o*-quinone-(+)-catechin and *o*-quinone-(−)-epicatechin in the absence of other nucleophilic species. Furthermore, *o*-quinone-(−)-epicatechin reacted faster than *o*-quinone-(+)-catechin with 3SH. Sulfur dioxide decreased the yield of adduct formation to a significant extent. Under chemical oxidation conditions, the rates and yields of adduct formation were lower than those observed in the presence of PPO, and *o*-quinone-caftaric acid was slightly less reactive with 3SH, compared to oxidized flavan-3-ols. The identification of *o*-quinone-caftaric acid/3SH and *o*-quinone-(+)-catechin/3SH adducts in a must matrix suggests that the proposed reaction mechanism is responsible for 3SH loss in dry wines during their vinification and aging process.

KEYWORDS: *volatile thiols, phenolic compounds, reactivity, oxidation, polyphenoloxidase, oxygen*

INTRODUCTION

Volatile thiols are well-known to be relevant to the characteristic aroma of several white and red wines. Volatile thiols are very potent odorant molecules that contribute to the complexity of the flavors of wines.[1−9] During the past 20 years, several volatile thiols have been identified. For example, 4-methyl-4-sulfanylpentan-2-one (4MSP),[1] which was first isolated in Sauvignon blanc wines, exhibits a strong box-tree odor that turns into a cat urine odor at higher concentrations. Together with 3-sulfanylhexan-1-ol (3SH)[4] and 3-sulfanylhexyl acetate (3SHA),[10] 4MSP can contribute to the typicality of varietal wines such as Sauvignon blanc, Semillon, Scheurebe, Petite Arvine, Gewürztraminer, and Muscat d'Alsace.[1,2,4,7,11] 3SH is the most abundant polyfunctional thiol in wine, present in relatively high concentrations (i.e., 100−3500 ng L^{-1}) as compared to 4MSP (i.e., 0.3−97 ng L^{-1}) and 3SHA (i.e., 0.1−777 ng L^{-1}).[6,12] Other thiols such as 2-furanmethanethiol (2FMT), 2-methyl-3-furanthiol, and benzenemethanethiol, which are formed during wine aging, can contribute to the empyreumatic nuances in a wine "bouquet".[3,13,14]

Thiols are reactive molecules, and they can be transformed into disulfides and/or consumed by oxidized phenolic compounds under wine oxidation conditions.[15−18] During barrel and bottle aging of red and white wines, a significant decrease of volatile odoriferous thiols such as 3SH has been noticed due to the presence of dissolved oxygen.[19,20] Moreover, experiments carried out under controlled oxidation conditions, in red wines, have shown that the disappearance of 3SH in oxygenated wine was not concomitant with the oxygen consumption, but occurred 48 h later.[19] Consequently, the degradation of a volatile thiol such as 3SH could not be exclusively related to its direct oxidation, but probably also to its reactivity with other species present in wine. Blanchard et al.[19] showed that oxidized (+)-catechin reacted with 3SH, resulting in a loss of the fruity varietal character, due to this compound, in red wines. Recently, under winelike oxidation conditions, Nikolantonaki et al.[17] established a clear difference between (+)-catechin and (−)-epicatechin in their reactivity toward aromatic volatile thiols such as 3SH, 4MSP, and 2FMT.

Received: October 20, 2011
Revised: February 11, 2012
Accepted: February 12, 2012
Published: February 12, 2012

The presence of (+)-catechin and (−)-epicatechin, together with Fe(III) catalyzing their oxidation into *o*-quinones, favors the disappearance of such thiols.[17] However, the presence of free SO_2 slows down volatile thiol consumption.[17]

Indeed, volatile thiols can undergo nucleophilic addition reactions with certain electrophiles. One of the most abundant and chemically compatible electrophiles in wine and must is the *o*-quinones formed from phenolic compounds by enzymatic or chemical oxidation. Chemical oxidation generally occurs in fermenting grape must and in aging wine, whereas enzymatic oxidation occurs primarily in freshly crushed grapes, but may also take place in fermenting must in which the amount of SO_2 added is insufficient to inactivate PPO completely.[21,22] As volatile thiols are nucleophiles, they can thus add to the electrophilic sites of *o*-quinones in a conjugate addition fashion according to the Michael-type addition scenario,[23] which leads to the formation of adducts differing from one another by the quinonoid carbon center(s) at which the thiol function has reacted. These adducts are nonvolatile, and their formation could likely underpin the diminishing of the wine aroma intensity. The efficacy of the addition reaction between volatile thiols and *o*-quinones derived from the oxidation of phenolic (catecholic) compounds strongly depends upon the nucleophilic strength of the thiol and the oxidation rate of each phenolic substrate. The nucleophilicity of thiols is mainly modulated by their steric hindrance, primary thiols being more reactive than tertiary thiols.[24] Such nucleophilic additions have already been reported in the literature concerning the addition of thiols to *o*-quinones derived from caftaric acid,[23,25] caffeic acid,[26] gallic acid,[27] (+)-catechin,[28] (−)-epicatechin,[29] and 4-methylcatechol.[30] Most of these precedents relate to reactions performed in an alkaline buffer[26,28] or organic medium.[27] However, in acidic white grape must, Singleton et al.[31] observed the reaction of a caftaric acid-derived *o*-quinone with the tripeptide thiol, glutathione (GSH), which led to the formation of what they called the "grape reaction product" (GRP, 2-*S*-glutathionyl caffeoyl tartrate). They also studied the reaction of this same *o*-quinone, generated using the PPO enzyme, with other thiols (i.e., cysteine, 2-mercaptoethanol).[31]

The main purpose of this study was to progress in the understanding of mechanisms underpinning the evolution of volatile thiols in dry white wines and their must matrix through the monitoring of their reactions with oxidized phenolic compounds formed under chemical and enzymatic pathways. Adducts between the major appropriate oxidized phenolic compounds in white must and wine [i.e., caftaric acid, (+)-catechin, and (−)-epicatechin] and 3SH were identified and characterized. The influence of the presence of transition metal ions (Fe(II) sulfate) and SO_2 on the formation of these adducts was also investigated.

MATERIALS AND METHODS

Reagents, Chemicals, and Materials. (+)-Catechin hydrate (98%), (−)-epicatechin (98%), formic acid, reagent grade, and L-tartaric acid were purchased from Sigma-Aldrich (Saint-Quentin Fallavier, France). 3-Sulfanylhexan-1-ol (>95%) was obtained from Lancaster (Bischheim, France). L-Caftaric acid was purchased from Dalton (Toronto, Canada). Sulfur dioxide (potassium bisulfate form) was at a concentration of 10% (w/v) in water (Laffort, Bordeaux, France). Hydrogen peroxide solution 35% in water and methanol (HPLC grade) were purchased from Merck (Darmstadt, Germany). Iron(II) sulfate and HPLC-grade methanol were purchased from VWR (Fontenay-sous-Bois, France). Pimaricin (natamycin) was purchased from DMS (Heerlen, Holland). Water was purified using a Milli-Q system (Millipore, Molsheim, France). The crude PPO extract was prepared as a powder from merlot grapes, harvested in 2008, as described by Singleton et al.[31]

Reactions in Model Must. (+)-Catechin, (−)-epicatechin, and caftaric acid (9 mM) were incubated with 3SH in excess (12 mM) at 30 °C with 5 g L^{-1} of crude enzymatic extract. Enzymatic reactions were carried out in an aqueous potassium hydrogen tartrate aerated medium (5 g L^{-1}, pH = 3.5, 5 mg L^{-1} dissolved oxygen, LDO HQ10, Hach, Düsseldorf, Germany). Aliquots of 10 mL of each reaction were prepared in amber vials. Samples were added with 50 mg L^{-1} of potassium bisulfate solution in order to stop the reactions. All reactions were performed in triplicate.

