Quantitative Studies on the Formation of Phenol/2-Furfurylthiol Conjugates in Coffee Beverages toward the Understanding of the Molecular Mechanisms of Coffee Aroma Staling

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To gain a more comprehensive knowledge of the contribution of recently identified phenol/thiol conjugates to the storage-induced degradation of odorous thiols, the concentrations of the sulfury-roasty smelling key odorant 2-furfurylthiol and the concentrations of the putative thiol-receptive di- and trihydroxybenzenes pyrogallol (**1**), hydroxyhydroquinone (**2**), catechol (**3**), 4-ethylcatechol (**4**), 4-methylcatechol (**5**), and 3-methylcatechol (**6**), as well as of the phenol/thiol conjugates 3-[(2-furylmethyl)sulfanyl]catechol (**7**), 3-[(2-furylmethyl)sulfanyl]-5-ethylcatechol (**8**), 4-[(2-furylmethyl)-sulfanyl]hydroxyhydroquinone (**9**), and 3,4-bis[(2-furylmethyl)sulfanyl]hydroxyhydroquinone (**10**) were quantitatively determined in fresh and stored coffee beverages by means of stable isotope dilution analyses (SIDA). Although **2** was found to be the quantitatively predominant trihydroxybenzene in freshly prepared coffee brew, this compound exhibited a very high reactivity and decreased rapidly during coffee storage to generate the conjugates **9** and **10**. After only 10 min, about 60% of the initial amount of 2-furfurylthiol in a coffee beverage reacted with **2** to give **9** and **10**. In contrast, conjugate **7** was found to be exclusively formed during coffee roasting because its initial concentration as well as the amount of its putative precursor, phenol **3**, was not affected by storage. It is interesting to note that the concentration of **8** was increased with increasing incubation time, but its putative precursor **4** was not affected, thus indicating another formation pathway most likely via the chlorogenic acid degradation product 4-vinylcatechol. This study demonstrates for the first time that the loss of 2-furfurylthiol during coffee storage is mainly due to the oxidative coupling of the odorant to hydroxyhydroquinone (**2**), giving rise to the conjugates **9** and **10**.

KEYWORDS: Coffee; aroma; 2-furfurylthiol; catechol; hydroxyhydroquinone; 4-ethylcatechol; 4-vinyl-catechol; quinones

INTRODUCTION

In addition to its stimulatory and potential health-promoting effects, the wide popularity of a freshly brewed roast coffee beverage is mainly based on its pleasant, attractive overall aroma. Unfortunately, the freshness of the brew cannot be preserved because the desirable aroma, in particular, the roasty-sulfury note, is rather unstable and changes shortly after preparation of the coffee brew (*1−4*).

In particular, the concentration of the key coffee odorant 2-furfurylthiol, contributing to the sulfury-roasty odor quality of coffee brew, was found to be drastically reduced upon coffee processing or storage (*3*). Comparative aroma dilution analysis revealed that besides 2-furfurylthiol, decreases of 3-methyl-2-butene-1-thiol, 3-mercapto-3-methylbutyl formate, 2-methyl-3-

furanthiol, and methanethiol are also responsible for the aroma change (*1−4*). Such thiols have also been identified to be involved in the aroma changes induced by the instant process (*5*) as well as by sterilization of canned coffee beverages (*6*).

Various coffee ingredients were investigated in previous studies for their influence on 2-furfurylthiol degradation; for example, 1,4-bis(5-amino-5-carboxy-1-pentylpyrazinium) radical cations (*7−9*), as part of the macromolecular coffee melanoidins, have been demonstrated to covalently bind 2-furfurythiol in model solutions (*2, 3*). Recent "in bean" model roast experiments confirmed for the first time that thermal degradation products liberated from chlorogenic acids are the key compounds involved in thiol degradation but not the melanoidins (*10*). Among the chlorogenic acid degradation products, in particular, the di- and trihydroxybenzenes pyrogallol (**1**) (**Figure 1**), hydroxyhydroquinone (**2**), catechol (**3**), 4-ethylcatechol (**4**), 4-methylcatechol (**5**), and 3-methylcatechol (**6**) have been demonstrated by means of LC-MS/MS and NMR studies to

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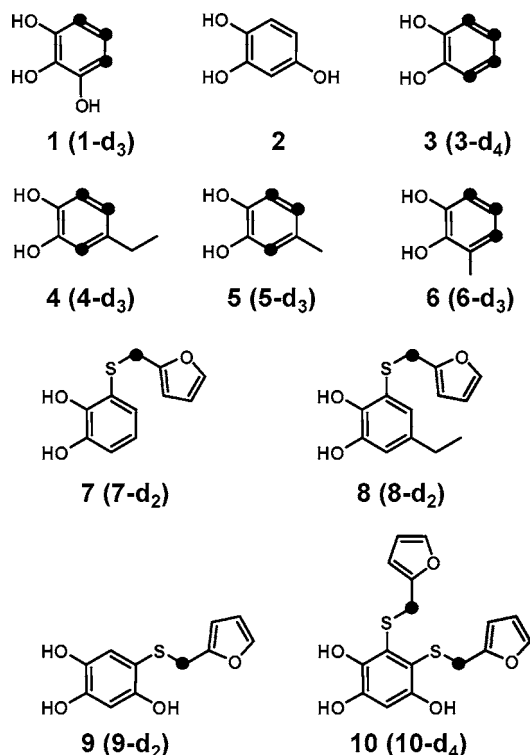† Universität Münster.
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Figure 1. Chemical structures of the di- and trihydroxybenzenes pyrogallol (**1**), hydroxyhydroquinone (**2**), catechol (**3**), 4-ethylcatechol (**4**), 4-methylcatechol (**5**), and 3-methylcatechol (**6**), and the phenol/thiol conjugates 3-((2-furylmethyl)sulfanyl)catechol (**7**), 3-((2-furylmethyl)sulfanyl)-5-ethylcatechol (**8**), 4-((2-furylmethyl)sulfanyl)hydroxyhydroquinone (**9**), and 3,4-bis((2-furylmethyl)sulfanyl))hydroxyhydroquinone (**10**). (•) The marked positions indicate the carbon atoms substituted with deuterium atoms in the stable isotope labeled standards (numbering given in parentheses).