Reactions in Model Wine. (+)-Catechin, (−)-epicatechin, and caftaric acid (9 mM) were dissolved in one component mixture in an aqueous potassium hydrogen tartrate (5 g L^{-1}, pH 3.5) containing 12% (v/v) of ethanol. The model solution was aerated (5 ± 0.1 mg L^{-1} dissolved oxygen, LDO HQ10, Hach, Düsseldorf, Germany), before the addition of each substrate, with medical grade compressed air (Air Liquide, France) for 10 min using a diffuser (Porex) with a pore diameter of 12 μm. Aliquots of 10 mL of each substrate were prepared in amber vials. Stock solutions of Fe(II) sulfate, SO_2, H_2O_2, and 3SH were prepared in degassed water immediately before use. The Fe(II) (50 mM) and SO_2 (0.78 mM) were introduced to the reaction medium with a syringe. In all cases, reactions were initiated upon addition of hydrogen peroxide (300 mM), followed by addition of 3SH (12 mM), to the model wine solution with a syringe. The temperature was set at 35 °C, and all reactions were performed in triplicate. Samples were added with 50 mg L^{-1} of potassium bisulfate solution in order to stop the reactions.

Identification of Adducts between 3-Sulfanylhexan-1-ol and *o*-Quinones Derived from (+)-Catechin, (−)-Epicatechin, and Caftaric Acid by Liquid Chromatography−Electrospray Ionization Mass Spectrometry. HPLC analyses were performed on a HPLC Finnigan system constituted of a Surveyor autosampler, a Finnigan ternary pump and a UV−vis 200 detector (Finnigan) coupled to a 1.2 Xcalibur data treatment system. Samples (20 μL) were injected on a reversed-phase Agilent Nucleosil C18 column (250 mm × 4 mm, 5 μm). The mobile phase was composed of solvent A [H_2O−HCOOH (996:4, v/v)] and solvent B [MeOH-HCOOH (996:4, v/v)]. The elution gradient was different for the three reaction media. The elution gradient used to monitor the reactions of caftaric acid/3SH and (+)-catechin/3SH was 0−5 min, 0−31% solvent B; 5−25 min, 31−38% solvent B; 25−40 min, 38−48% solvent B; and 40−53 min, 100% solvent B, followed by washing and reconditioning of the column. The elution gradient used to monitor the reactions of (−)-epicatechin/3SH was 0−5 min, 0−35% solvent B; 5−25 min, 35−45% solvent B; 25−50 min, 45−70% solvent B; and 50−55 min, 100% solvent B, followed by washing and reconditioning of the column. The gradient was applied at a flow rate of 1 mL min^{-1}.

The ion trap mass spectrometer was a LCQ Deca (Thermo Finnigan) equipped with an electrospray ionization source. 200 μL of the total solvent flow was injected into the mass spectrometer source with a split. Analyses were carried out in the negative ion mode. The source parameters were as follows: spray voltage (3.7 kV), capillary voltage (70.0 V), sheath gas (65 arbitrary units), auxiliary gas (20 arbitrary units), and capillary temperature (250 °C). Nitrogen was used as the nebulizing gas. Helium was used as the damping gas. The model solutions were first analyzed in full MS mode (m/z 100−1000) and then in selected ion mode using the following molecular ion mass m/z: 289 for (+)-catechin and (−)-epicatechin; 311 for caftaric acid; 421 for adducts **1a**/**b**, **2**, **4a**/**b**, and **5**; 553 for adducts **3** and **6**; and 443 for adduct **7**.

Synthesis of Adducts between 3-Sulfanylhexan-1-ol and *o*-Quinones Derived from (+)-Catechin, (−)-Epicatechin, and Caftaric Acid. 250 mg of (+)-catechin, (−)-epicatechin, or caftaric acid in 500 mL of a model medium, which consisted of aerated water (5 ± 0.1 mg L^{-1} dissolved oxygen, LDO HQ10, Hach, Düsseldorf, Germany), tartaric acid 5 g L^{-1} at pH 3.50, was treated with 10 g L^{-1} of crude polyphenoloxidase in the presence of 1 g L^{-1} of 3SH. The reaction mixtures were kept at room temperature for 48 h until the