generate 25 covalent phenol/thiol conjugates when these phenols were incubated in the presence of air oxygen and transition metals (*11*). Moreover, LC-MS/MS analysis performed in the multiple reaction monitoring mode led for the first time to the identification of four phenol/thiol conjugates, namely, 3-[(2-furylmethyl)sulfanyl]catechol (**7**) (**Figure 1**), 3-[(2-furylmethyl)sulfanyl]-5-ethylcatechol (**8**), 4-[(2-furylmethyl)sulfanyl]-hydroxyhydroquinone (**9**), and 3,4-bis[(2-furylmethyl)sulfanyl]-hydroxyhydroquinone (**10**) in coffee brew (*12*).

To investigate the contribution of phenol/thiol conjugate formation to the decrease of 2-furfurylthiol concentration during coffee storage, this study was conducted to quantify the concentrations of the putative thiol-trapping di- and trihydroxy-benzenes **1**–**6** and the key odorant 2-furfurylthiol, as well as the phenol/thiol conjugates **7**–**10** in coffee beverages by means of stable isotope dilution analyses (SIDA).

MATERIALS AND METHODS

Chemicals. The following compounds were obtained commercially: hydroxyhydroquinone, pyrogallol, catechol, 4-methylcatechol, 3-methylcatechol, 2-furfurylthiol, iron(III) chloride, furoyl chloride, lithium aluminum deuteride, 4-vinylpyridine (Sigma-Aldrich, Steinheim, Germany); diethyl ether, thiourea, hydrochloric acid, acetonitrile, ethyl acetate, formic acid, l-cysteine, sodium hydroxide, sodium sulfate (Merck KGA, Darmstadt, Germany); and 4-ethylcatechol (Lancaster, Eastgate, U.K.). Acetonitrile was of HPLC grade, and water was of Millipore grade. [^{2}H]$_3$Pyrogallol (**1-d$_3$**), [^{2}H]$_4$catechol (**3-d$_4$**), [^{2}H]$_3$-4-ethylcatechol (**4-d$_3$**), [^{2}H]$_3$-4-methylcatechol (**5-d$_3$**), and [^{2}H]$_3$-3-methylcatechol (**6-d$_3$**) were synthesized by closely following a procedure developed earlier (*13*). 3-[(2-Furylmethyl)sulfanyl]catechol (**7**), 3-[(2-

furylmethyl)sulfanyl]-5-ethylcatechol (**8**), 4-[(2-furylmethyl)sulfanyl]-hydroxyhydroquinone (**9**), and 3,4-bis[(2-furylmethyl)sulfanyl]hydroxy-hydroquinone (**10**) were synthesized as reported recently (*12*). [^{2}H]$_2$-2-Furfurylthiol was synthesized following the procedure reported in the literature (*14*). Coffee beans (Arabica, Colombia), espresso coffee, and instant coffee were obtained from the food industry. Light (CTN value 150), medium (CTN value 86), and dark roasted coffee beans (CTN value 56) were obtained from the food industry.

Roasting and Grinding of Coffee Beans. Prior to roasting, coffee beans were freeze-dried for 48 h to adjust to the same humidity of 6.0%. The raw coffee beans (50 g) were then roasted at 240 °C by means of a Probat BRZ II-type batch roaster (Emmerich, Germany) for 4.5 min and, after cooling to room temperature, were ground by means of a batch mill (IKA, Staufen, Germany).

Preparation of Coffee Beverages. For the preparation of the coffee brew, the powder obtained from the freshly roasted coffee (54 g) was percolated with hot water (1.1 L, 88−92 °C) using a Kaffeefilterpapier no. 4 drip filter (Aldi Einkauf GmbH & Co. oHG, Essen, Germany). Espresso coffee was prepared from a fully automatic coffee machine (60 mL/cup) using commercially available espresso beans. For the preparation of instant coffee and instant espresso, a sample (1.5 g) of the instant powder was dissolved in hot water (100 mL, 88−92 °C). After preparation, the brews were immediately cooled to room temperature in an ice bath and used for analysis.