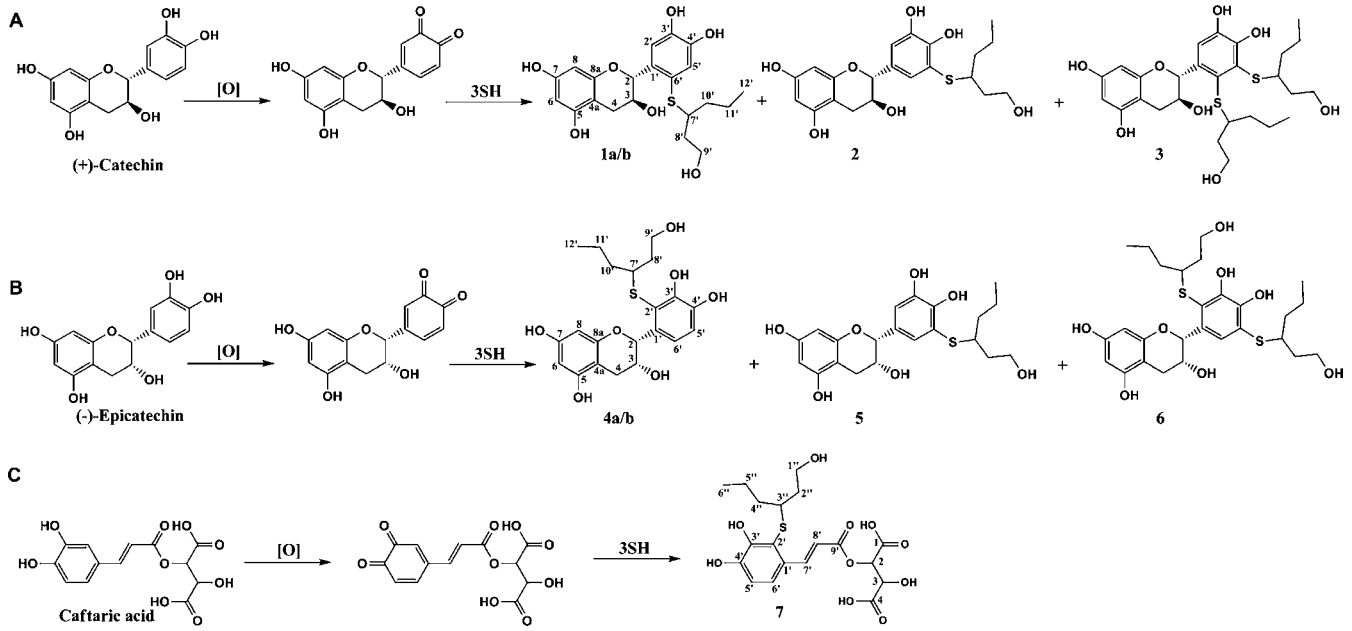

Figure 1. Structures of adducts between 3-sulfanylhexan-1-ol and *o*-quinones derived from (A) (+)-catechin [6′-(7′-sulfanylhexan-9′-ol)−catechin (**1a**/**b**); 5′-(7′-sulfanylhexan-9′-ol)−catechin (**2**); 5′-6′-(7′-sulfanylhexan-9′-ol)−catechin (**3**)], (B) (−)-epicatechin [2′-(7′-sulfanylhexan-9′-ol)−epicatechin (**4a**/**b**); 5′-(7′-sulfanylhexan-9′-ol)−epicatechin (**5**); 2′-5′-(7′-sulfanylhexan-9′-ol)−epicatechin (**6**)], and (C) caftaric acid [2′-(3″-sulfanylhexan-1″-ol)−caftaric acid (**7**)] formed under chemical and enzymatic oxidation in acidic conditions.

total consumption of the substrate, as monitored by HPLC−UV analysis. The crude reaction mixtures, after filtration on a fiber glass membrane, were washed three times with 250 mL of chloroform in order to eliminate the residual 3SH. After washing, the extracts were concentrated to dryness under vacuum. The residues were then dissolved to 100 mL of ultrapure water, and the aqueous solutions were frozen and freeze-dried.

Semipreparative HPLC Purification. The purification of each reactional medium was carried out on a HP 1090 (Agilent Technologies, Palo Alto, CA) HPLC system equipped with an HP 1090 UV detector (Agilent Technologies, Palo Alto, CA) fixed at 280 nm. Acquisitions were performed using Chemstation software. Separations were performed using a Nucleosil C-18 column (21.4 × 250 mm, 5 μm) with a guard column filled with the same phase. The solvents and the gradient elution were the same described above. The flow rate was set at 4 mL min^{-1}, and the injection volume was set to 250 μL of each crude reaction mixture (80 mg L^{-1}).

NMR. Detailed descriptions of NMR spectra of all the new isolated compounds are given in the Supporting Information.

Kinetic Study in Model Wine and in Must-like Solutions by HPLC−UV. The equipment used for the HPLC monitoring conditions for the incubation in a model wine solution or in must solution was similar to that used for the identification of the adducts. The formations of the *o*-quinone-(+)-catechin/3SH and *o*-quinone-(−)-epicatechin/3SH adducts were monitored at 280 nm, whereas that of the *o*-quinone-caftaric acid/3SH adducts was monitored at 320 nm. External calibration curves for the synthesized adducts in methanol (50%), in the range of 0.05−2 mM, were run and recorded in triplicate. Calibration curves for the quantitative analyses of **1a**, **1b**, **2**, **3**, **4a**, **4b**, **5**, and **6** were established at 280 nm and at 320 nm for **7**. Calibration curves were determined using a methanolic solution (50%) spiked with dilute methanol solutions containing **1a**, **1b**, **2**, **4a**, **4b**, **5**, and **7** at final concentrations ranging from 0 to 10 mM. The quantification limit was calculated as the minimum concentration that generated a peak signal 10 times higher than the signal from background noise. **1a**, **1b**, **2**, **4a**, **4b**, **5**, and **7** concentrations were then determined from the regression equations, and the results were expressed in mM. **3** and **6** concentrations were expressed in mM equivalent **2** and **5**, respectively.

Kinetic Study in White Native Must Matrix by HPLC/ESI-MS. 50 mL of samples were taken at regular intervals (every hour for the first 12 h and once per day for the next four days) throughout the incubations and immediately extracted once with ethyl acetate (50 mL). The organic phases were evaporated under reduced pressure, the extracts were dissolved in 2 mL of methanol, and the solution was filtered through a 0.45 μm filter. 20 μL of samples were injected in HPLC/ESI-MS using the analytical conditions described above.

RESULTS AND DISCUSSION

Our experimental approach was based on first evaluating the outcome of the reactions between the selected phenolic compounds [i.e., caftaric acid, (+)-catechin, and (−)-epicatechin] and 3SH on an analytical scale in an acidic aqueous solution under enzymatic oxidation conditions. The reaction progress was monitored by high-performance liquid chromatography coupled to electrospray ionization mass spectrometry (HPLC/ESI-MS) analysis. Products were identified on the basis of their retention time and molecular mass. Reactions were also performed on a semipreparative scale under otherwise identical conditions in order to obtain pure products in sufficient amount for their full structural characterization by NMR spectroscopy. All these reactions were also carried out in a model wine system, consisting of a 12% (v/v) aqueous ethanol solution containing 5 g L^{-1} of tartaric acid at pH 3.5, in order to analytically determine the kinetics of adduct formation in a winelike medium under chemical oxidation conditions.

Adducts between 3-Sulfanylhexan-1-ol and *o*-Quinones derived from (+)-Catechin and (−)-Epicatechin. To determine the structure of the products formed through a chemical reaction between flavan-3-ols and 3SH in wine and must during fermentation, (+)-catechin and (−)-epicatechin were separately incubated with 3SH in an aqueous acidic medium in the presence of crude PPO. The reaction progress was monitored by HPLC/ESI-MS analysis. One hour after incubation, the HPLC/ESI-MS results showed the formation of four major products, **1a**/**b**, **2**, and **3** (t_R = 26.0, 27.1, 34.8, and

dx.doi.org/10.1021/jf204295s | J. Agric. Food Chem. 2012, 60, 2647−2656

46.5 min, respectively). After 20 h, when the total consumption of the (+)-catechin substrate had occurred, the reaction was stopped in order to prevent the oxidation of the newly formed products. Thus, the products from the (+)-catechin/3SH reaction mixture were directly separated by semipreparative HPLC and the **1a**/**b**, **2**, and **3** products were obtained in a 18:18:59:4 ratio and with respective yields of 17, 17, 55, and 4%. The analogous compounds **4a**/**b**, **5**, and **6** (t_R = 28.8, 30.8, 37.6, and 44.8 min, respectively) were obtained from the reaction using (−)-epicatechin and 3SH in a 18:18:59:4 ratio and with a yield of 17%:17%:56%:4%, respectively.

The HPLC/ESI-MS analysis of the purified compounds **1a**/**b**, **2**, **4a**/**b**, and **5**, performed in negative ion mode, showed a molecular ion mass of m/z = 421 ($[M - H]^-$) indicating that these compounds could result from the nucleophilic addition of 3SH onto the *o*-quinone species derived from the oxidation of (+)-catechin or (−)-epicatechin (Figures 1A and 1B) in a similar manner as that previously reported for the addition of glutathione or cysteine onto the oxidized forms of caftaric acid, caffeic acid, (−)-epicatechin and (−)-epigallocatechin.[23,26,28−30] The presence of three different products with the same molecular ion mass (i.e., m/z = 421) in each reaction mixture could result from a nucleophilic attack by 3SH on three different electrophilic carbon centers (i.e., C2′, C5′, and C6′) of the B-ring of the *o*-quinones derived from (+)-catechin and (−)-epicatechin. Moreover, high resolution ESI-MS analyses showed that **1a**/**b**, **2**, **4a**/**b**, and **5** have the same molecular formula, i.e., $C_{21}H_{26}O_7S$. The HPLC/ESI-MS analysis of the other two products, **3** and **6**, revealed a molecular ion mass of m/z = 553, which indicated a double addition of 3SH onto the oxidized flavan-3-ols (Figures 1A and 1B). Thus, the formation of **3** and **6** results from a second nucleophilic addition of 3SH onto the *o*-quinones deriving from the oxidation of the initially formed adducts **1a**/**b**, **2**, **4a**/**b**, and **5**. Also, in this case, according to the HPLC−UV chromatogram, peaks **3** and **6** represented a mixture of products with the same molecular formula, i.e., $C_{27}H_{38}O_8S_2$. However, the HPLC analysis did not allow the separation of the two diastereoisomers resulting from the use of racemic 3SH.

The complete structural elucidation of compounds **1a**/**b**, **2**, **4a**/**b**, and **5** was achieved by 1D and 2D NMR analysis. The quantities of the HPLC purified compounds, **3** and **6**, were limited, and their low yield precluded further characterization by NMR. The structure of **1a** was determined by one-dimensional ^1H and ^{13}C and two-dimensional HMQC and HMBC NMR experiments. The key point in this structural determination was the identification of the connection between the 3SH and (+)-catechin moieties that was established using long-range proton−carbon HMBC correlation data. The HMBC spectrum shows an intense correlation between proton H-7′ (δ_H 3.32 ppm) of the 3SH unit and carbon C-6′ (δ_C 121.4 ppm) of the (+)-catechin aromatic B-ring. The latter was assigned from its HMBC correlation with proton H-2 (δ_H 6.86 ppm) of the (+)-catechin C-ring, which was itself identified by its characteristic chemical shift. Moreover, the identification of this connecting link between the 3SH and (+)-catechin moieties was also supported by the presence of a broad singlet integrating for two protons in the aromatic region at 6.86 ppm. This resonance signal was attributed to protons H-2′ and H-5′ of the (+)-catechin aromatic B-ring. The other protons and carbons of the aliphatic chain of the 3SH moiety and those of the (+)-catechin moiety were all assigned from HMQC and HMBC correlation data. Thus, these 1D and 2D NMR analyses unambiguously established the structure of **1a** as 6′-(7′-sulfanylhexan-9′-ol)−catechin, which features a 3SH moiety connected to the (+)-catechin moiety through a thioether linkage at position 6′ of the (+)-catechin aromatic B-ring. Similar correlations were also observed on the HMBC spectrum of **1b**, hence revealing that **1b** also has a 3SH moiety linked to the (+)-catechin moiety through a thioether linkage at position 6′ of the (+)-catechin aromatic B-ring. Overall, **1a** and **1b** are two 6′-(7′-sulfanylhexan-9′-ol)−catechin diastereoisomers resulting from the addition of a (*R*)- or (*S*)-3-sulfanylhexan-1-ol enantiomer on the oxidized (+)-catechin moiety.

The NMR spectra of **2** and **5** also revealed important similarities, and both spectra appeared to be more complex with more signals than those of **1a**/**b**. Nevertheless, a closer analysis showed that most of the signals were duplicated, especially those corresponding to the carbons and protons around the chiral center of 3SH (i.e., C-7′). In fact, after having assigned all the signals observed in the proton and carbon spectra using two-dimensional NMR analyses (HMQC, HMBC, and COSY), it appeared that **2** was not a single compound, but an inseparable mixture of the two diastereoisomers resulting from the use of racemic 3SH. Despite the presence of these two diastereoisomers, it was possible to determine the connecting link between the 3SH and (+)-catechin moieties using the long-range proton−carbon correlations. The HMBC map showed an intense correlation between 3SH proton H-7′ at 3.18 ppm and carbon C-5′ of the (+)-catechin aromatic B-ring resonating at 120.6 ppm. Moreover, the presence of two doublets with a small coupling constant at 6.81 ppm (J = 1.67 Hz) and at 6.91 ppm (J = 1.67 Hz), which were attributed to the resonances of protons H-2′ and H-6′ of the (+)-catechin aromatic B-ring, confirmed the absence of a proton on carbon C-5′ of this ring. In conclusion, the compound mixture **2** was identified as a mixture of the two 5′-(7′-sulfanylhexan-9′-ol)−catechin diastereoisomers that resulted from the nucleophilic addition of the two 3SH enantiomers onto carbon-C-5′ of the (+)-catechin aromatic B-ring. Similar correlations were also observed on the HMBC spectrum of **5**, hence revealing that **5** is also a mixture of the two 5′-(7′-sulfanylhexan-9′-ol)−epicatechin diastereoisomers in which the 3SH moiety is connected to the (−)-epicatechin moiety through a thioether linkage at position 5′ of the (−)-epicatechin aromatic B-ring.

The complete structural elucidation of compounds **4a**/**b** was also achieved by 1D and 2D NMR analyses. The aromatic region in the ^1H NMR spectrum of **4a** revealed two mutually coupled doublets, as confirmed by COSY experiments, at 6.86 ppm (J = 8.40 Hz) and 7.16 ppm (J = 8.40 Hz). These signals were assigned to protons H-5′ and H-6′ of the (−)-epicatechin aromatic B-ring. These strictly mutually coupled aromatic B-ring protons are a consequence of the absence of protons on carbon C-2′ of the same ring, which indicates that the 3SH moiety is, in this case, connected to this position of the (−)-epicatechin unit. Moreover, this linkage was confirmed by a three-bond correlation between proton H-7′ (δ_H 3.36 ppm) of the 3SH moiety and carbon C-2′ (δ_C 118.5 ppm) of the aromatic B-ring in the HMBC map. This latter carbon was unambiguously assigned from its diagnostic three-bond correlations with protons H-2 (δ_H 5.48 ppm) and H-6′ (δ_H 7.16 ppm). The other protons and carbons were assigned to the aliphatic chain of the 3SH moiety and to those of the (−)-epicatechin moiety by HMQC and HMBC experiments. These NMR analyses unambiguously established the structure