Synthesis and Preparative Separation of ^{2}H-Labeled Phenol/Thiol Conjugates 7-d$_2$, 8-d$_2$, 9-d$_2$, and 10-d$_4$. Following a procedure reported recently for the synthesis of the nonlabeled analogues (*12*), an aqueous solution of iron(III) chloride (0.5 mmol; 50 mL) was dropped into a binary solution of [^{2}H]$_2$-2-furfurylthiol (0.45 mmol) and either catechol, 4-ethylcatechol, or hydroxyhydroquinone (0.5 mmol each) in water (200 mL) over a period of 30 min with stirring. After additional stirring for 30 min, ethyl acetate (100 mL) was added and stirring was continued for an additional 10 min. The organic layer was separated, and the aqueous phase was extracted with additional ethyl acetate (3 × 150 mL). The combined organic fractions were dried over anhydrous Na$_2$-SO$_4$, filtered, and, finally, freed from solvent in a vacuum to obtain the crude reaction products. The residue was dissolved in a mixture (6 mL; 1:1, v/v) of acetonitrile and 1% formic acid in water and separated by preparative HPLC on a 250 × 21.2 mm i.d., Phenyl-Hexyl Luna column (Phenomenex, Aschaffenburg, Germany). Monitoring the effluent at a wavelength of 280 nm, chromatography was performed starting with a mixture (85:15, v/v) of aqueous formic acid (1% in water; solvent A) and acetonitrile (solvent B) for 2 min, increasing B to 60% within 18 min, followed by an increase of B to 100% within 5 min, and, finally, maintaining B at 100% for 3 min. In the case of **9-d$_2$** and **10-d$_4$**, chromatography was performed by extending the time to increase B from 15 to 60% from 18 to 23 min. The effluent containing the title compounds [^{2}H]$_2$-3-[(2-furylmethyl)sulfanyl]catechol (**7-d$_2$**), [^{2}H]$_2$-3-[(2-furylmethyl)sulfanyl]-5-ethylcatechol (**8-d$_2$**), [^{2}H]$_2$-4-[(2-furylmethyl)sulfanyl]hydroxyhydroquinone (**9-d$_2$**), and [^{2}H]$_2$-3,4-bis[(2-furylmethyl)sulfanyl]hydroxyhydroquinone (**10-d$_4$**) was collected, the solvent amount was reduced in vacuum to about 20 mL, and, after addition of water (100 mL), the aqueous solutions were extracted with ethyl acetate (3 × 100 mL). The combined ethyl acetate fractions were washed with water (20 mL) and dried over anhydrous sodium sulfate. After removing the solvent in vacuum, the compounds **7-d$_2$** (0.07 mmol, 16% yield), **8-d$_2$** (0.08 mmol, 18% yield), **9-d$_2$**(0.04 mmol, 9% yield), and **10-d$_4$** (0.04 mmol, 9% yield) were obtained as colorless oils and stored as solutions in acetonitrile at −18 °C until use.

[^{2}H]$_2$-3-[(2-Furylmethyl)sulfanyl]catechol, 7-d$_2$ (Figure 1): LC-MS (ESI$^-$), *m/z* 223 (100, [M − H]$^-$); MS/MS (−30 V), *m/z* 140 (100), 114 (8); ^{1}H NMR (400 MHz, CDCl$_3$), δ 5.99 [d, 1H, *J* = 3.2 Hz, H−C(9)], 6.23 [dd, 1H, *J* = 1.8, 3.2 Hz, H−C(10)], 6.58 [t, 1H, *J* = 7.9 Hz, H−C(5)], 6.71 [dd, 1H, *J* = 1.5, 8.0 Hz, H−C(6)], 6.73 [dd, 1H, *J* = 1.5, 8.0 Hz, H−C(4)], 7.35 [dd, 1H, *J* = 0.9, 1.8 Hz, H−C(11)]; ^{13}C NMR (100 MHz, CDCl$_3$, HMQC, HMBC), δ 33.6 [C(7)], 107.2 [C(9)], 110.0 [C(10)], 115.0 [C(4/6)], 119.2 [C(5)], 119.5 [C(3)], 124.5 [C(4/6)], 141.5 [C(11)], 145.0 [C(1/2)], 145.3 [C(1/2)], 151.3 [C(8)].

[^{2}H]$_2$-3-[(2-Furylmethyl)sulfanyl]-5-ethylcatechol, 8-d$_2$ (Figure 1): LC-MS (ESI$^-$), *m/z* 251 (100, [M − H]$^-$); MS/MS (−30V), *m/z* 168

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(100), 153 (11), 140 (3), 134 (3); ^{1}H NMR (400 MHz, MeOD), δ 1.10 [t, 3H, J = 7.6 Hz, H$-$C(13)], 2.40 [q, 2H, J = 7.6 Hz, H$-$C(12)], 5.96 [d, 1H, J = 3.2 Hz, H$-$C(9)], 6.22 [dd, 1H, J = 1.8, 3.1 Hz, H$-$C(10)], 6.51 [d, 1H, J = 1.8 Hz, H$-$C(5)], 6.57 [d, 1H, J = 1.8 Hz, H$-$C(3)], 7.34 [dd, 1H, J = 0.8, 1.8 Hz, H$-$C(11)]; ^{13}C NMR (100 MHz, MeOD, HMQC, HMBC), δ 14.7 [C(13)], 27.6 [C(12)], 30.4 [C(7)], 107.2 [C(9)], 109.9 [C(10)], 114.8 [C(3)], 119.3 [C(6)], 123.7 [C(5)], 135.5 [C(4)], 141.8 [C(11)], 143.1 [C(1), C(2)], 151.3 [C(8)].

[^{2}H]$_2$-4-[(2-Furylmethyl)sulfanyl]hydroxyhydroquinone, 9-d$_2$ (Figure 1): LC-MS (ESI$^-$), m/z 239 (100, [M $-$ H]$^-$); MS/MS ($-$30 V), m/z 156 (100), 128 (6), 123 (45); ^{1}H NMR (400 MHz, MeOD), δ 5.91 [d, 1H, J = 3.2 Hz, H$-$C(9)], 6.21 [dd, 1H, J = 2.0, 3.0 Hz, H$-$C(10), 6.33 [s, 1H, H$-$C(3)], 6.62 [s, 1H, H$-$C(6)], 7.33 [dd, 1H, J = 0.7, 1.9 Hz, H$-$C(11)]; ^{13}C NMR (100 MHz, MeOD, HMQC, HMBC), δ 31.8 [C(7)], 102.3 [C(3)], 107.2 [C(9)], 109.6 [C(10)], 121.7 [C(6)], 122.1 [C(5)], 138.0 [C(1)], 141.8 [C(11)], 147.5 [C(2)], 151.3 [C(4)], 151.4 [C(8)].

[^{2}H]$_4$-3,4-Bis[(2-furylmethyl)sulfanyl]hydroxyhydroquinone, 10-d$_4$ (Figure 1): LC-MS (ESI$^-$), 353 (100, [M $-$ H]$^-$); MS/MS ($-$30 V), m/z 270 (6), 187 (100); ^{1}H NMR (400 MHz, MeOD), δ 5.83 [d, 1H, J = 3.1 Hz, H$-$C(9/9')], 5.93 [d, 1H, J = 2.9 Hz, H$-$C(9/9')], 6.19 [dd, 1H, J = 1.8, 3.1 Hz, H$-$C(10/10')], 6.20 [dd, 1H, J = 1.8, 3.1 Hz, H$-$C(10/10')], 6.36 [s, 1H, H$-$C(3)], 7.32 [m, 2H, H$-$C(11,11')]; ^{13}C NMR (100 MHz, MeOD, HMQC, HMBC), δ 30.9 [C(7/7')], 31.8 [C(7/7')], 102.7 [C(3)], 107.3 [C(9/9')], 107.5 [C(9/9')], 110.1 [C(10,-10')], 111.1 [C(5)], 123.7 [C(6)], 140.8 [C(1)], 141.8 [C(11,11')], 147.5 [C(2)], 151.2 [C(8,8')], 152.3 [C(4)].

Quantitation of Di- and Trihydroxybenzenes in Coffee Brews by Means of SIDA. An aliquot (20 mL) of a freshly prepared filtered coffee brew was maintained in a temperature-controlled, septum-sealed vessel (180 mL) at 30 °C in either the absence or the presence of an aqueous solution of the odorant 2-furfurylthiol (1.0 mL; 500 μg in 0.1 mol/L phosphate buffer; pH 5.7). As reported earlier (*13*), the coffee brew incubated for 0, 10, 20, or 60 min, respectively, was placed into an 80 mL centrifugation tube with a screw cap (Schott AG, Mainz, Germany), defined amounts of the internal standards **1-d$_3$**, **3-d$_4$**, **4-d$_3$**, **5-d$_3$**, and **6-d$_3$** were added, and, after the tube had been closed, the sample was equilibrated for 5 min at room temperature with stirring. The samples were then extracted with diethyl ether (20 mL) and centrifuged (3000 rpm) for 10 min; the organic phase was freed from the solvent in vacuum; and the residue was taken up in methanol/water (1:1, v/v; 1 mL), membrane filtered (0.45 μm), and, finally, analyzed by means of LC-MS/MS. Following the same procedure, the di- and trihydroxybenzenes were quantitatively determined in instant coffee and instant espresso beverages (20 mL).

Solutions of the deuterated standards **1-d$_3$**, **3-d$_4$**, **4-d$_3$**, **5-d$_3$**, and **6-d$_3$** and the analytes **1**$-$**6** were prepared in five concentration ratios from 0.2 to 5.0 prior to analysis. Calibration curves were prepared by plotting peak area ratios of analyte to internal standard against concentration ratios of each analyte to the internal standard using linear regression. Hydroxyhydroquinone (**2**) was calibrated with **1-d$_3$** as internal standard over a range of concentration ratios from 0.2 to 5.0.

The following mass transitions and retention times (both given in parentheses) were recorded for the deuterium-labeled internal standards and the nonlabeled analytes: **1** (125$\rightarrow$69; 5.52 min), **1-d$_3$** (128$\rightarrow$72; 5.36 min), **2** (125$\rightarrow$79; 3.77), **3** (109$\rightarrow$91; 19.79 min), **3-d$_4$** (113$\rightarrow$94; 19.19 min), **4** (137$\rightarrow$122; 21.32 min), **4-d$_3$** (140$\rightarrow$125; 21.29 min), **5** (123$\rightarrow$108; 19.49 min), **5-d$_3$** (126$\rightarrow$111; 19.41 min), **6** (123$\rightarrow$108; 20.08 min), **6-d$_3$** (126$\rightarrow$111; 19.97 min). After sample injection (5 μL), chromatographic separation was carried out on a 150 × 2.5 mm i.d., Phenyl-Hexyl Luna column (Phenomenex, Aschaffenburg, Germany) with gradient elution at a flow rate of 0.25 mL/min. Eluent A was 0.5% formic acid in methanol, and eluent B was 0.5% formic acid in water. For chromatography, eluent A was kept isocratically at 2% for 3 min, then increased linearly to reach 15% after 10 min, followed by a linear increase to 100% within additional 10 min, and, finally, maintained isocratically at 100% for 9 min.

Quantitation of 2-Furfurylthiol in Coffee Brew after Derivatization with 4-Vinylpyridine by Means of SIDA. An aliquot of the freshly prepared and cooled coffee brew (100 mL) was spiked with defined amounts of the internal standard [^{2}H]$_2$-2-furfurylthiol dissolved in methanol, freshly distilled 4-vinylpyridine (10 mmol) was added, and the pH value was adjusted to 7.5 with aqueous sodium hydroxide (2 mol/L). After 3 h of stirring in the dark at room temperature, L-cysteine was added (10 mmol), and the solution was stirred for another 1 h in the dark and, then, extracted with diethyl ether (5 × 30 mL). The combined diethyl ether fractions were extracted with aqueous sodium hydroxide solution (2 × 20 mL; 1 mol/L), the aqueous layer was discarded, and the organic layer was extracted with aqueous hydrochloric acid (3 × 20 mL; 1 mol/L). The acidic aqueous fraction was adjusted to pH 10 with sodium hydroxide (1 mol/L) and extracted with diethyl ether (5 × 30 mL); the combined diethyl ether fractions were washed with brine, dried over anhydrous sodium sulfate, and, finally, concentrated to about 100 μL in a vacuum. An aliquot (1.0 μL) was used for mass chromatography by means of HRGC-MS analysis of the nonlabeled (m/z 220) and labeled 4-[2-(2-furylmethyl-sulfanyl)ethyl] pyridine (m/z 222), respectively. Solutions of [^{2}H]$_2$-2-furfurylthiol and 2-furfurylthiol were prepared in three concentration ratios from 0.2 to 5 prior to derivatization and analysis. Calibration curves were prepared by plotting peak area ratios of analyte to internal standard against concentration ratios of each analyte to the internal standard using linear regression. Analyte concentrations in coffee samples are calculated from the corresponding linear equation.