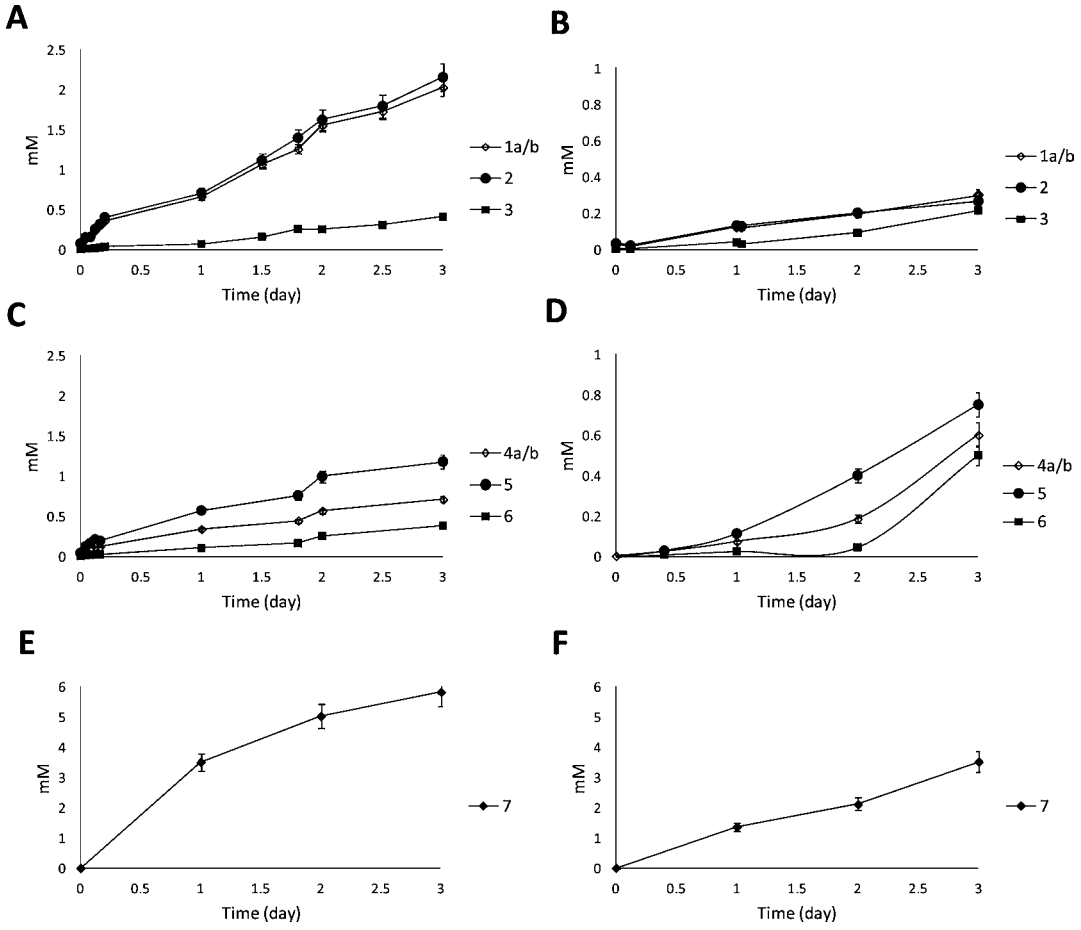

Figure 2. Kinetic formation rate of adducts between 3-sulfanylhexan-1-ol and *o*-quinones derived from (+)-catechin, (−)-epicatechin and caftaric acid in model must under enzymatic oxidation conditions. (A) **1a**/**b**, **2**, and **3** formation in the presence of polyphenoloxidase (PPO); (B) **1a**/**b**, **2**, and **3** formation in the presence of PPO and SO_2; (C) **4a**/**b**, **5**, and **6** formation in the presence of PPO; (D) **4a**/**b**, **5**, and **6** formation in the presence of PPO and SO_2; (E) **7** formation in the presence of PPO; (F) **7** formation in the presence of PPO and SO_2. The compound numbering is defined in Figure 1. **1a**/**b** and **4a**/**b** present the summary of the **1a** and **1b** and the **4a** and **4b** diastereoisomers, respectively.

of **4a** as 2′-(7′-sulfanylhexan-9′-ol)−epicatechin, which features a 3SH moiety connected to the (−)-epicatechin moiety through a thioether linkage at position 2′ of the (−)-epicatechin aromatic B-ring. Similar NMR data were also observed for **4b**, thus revealing that **4b** also has a 3SH moiety connected to the (−)-epicatechin moiety through the same type of thioether linkage. Overall, **4a**/**b** are two 2′-(7′-sulfanylhexan-9′-ol)−epicatechin diaseteroisomers resulting from the addition of the (*R*)- or (*S*)-3-sulfanylhexan-1-ol enantiomer on the oxidized (−)-epicatechin.

Adducts between 3-Sulfanylhexan-1-ol and *o*-Quinones Derived from Caftaric Acid. The procedure used for the hemisynthesis of adducts between *o*-quinone-caftaric acid and 3SH was identical to the procedure used for the oxidized flavan-3-ols, *o*-quinone-(+)-catechin and *o*-quinone-(−)-epicatechin. The progress of the reaction was monitored by HPLC/ESI-MS. After 1 h of incubation, the reaction was stopped while the total consumption of the phenolic substrate occurred and the reaction mixture was separated by semipreparative HPLC to furnish one reaction product, **7**, as an amorphous yellowish powder with a 75% yield. HPLC/ESI-MS analysis of **7**, in negative ion mode, showed a molecular ion mass of m/z = 443 ($[M - H]^-$) indicating that **7** could result from the nucleophilic addition of 3SH onto oxidized caftaric acid in a manner similar to what was observed for *o*-quinone-(+)-catechin and *o*-quinone-(−)-epicatechin (Figure 1C). Moreover, high resolution ESI-MS analyses supported the evidence for the formation of such an adduct, since the molecular formula of **7** was thus established to be $C_{19}H_{24}O_{10}S$.

The full characterization of **7** revealed that it was also a mixture of two diastereoisomers. However, it was again possible to easily determine the connecting link between the 3SH and caftaric acid moieties. The ^{1}H NMR spectrum of **7** revealed two mutually coupled doublets in the aromatic region, as confirmed by a COSY experiment, at 6.95 ppm (J = 8.32 Hz) and at 7.38 ppm (J = 8.32 Hz). These resonances were attributed to protons H-5′ and H-6′ of a 2′-substituted caftaric acid aromatic ring. This substitution was also supported by the observation of long-range proton−carbon correlations on the HMBC map between proton H-3″ (δ_H 3.15 ppm) of the 3SH moiety and carbon C2′ (δ_C 117.1 ppm) of the caftaric acid aromatic ring. This carbon resonance was assigned from its correlations with the characteristic aromatic proton H-6′ (δ_H 7.38 ppm) and the ethylenic proton H-7′ (δ_H 8.47 ppm). The other protons and carbons of the aliphatic chain of the 3SH moiety and those of the caftaric acid moiety were assigned using HMQC and HMBC correlation data. Thus, **7** was confirmed to be a mixture of the two diaseteroisomers of 2′-(3″-sulfanylhexan-1″-ol)−caftaric acid that resulted from the nucleophilic addition of

dx.doi.org/10.1021/jf204295s | J. Agric. Food Chem. 2012, 60, 2647−2656

racemic 3SH at the C-2′ center of the *o*-quinone derived from the oxidation of caftaric acid.

Reactivity of 3-Sulfanylhexan-1-ol with (+)-Catechin, (−)-Epicatechin, and Caftaric Acid under Enzymatic Oxidation Conditions. In order to gain a better understanding of the mechanism of 3SH addition onto oxidized (+)-catechin, (−)-epicatechin, and caftaric acid, the kinetics of formation of all the adducts characterized by NMR were examined by HPLC analysis with UV detection. The addition mechanism involved production of the *o*-quinone from either (+)-catechin, (−)-epicatechin, or caftaric acid by PPO-catalyzed oxidation of their catechol unit, followed by the conjugate addition of 3SH. Among these three substrates that were incubated with 3SH in equal concentrations (9 mM) in the presence of PPO, *o*-quinone-caftaric acid led to the greater production of adducts with 3SH (5 mM; Figure 2E), followed by *o*-quinone-(+)-catechin (4.5 mM of adduct with 3SH; Figure 2A) and then by *o*-quinone-(−)-epicatechin (2.2 mM of adduct with 3SH; Figure 2C). Indeed, and in agreement with a previously published article,[32] the caftaric acid consumption was about 69% after 3 days of incubation (Figure 3). This consumption was 10% and 45% greater than that of (+)-catechin and (−)-epicatechin, respectively. The above results could be related to the fact that caftaric acid is known to be a better substrate than (+)-catechin and (−)-epicatechin for grape PPO (Figure 3), and thus its oxidation rate was well correlated with the yield of adduct **7** (80%).