Quantitation of Phenol/Thiol Conjugates 7$-$10 in Coffee Brews by Means of SIDA. Aliquots (100 mL) of coffee brews freshly prepared from light, medium, and dark roasted coffee beans, instant coffee, instant espresso, or aliquots (60 mL) of espresso-type coffee prepared from a fully automatic coffee machine were used as samples. These samples either were used directly for quantitative analysis of compounds **7**$-$**10** or were incubated for 0, 10, 20, or 60 min, respectively, in a temperature-controlled, septum-sealed vessel (180 mL) at 30 or 60 °C in the absence or presence of an added aqueous solution of 2-furfurylthiol (0.2$-$10 mL; 500 μg/mL in 0.1 mol/L phosphate buffer; pH 5.70). To these samples were added defined amounts of the internal standards **7-d$_2$**, **8-d$_2$**, **9-d$_2$**, and **10-d$_4$**, and the samples were equilibrated for 10 min at room temperature with stirring under an atmosphere of argon. After dilution with water (1:5, v/v) and addition of sodium chloride (20 g), each solution was extracted with ethyl acetate (3 × 200 mL); the combined organic layers were freed from solvent in vacuum; and the residue was taken up in a mixture (4 mL; 1:1, v/v) of acetonitrile and aqueous formic acid (1% in water), membrane filtered (0.45 μm), and, finally, analyzed by means of HPLC-MS/MS.

Solutions of the deuterated standards and the analytes were prepared in five concentration ratios from 0.2 to 5.0 prior to analysis. Calibration curves were prepared by plotting peak area ratios of analyte to internal standard against concentration ratios of each analyte to the internal standard using linear regression.

The following mass transitions, retention times, declustering potential (DP), collision energy (CE), and cell exit potential (CXP) (given in parentheses) were used for the deuterium-labeled internal standards and the nonlabeled analytes: **7** (221$\rightarrow$140; 16.34 min; DP/CE/CXP $-$60/$-$40/$-$15), **7-d$_2$** (223$\rightarrow$140; 16.13 min; DP/CE/CXP $-$60/$-$40/$-$15), **8** (249$\rightarrow$168; 26.48 min; DP/CE/CXP $-$55/$-$20/$-$10), **8-d$_2$** (251$\rightarrow$168; 26.44 min; DP/CE/CXP $-$55/$-$20/$-$10), **9** (237$\rightarrow$156; 18.06 min; DP/CE/CXP $-$60/$-$25/$-$12), **9-d$_2$** (239$\rightarrow$156; 18.03 min; DP/CE/CXP $-$60/$-$25/$-$12), **10** (349$\rightarrow$268; 26.76 min; DP/CE/CXP $-$60/$-$20/$-$16), and **10-d$_4$** (353$\rightarrow$270; 26.72 min; DP/CE/CXP $-$60/$-$20/$-$16). After sample injection (5 μL), chromatographic separation was carried out on a 150 × 2.5 mm i.d., Phenyl-Hexyl Luna column (Phenomenex) with gradient elution at a flow rate of 0.25 mL/min. Eluent A was acetonitrile, and eluent B was 1% formic acid in water. For chromatography, eluent A was held at 15% for 5 min, then increased linearly to 80% within 30 min, then to 100% within 1 min, and, finally, maintained at 100% for 9 min.

High-Performance Liquid Chromatography$-$Tandem Mass Spectrometry (HPLC-MS/MS). The Agilent 1100 series HPLC system consisted of a pump, a degasser, and an autosampler (Agilent, Waldbronn, Germany) and was connected to a 4000 Q Trap triple-quadrupole/linear ion trap mass spectrometer (Applied Biosystems/MDS Sciex, Darmstadt, Germany) with an electrospray ionization (ESI) device running in negative ionization mode. The nebulizer and the
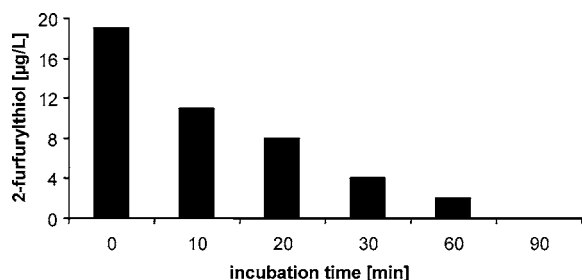
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Figure 2. Influence of storage time on the concentration of 2-furfurylthiol in a coffee beverage incubated for up to 90 min at 30 °C in a septum-sealed vessel.

curtain gas were set to 35 and 45 psi, respectively, and the detection was performed in the multiple reaction monitoring (MRM) mode, recording the transition from the negative pseudo-molecular ion [M − H]⁻ to the main fragment generated after collision-induced dissociation. The quadrupoles operated at unit mass resolution. Sciex Analyst software (v1.4) was used for instrumentation control and data collection.