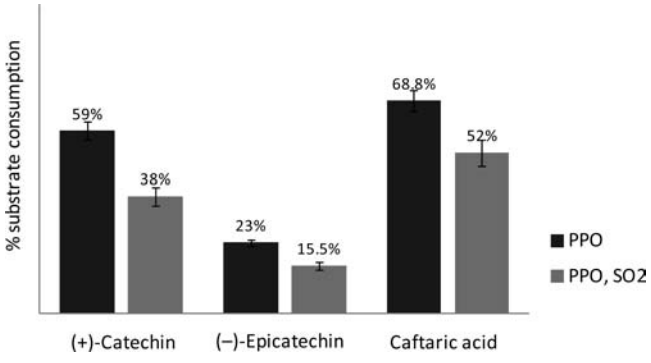

Figure 3. (+)-Catechin, (−)-epicatechin, and caftaric acid consumption (expressed in percentages) under enzymatic oxidation conditions after 3 days of incubation with 3-sulfanylhexan-1-ol and either in the presence or in the absence of SO_2.

For the three substrates, no lag period was observed at the beginning of the reaction (Figure 2). The addition of 3SH to both *o*-quinone-(+)-catechin and *o*-quinone-(−)-epicatechin followed a linear trend, whereas the *o*-quinone-caftaric acid/3SH adduct formation kinetics could be divided into two time periods. During the first period (phase I), from T_0 to T_{1day}, the formation rate of **7** was high and reached 80% of its final yield, and during phase II, from T_{1day} to T_{3days}, the reaction rate was two times slower than in phase I. In both kinetic phases, the formation of **7** followed a linear regression. The HPLC−UV monitoring of the reaction mixtures containing the starting flavan-3-ols showed that the formation of adducts between 3SH and either *o*-quinone-(+)-catechin or *o*-quinone-(−)-epicatechin had a similar rate. The rates of addition of 3SH to the 5′ or 6′ position of the B-ring of the oxidized (+)-catechin were also similar (≈2 mM). In the case of *o*-quinone-(−)-epicatechin, the attack of 3SH at the 5′ position (**5**, 1.2 mM) was favored over the 2′ position (**4a**/**b**, 0.7 mM). The formation rate of **7** was slightly higher than that of **2** and double that of **5**. In all flavan-3-ol model solutions, (+)-catechin/3SH and (−)-epicatechin/3SH, we observed the multiple addition of 3SH with no difference to the yield of the double adducts after three days of incubation (≈0.4 mM). The absence of multiple adducts in the case of *o*-quinone-caftaric acid could be explained by the fact that **7** is no longer a substrate for PPO, as previously observed with 2-*S*-glutathionylcaftaric acid (GRP).[31]

The addition of sulfur dioxide in the reaction media directly influenced the rates and yields of all adducts formed (Figures 2B; 2D; 2F). Under enzymatic oxidation conditions, SO_2reduces PPO activity, but at the same time, reacts with *o*-quinones to form *o*-dihydroxyphenols and sulfones.[33,34] When SO_2 was introduced in the must model system containing 3SH, we observed the suppression of every phenolic compound's

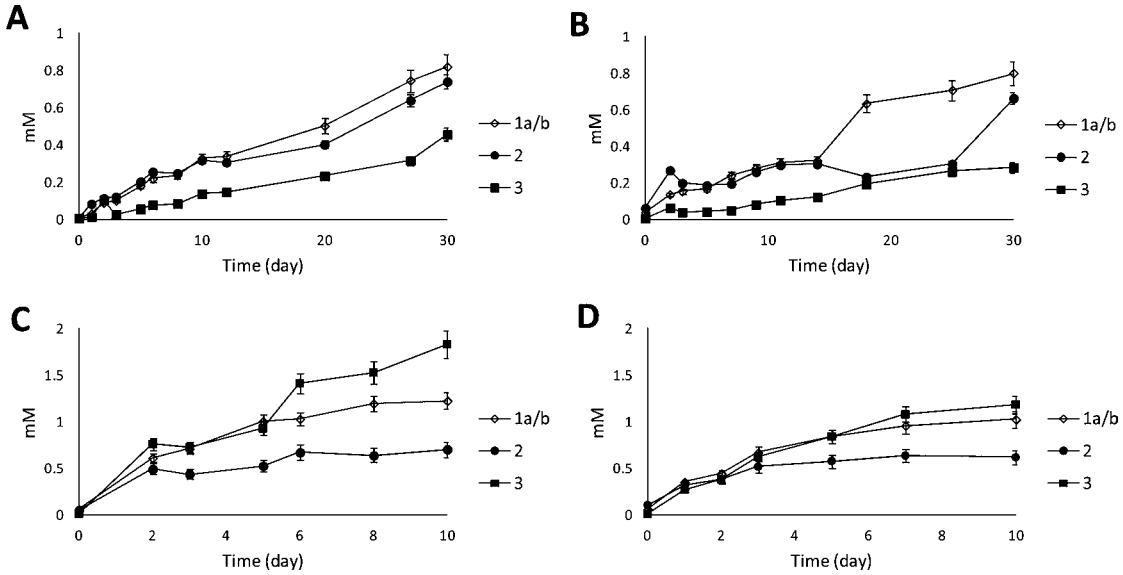

Figure 4. Kinetic formation rate of **1a**/**b**, **2**, and **3** under chemical oxidation conditions: (A) control; (B) with SO_2; (C) with iron(II) sulfate; (D) with iron(II) sulfate and SO_2. The compound numbering is defined in Figure 1. **1a**/**b** presents the summary of the **1a** and **1b** diastereoisomers.