High-Resolution Gas Chromatography−Mass Spectrometry (HRGC-MS). HRGC-MS analysis of deuterium-labeled and nonlabeled 2-furfurylthiol as the corresponding 4-[2-(2-furylmethylsulfanyl)ethyl]pyridines was performed on a 30 m × 0.25 mm i.d. J&W DB-FFAP, 0.25 μm, fused silica capillary (Agilent, Waldbronn, Germany) located in a HP6890-type gas chromatograph (Agilent) coupled to a HP5973-type mass spectrometric detector (Agilent) operating in the chemical ionization mode with isobutane as the reactant gas. Using helium as the carrier gas at a flow rate of 1.8 mL/min, chromatographic separation was performed by keeping the oven temperature at 40 °C for 2 min, raising it to 50 °C at a rate of 40 °C/min, holding it for 2 min, then raising it to 240 °C at a rate of 6 °C/min, and holding it for 10 min.

Nuclear Magnetic Resonance (NMR) Spectroscopy. NMR spectra were recorded on a Bruker DMX-400 spectrometer (Bruker, Rheinstetten, Germany). Using methanol-d_4 and CDCl₃ as solvents, chemical shifts were determined using tetramethylsilane (TMS) as the internal standard.

RESULTS AND DISCUSSION

To investigate the contribution of phenol/thiol reactions to the decrease of 2-furfurylthiol concentration during storage of coffee, we first studied the influence of storage time on the concentrations of the odorant 2-furfurylthiol and the putative thiol-receptive di- and trihydroxybenzenes **1**−**6** as well as the phenol/thiol-conjugates **7**−**10** in coffee beverages by means of stable isotope dilution analyses.

Influence of Storage Time on the Concentration of 2-Furfurylthiol in Coffee Beverages. To visualize the time course of 2-furfurylthiol degradation during storage of a coffee beverage on a quantitative basis, the odorous thiol was quantitatively determined by means of a SIDA by spiking the coffee samples with distinct amounts of the internal standard [²H]₂furfurylthiol (*14*) prior to workup and analysis. To overcome problems with the instability of 2-furfurylthiol and to measure accurately the actual concentration of the "free" thiol in coffee samples, the analyte 2-furfurylthiol as well as the internal standard [²H]₂-2-furfurylthiol were trapped by the addition of 4-vinylpyridine as their stable thioether derivatives following an analytical procedure reported earlier (*15*) and then analyzed quantitatively by HRGC-MS.

To quantify the concentrations of 2-furfurylthiol in coffee at various storage times, aliquots of a freshly prepared coffee brew were incubated for 0, 10, 20, 30, 60, and 90 min at 30 °C prior to analysis (**Figure 2**). Starting with a concentration of 19 μg/L in the freshly prepared coffee beverage, the amount of 2-furfurylthiol decreased rapidly with increasing storage time, for

Table 1. Influence of the Incubation Time on Concentrations of Pyrogallol (**1**), Hydroxyhydroquinone (**2**), Catechol (**3**), 4-Ethylcatechol (**4**), 4-Methylcatechol (**5**), and 3-Methylcatechol (**6**) in Coffee Beverages

incubation time (min)	concentration[a] (mg/L) of di- and trihydroxybenzene					
	1	**2**	**3**	**4**	**5**	**6**
	Without Additive					
0	2.80	30.04	4.72	1.17	0.30	1.22
	(±0.25)	(±2.50)	(±0.30)	(±0.22)	(±0.05)	(±0.15)
10	2.86	22.03	4.77	1.15	0.37	1.25
20	2.72	17.53	4.66	1.13	0.35	1.29
60	2.78	1.04	4.45	1.12	0.32	1.21
	After Addition of 2-Furfurylthiol					
0	2.80	30.04	4.72	1.17	0.30	1.22
10	2.69	12.20	4.73	1.16	0.35	1.20
20	2.88	9.73	4.75	1.21	0.29	1.30
60	2.82	<0.1	4.73	1.19	0.30	1.28

[a] Data are given as the mean of replicates.

example, >50% of the initial amount of the thiol was degraded after only 20 min of storage, and after 90 min not even trace amounts of 2-furfurylthiol were detectable. To investigate whether the decrease in 2-furfurylthiol concentration is somehow related to the degradation of di- and trihydroxybenzenes, the phenols **1**−**6** were quantitatively determined in coffee beverages differing in their storage time.

Influence of the Storage Time on the Concentrations of Di- and Trihydroxybenzenes in Coffee Beverages. Coffee brews were maintained at 30 °C for 0, 10, 20, and 60 min and, after the samples had been spiked with defined amounts of the internal standards **1-d₃, 3-d₄, 4-d₃, 5-d₃,** and **6-d₃** (**Figure 1**), the analytes **1**−**6** as well as the internal standards were analyzed by means of HPLC-MS/MS running in the MRM mode. As shown in **Table 1**, hydroxyhydroquinone (**2**) was found to be the quantitatively predominant trihydroxybenzene with a concentration of about 30 mg/L in the freshly prepared coffee beverage. With increasing storage time, the concentration of **2** decreased rapidly, thus reaching a concentration of only 1 mg/L after 60 min of storage. In comparison, the concentrations of catechol (**3**) and 4-ethylcatechol (**4**) decreased only slightly, and the levels of pyrogallol (**1**), 4-methylcatechol (**5**), and 3-methylcatechol (**6**) were found to be rather constant during the first 60 min of storage. In conclusion, these data demonstrate for the first time the extraordinarily high reactivity of hydroxyhydroquinone (**2**) in coffee.