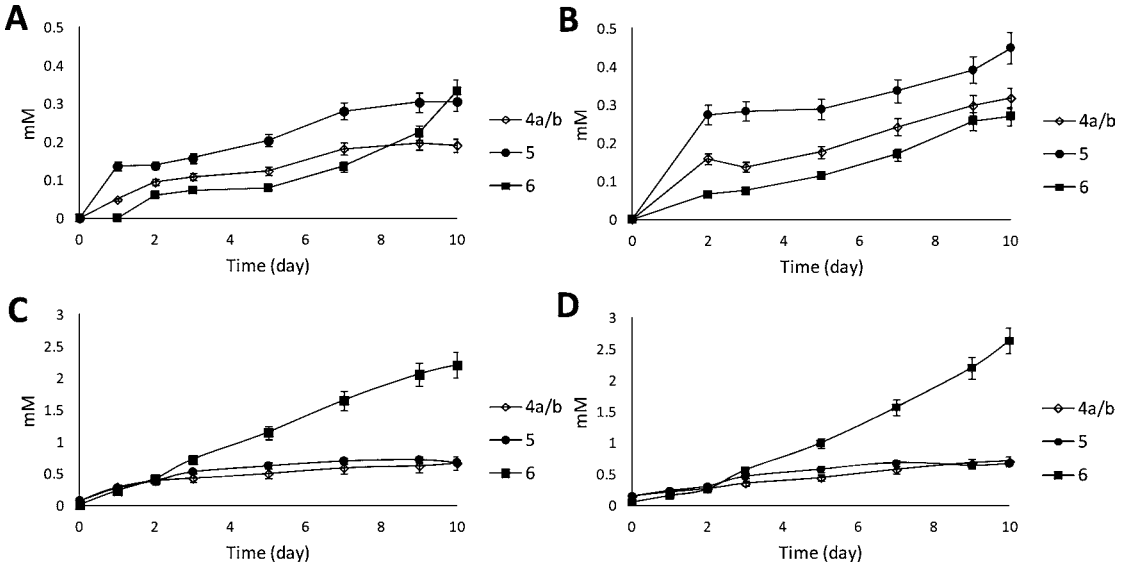

Figure 5. Kinetic formation rate of **4a**/**b**, **5**, and **6** under chemical oxidative conditions: (A) control; (B) with SO_2; (C) with iron(II) sulfate; (D) with iron(II) sulfate and SO_2. The compound numbering is defined in Figure 1. **4a**/**b** presents the summary of the **4a** and **4b** diastereoisomers.

consumption (Figure 3). Consequently, the formation rates and yields of all adducts decreased significantly. In the case of the flavanol substrates, the addition of SO_2 significantly slowed down the beginning of the reactions, with an equal duration in both cases (T_0 to T_{1day}). After one day of incubation, the production of the *o*-quinone-(−)-epicatechin/3SH adducts (**4a**/**b**, **5**) followed an exponential regression and that of the *o*-quinone-(+)-catechin/3SH adducts (**1a**/**b**, **2**) followed a linear curve (Figures 2B and 2D). The addition of SO_2 into the caftaric acid/3SH reaction mixture affected the kinetic profile of the reaction which displayed a linear regression fitting. In that case, the final yield of adduct **7** was two times less abundant than when obtained in the absence of SO_2, and this diminution was proportional to that of the caftaric acid consumption (Figure 2F).

Reactivity of 3-Sulfanylhexan-1-ol with (+)-Catechin, (−)-Epicatechin, and Caftaric Acid under Chemical Oxidation Conditions. The reactions of *o*-quinone-(+)-catechin, *o*-quinone-(−)-epicatechin, and *o*-quinone-caftaric acid with 3SH were also investigated in an air-saturated winelike solution. In all cases, the reactions were initiated by the addition of hydrogen peroxide into the reaction mixtures in order to initiate the Fenton reaction. Under these chemical oxidation conditions, the yields and rates of adduct formation were significantly lower than those observed under enzymatic oxidation conditions. It has been stated[35] that the production of *o*-quinones is faster under enzymatic oxidation than under autoxidation conditions, but once *o*-quinones were formed, their reactivity toward thiol addition was the same whether they originated from enzymatic or chemical oxidation. In wine acidic conditions (Figures 4 and 5), the rate of formation of the *o*-quinone-(−)-epicatechin/3SH adducts (**4a**/**b**, **5**, and **6**) was 13% higher than the one observed for the formation of the *o*-quinone-(+)-catechin/3SH adducts (**1a**/**b**, **2**, and **3**) at T_{10day}.

Using equimolar amounts of each flavan-3-ol (9 mM) with an excess of 3SH (12 mM), the thiol function addition to the B-ring of the oxidized (+)-catechin occurred equally at both the 2′ and 5′ positions (i.e., **1a**/**b**, 0.81 mM; **2**, 0.73 mM, Figure 4A). However, 3SH preferentially added to the 5′ position of the (−)-epicatechin-derived *o*-quinone (i.e., 0.35 mM for **5** vs 0.19 mM for **4a**/**b**) (Figure 5A). 3SH bisadducts were produced with significant difference (56%) in terms of yields after ten days of reaction (i.e., **3**, 0.13 mM, and **6**, 0.31 mM). The kinetic study of *o*-quinone-(−)-epicatechin/3SH adduct formation was stopped after T_{10day} since (−)-epicatechin autoxidation products dominated over the adduct formation (data not shown). This phenomenon was observed and amplified in the presence of iron(II) for both the (+)-catechin and (−)-epicatechin reaction mixtures.

The effect of SO_2 addition was also examined under these chemical oxidation conditions as shown in Figures 4 and 5. Clearly, the presence of SO_2 did not prevent the formation of 3SH adducts. However, the rate of the *o*-quinone-(+)-catechin/3SH reaction measured at T_{10day} was approximately 16% slower than that in the absence of SO_2 (Figures 4A and 4B). This result could explain the presence of a lag phase at the beginning of this reaction, in which the consumption of (+)-catechin was limited. Surprisingly, the presence of SO_2 did not modify the rate and yield of the *o*-quinone-(−)-epicatechin/3SH reaction at all (Figure 5B). According to the literature, catechols (i.e., 4-methylcatechol, caffeic acid, (+)-catechin) that are used/present at concentrations similar to those of our model reactions (i.e., 9 mM) appeared to be oxidized at a faster rate than SO_2, which then reacted with the hydrogen peroxide produced during the oxygen reduction.[36,37]

However, the kinetic study of the production of 3SH adducts derived from the oxidation of caftaric acid (Figure 6) again revealed the special and mutual reactivity of 3SH and the caftaric acid-derived *o*-quinone, as previously observed under enzymatic oxidation conditions. Under the control chemical oxidation conditions (H_2O_2, at 35 °C), the formation of **7** was observed, with a maximum yield at T_{2day}. This result also indicated a rate of formation for **7** that was much greater than those observed for **2** and **5** (i.e., 5-fold greater than for **5** and 15-fold greater than for **2**). Moreover, once the maximum yield of **7** was reached, no further conversion was observed.

The effect of iron(II) on the formation rates and yields of **1a**/**b**, **2**, **3**, **4a**/**b**, **5**, **6**, and **7** in the wine model media was then investigated. Similar experiments to those described above were performed in an oxygen saturated system, in which a 6-fold

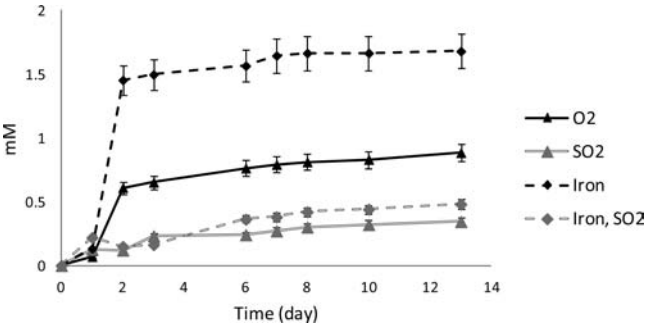

Figure 6. Kinetic formation rate of 7 under chemical oxidation conditions. Impact of sulfur dioxide and iron(II) sulfate. The compound numbering is defined in Figure 1.

molar excess of H_2O_2 was used relative to the quantity of iron(II) sulfate added. The presence of iron increased the final yields of all 3SH monoadducts derived from the oxidation of (+)-catechin (i.e., **1a**/**b** and **2**) (Figure 4C), (−)-epicatechin (i.e., **4a**/**b** and **5**), (Figure 5C) and caftaric acid (i.e., **7**) (Figure 6). However, the reaction rate was dependent upon the nature of the starting phenolic compound. The formation rates of **2** and **5** were increased about 2-fold and those of **7** and **1a**/**b** 2.5- and 4-fold, respectively. The kinetic profile of the formation of **7** was similar to that observed in the absence of iron salts (Fe(II) sulfate). However, iron increased the yield of **7**, by promoting *o*-quinone-caftaric acid production, but not the rate of thiol addition. In the presence of iron(II) sulfate, the addition of 3SH at position 6′ of the *o*-quinone-(+)-catechin was favored compared to that at position 5′ (Figure 4C). In contrast, no preference for the addition site of 3SH on *o*-quinone-(−)-epicatechin was observed in the presence of iron(II) sulfate (Figure 5C). Under these oxidative conditions (Fe(II), H_2O_2) a major production of multiple substitution adducts appeared with both *o*-quinone-(+)-catechin and *o*-quinone-(−)-epicatechin. Single adducts between 3SH and *o*-quinone-(+)-catechin or *o*-quinone-(−)-epicatechin appeared to be easily oxidized in the presence of Fenton reagents (Fe(II), H_2O_2), resulting in the formation of multiadducts. Moreover, the production of **6** was 17% higher than that of **3** after 10 days of incubation, indicating that *o*-quinone-(−)-epicatechin/3SH adducts could undergo coupled oxidation reactions more easily than the adducts of *o*-quinone-(+)-catechin/3SH.

However, the catalytic role played by iron(II) in oxidizing polyphenolic flavanoids was clearly demonstrated during the incubation period. In the presence of SO_2, the concentrations of **1a**/**b**, **2**, and **3** dropped slightly (Figure 4D), whereas the rates of formation of **4a**/**b**, **5** and **6** appeared unchanged (Figure 5D). In the presence of iron(II) and H_2O_2 and (−)-epicatechin, SO_2 became partially ineffective as an antioxidant.[17] This phenomenon might be related to the rapid decrease of free SO_2 and the resulting increase in sulfate in the medium due to the production of hydrogen peroxide: $H_2O_2 + HSO_3^- \rightarrow SO_4^{2-} + H^+ + H_2O$.[36] Moreover, as indicated in the literature, the autoxidation of SO_2 is initiated by coordination to a transition metal, such as Fe(III), to form a sulfite complex.[36] Electron transfer within the complex results in the formation of a sulfite radical with the release of Fe(II). The sulfite radical is then able to add to oxygen in order to produce the highly oxidizing peroxomonosulfite radical, which is capable of oxidizing sulfite to regenerate sulfite radicals and so continue the radical chain reaction.[36] Nevertheless, the level of caftaric acid oxidation surprisingly dropped sharply in the presence of SO_2 and a lag phase was observed for the formation of **7**, with the consequence of a maximum yield noticed at T_{6day} instead of T_{2day} (Figure 6).

Identification of Adducts between 3-Sulfanylhexan-1-ol and *o*-Quinones Derived from (+)-Catechin and Caftaric Acid in Must Supplemented with 3-Sulfanylhexan-1-ol. After the structural characterization of the adducts **1a**/**b**, **2**, **3**, **4a**/**b**, **5**, **6**, and **7**, and the mechanistic study performed in the model solution, the identification of these adducts in an enological matrix was then investigated. Considering the fact that under enzymatic oxidation conditions the yield of adducts was higher than under chemical oxidation conditions, the analysis was performed in a white native Sauvignon blanc must (Graves, 2008 vintage) freshly extracted at an industrial scale during grape pressing with a conventional white vinification process. Aliquots of 300 mL of must, containing 22 mg L^{-1} of (+)-catechin, 2.7 mg L^{-1} of (−)-epicatechin, 124 mg L^{-1} of caftaric acid, and 20 mg L^{-1} of free SO_2 were either supplemented or not with 3SH (100 μg L^{-1}) and 100 mg L^{-1} of pimaricine (natamycin), as an alcoholic fermentation inhibitor. Then, the mixtures were incubated at room temperature under stirring and while exposed to air for a period of 5 days. Each experiment was performed in duplicate.

The formation of the adducts of interest in this native must matrix was first verified and the kinetics of their formation was then monitored by HPLC coupled to mass spectrometry analysis. Acquisitions were carried out in negative ion mode with a full scan on the mass range 100−1000. After 24 h of incubation, adducts **2** and **7** were indeed generated as the major HPLC/ESI-MS-detected known products. The monitoring of their production in this complex matrix permitted us to observe evidence of a level of formation of the *o*-quinone-(+)-catechin/3SH adduct which was higher than that of the *o*-quinone-caftaric acid/3SH adduct, even though the initial concentration of (+)-catechin (i.e., 22 mg L^{-1}) was six times lower than that of caftaric acid (i.e., 120 mg L^{-1}) in the must matrix (Figure 7).

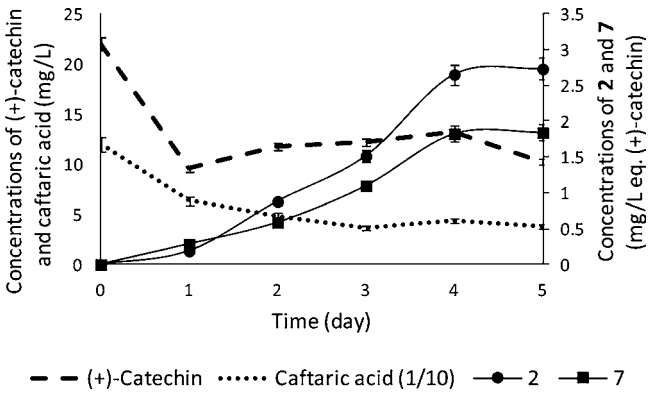

Figure 7. Kinetic formation rate of **2** and **7** as well as (+)-catechin and caftaric acid consumption in white must under enzymatic oxidative conditions. The compound numbering is defined in Figure 1.

Caftaric acid was also noticed to be oxidized more rapidly than (+)-catechin in the must, but its consumption was not proportional to the formation of **7**. It would thus seem that the caftaric acid-derived *o*-quinones in such a matrix have a greater affinity to other nucleophiles than to 3SH. The disappearance of caftaric acid could alternatively be related to the formation of different products with other phenolic

compounds that are formed more easily such as caftaric acid oligomers.[32,38] Thus, among the phenolic compounds investigated herein, oxidized flavan-3-ols seemed to be the most efficient trapping agents for volatile thiols. The consequences of such mechanisms during white wine vinification should have a significant impact on the level of volatile thiols in wines.

■ ASSOCIATED CONTENT

Supporting Information

Detailed descriptions of NMR spectra of all the new isolated compounds. This material is available free of charge via the Internet at http://pubs.acs.org.

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Funding

We thank the Bordeaux Wine Council (CIVB) and Aquitaine Council for their financial support.

Notes

The authors declare no competing financial interest.

■ ACKNOWLEDGMENTS

We express our thanks to Dr. Cédric Saucier, Dr. Dorothée Lefeuvre, and Laura Montero for contributing to some preliminary data for this study.

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