To answer the question as to what extent the concentrations of di- and trihydroxybenzenes in coffee are influenced by the presence of thiols, the odor-active 2-furfurylthiol (500 μg/20 mL coffee brew) was added to coffee prior to incubation at 30 °C. Quantitative analysis of the phenols **1**−**6** by means of SIDA revealed that the concentration of hydroxyhydroquinone (**2**) decreased even more rapidly in the presence of added 2-furfurylthiol compared to the coffee brew incubated without added thiol; for example, after 20 min, >70% of compound **2** was already degraded (**Table 1**). It is interesting to note that again the concentrations of the other di- and trihydroxybenzenes (**1, 3**−**6**) were not significantly influenced during storage. These findings clearly confirmed the high reactivity of compound **2** and further strengthened trihydroxybenzene as a putative key candidate in thiol binding. Although hydroxyhydroquinone has been reported earlier as an ingredient of roasted coffee (*16*−*18*), this is the first report demonstrating the exceptional instability and high reactivity of that phenol in coffee.
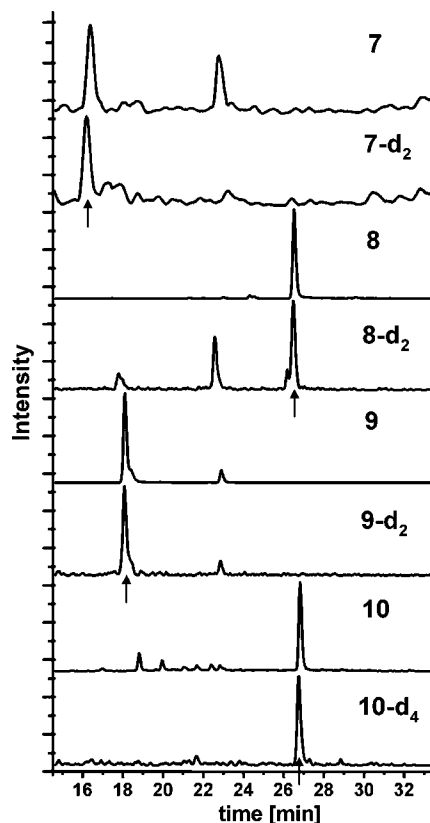
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Figure 3. HPLC-MS/MS analysis of phenol/thiol conjugates **7–10** and corresponding ^{2}H-labeled internal standards in a coffee brew maintained for 10 min at 30 °C.

Table 2. Influence of Incubation Time on Concentrations of Phenol/Thiol Conjugates **7–10** in Coffee Brews

incubation time (min) at 30 °C	concentration^a (μg/L) of conjugate			
	7	**8**	**9**	**10**
Without Additive				
0	0.38	0.62	4.21	< 0.01
10	0.36	3.92	28.89	9.49
20	0.36	5.07	13.57	0.72
	(±0.05)	(±0.62)	(±1.55)	(±0.10)
60	0.40	2.34	7.51	0.26
After Addition of 2-Furfurylthiol (0.5 mg/100 mL)				
0	0.38	0.62	4.21	<0.01
10	0.42	110.49	582.72	31.20
20	0.40	117.73	607.49	34.79
60	0.48	139.86	873.65	52.92
60^b	0.52	113.52	1142.92	19.08
After Addition of 2-Furfurylthiol (0.1–5 mg/100 mL)				
20	0.43	27.02	107.49	0.84
20	0.40	117.73	607.49	34.79
20	0.39	195.87	5409.83	1669.43

^{a} Data are given as the mean of replicates. ^{b} The experiment was performed at 60 °C.

Influence of Storage Time on the Concentrations of Phenol/Thiol Conjugates in Coffee Beverages. To gain insight into the amounts of phenol/thiol conjugates present in roasted coffee and generated during the storage of coffee brew, we aimed to quantitatively determine compounds **7–10** by means of a SIDA with HPLC-MS/MS detection. To achieve this, the deuterated internal standards **7-d₂**, **8-d₂**, **9-d₂**, and **10-d₄** (**Figure 1**) were first synthesized by iron(III)-mediated oxidative coupling of [²H]₂furfurylthiol with the phenols **2**, **3**, and **4**, respectively, using the procedure reported recently for the nonlabeled analogues (*12*). For the quantitative analysis of the conjugates **7**, **8**, **9**, and **10**, coffee brews were spiked with defined amounts of the internal standards **7-d₂**, **8-d₂**, **9-d₂**, and **10-d₄**. After equilibration, the coffee samples were extracted with ethyl acetate, and the extracts obtained were analyzed by RP-HPLC-MS/MS monitoring specific mass transitions by means of the MRM mode. As an example, mass chromatograms showing the analysis of the target compounds **7–10** in a coffee brew incubated for 10 min at 30 °C are shown in **Figure 3**.

To quantify the amounts of the phenol/thiol conjugates produced during storage of a coffee brew, a freshly prepared coffee brew was incubated for 0, 10, 20, and 60 min at 30 °C, then spiked with the labeled internal standards, and analyzed by means of HPLC-MS/MS (MRM). As shown in **Table 2**, conjugate **9** was present in the highest concentrations of 4.21 μg/L, followed by compounds **7** and **8** with concentrations below 1 μg/L, and conjugate **10** was just detectable in trace amounts. Whereas the concentration of compound **7** was rather constant independent of the incubation time, the amounts of conjugates **8–10** were strongly influenced by the storage time. For example, the concentration of the hydroxyhydroquinone conjugate **9** increased by a factor of 7 during the first 10 min, thereafter decreasing again with increasing storage time. In comparison,

compound **8** went to a maximum of 5.07 μg/L after the coffee brew had been maintained for 20 min at 30 °C.

Because none of these compounds were detectable in raw coffee beans (data not shown), it has to be concluded that the catechol/2-furfurylthiol conjugate **7** is thermally generated upon coffee roasting and is not a reaction product formed by storage-induced oxidative coupling reactions between catechol and the thiol. In contrast, just minor amounts of the compounds **8–10** are already present in the freshly prepared roasted coffee brew, but the majority of these conjugates seem to be formed upon storage of the coffee beverage. Furthermore, our data imply that the hydroxyhydroquinone conjugates **9** and **10**, once formed, are further converted in the course of secondary reactions. Preliminary model experiments with these conjugates demonstrated that, due to their high reactivity, they are rapidly transformed into highly complex components of unknown structure (data not shown).

Because the addition of exogeneous 2-furfurylthiol was found to induce an accelerated degradation of hydroxyhydroquinone (**Table 1**), the following experiment was done to investigate the influence of 2-furfurylthiol addition on the generation of the phenol/thiol conjugates **7–10** in coffee. To achieve this, coffee brews were spiked with 2-furfurylthiol (500 μg/20 mL coffee brew) and then incubated for 0, 10, 20, and 60 min at 30 °C or for 60 min at 60 °C, respectively, prior to quantitative analysis. The addition of 2-furfurylthiol resulted in rather high concentrations of the conjugates **8–10**; for example, after 20 min at 30 °C, about 46 times higher amounts of **9** were found when compared to the coffee brew stored without added thiol (**Table 2**). The concentrations of these compounds did not reach a maximum up to an incubation time of 60 min. The 2-furfurylthiol-induced increase of the concentration of compound **9** (**Table 2**) correlated with the strong decrease of the concentration of its precursor **2** induced by the addition of the thiol (**Table 1**). When the coffee spiked with 2-furfurylthiol was incubated for 60 min at 60 °C, the concentration of **9** was still higher than that in the corresponding experiment performed at 30 °C, whereas the concentrations of **8** and **10** were somewhat lower, thus implying that the formation of the conjugate **9** seemed to be favored at elevated temperatures (**Table 2**). In this experiment, compound **9** alone bound about 11% of the

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Table 3. Correlation of the Concentrations of 2-Furfurylthiol, Di- and Trihydroxybenzenes (**1**–**6**), and Phenol/Thiol Conjugates (**7**–**10**) during Storage of Coffee Beverages for up to 60 min at 30 °C

incubation time (min)	concentration[a] (μmol/L) of di- and trihydroxybenzenes					
	1	**2**	**3**	**4**	**5**	**6**
0	22.2	238.4	42.9	8.5	2.4	9.8
10	22.7	174.8	43.4	8.3	3.0	10.1
20	21.6	139.2	42.3	8.2	2.8	10.4
60	22.1	8.2	40.5	8.1	2.6	9.8

incubation time (min)	concentration[a] (μmol/L) of phenol/thiol conjugate			
	9	**10**	**7**	**8**
0	0.018	0.000	0.002	0.002
10	0.121	0.027	0.002	0.016
20	0.057	0.002	0.002	0.020
60	0.032	0.001	0.002	0.009

incubation time (min)	concentration[a] (μmol/L) of 2-furfurylthiol
0	0.17
10	0.10
20	0.07
60	0.02

[a] Data are given as the mean of replicates.

2-furfurylthiol amount added prior to incubation. In contrast, the concentration of conjugate **7** was not influenced by the addition of 2-furfurylthiol.

In a final experiment, the influence of the concentration of 2-furfurylthiol on the formation of phenol/thiol conjugates was investigated by adding increasing amounts of the thiol (1, 5, and 10 mg/L coffee) to a coffee brew prior to incubation for 20 min at 30 °C (**Table 2**). Whereas the concentration of conjugate **9** was 50 times higher after the addition of 50 mg of 2-furfurylthiol compared to the coffee sample spiked with just 1 mg of the thiol, the concentration of **8** increased only by a factor of 7. It is important to note that conjugate **7** was not influenced by the addition of 2-furfurylthiol, thus undoubtedly confirming that this conjugate is most likely generated upon coffee roasting, rather than during the storage of coffee brews.

Quantitative Correlation between the Concentrations of 2-Furfurylthiol, Di- and Trihydroxybenzenes, and Phenol/Thiol Conjugates. To visualize the correlation between the concentrations of the odorant 2-furfurylthiol and the phenol/thiol conjugates, as well as their precursor phenols, during the storage of a coffee beverage, the concentrations of all these compounds in coffee beverages maintained for up to 60 min at 30 °C are compiled in **Table 3** on a micromolar basis. Primarily, it is obvious that the thiol-trapping di- and trihydroxybenzenes **1**−**6** are present in excessive amounts when compared to the concentrations of endogeneous 2-furfurylthiol in the coffee brew. Although there is a huge pool for putative thiol trapping phenols, the stability and lower reactivity of pyrogallol (**1**), 4-methylcatechol (**5**), and 3-methylcatechol (**6**) upon storage is reflected in the absence of any of the corresponding thiol conjugates in coffee (*12*).

Among the di- and trihydroxybenzenes, hydroxyhydroquinone (**2**) seems to play a key role in thiol binding. Considering the starting amount of 0.018 μmol/L of conjugate **9** in the freshly prepared coffee brew and an amount of 0.121 μmol/L of this conjugate after 10 min of incubation, the difference of 0.103 μmol/L of **9** can be estimated to be generated from the thiol

and precursor **2** within the first 10 min of incubation. This accounts for about 60% of the initial amount of 2-furfurylthiol in the freshly prepared coffee beverage (**Table 3**). Again, conjugate **7** was found to be exclusively formed during coffee roasting because its initial concentration of 0.002 μmol/L was not affected at all by storage. This finding correlated well with the concentration of its putative precursor catechol (**3**), which also stayed constant upon storage.

Formation Pathway of Conjugates 9 and 10. Deduced from the results presented above, a reaction pathway is proposed in **Figure 4** for the formation of the conjugates **9** and **10** from their precursors. As demonstrated by the data of recent quantitative analyses (*13*), hydroxyhydroquinone (**2**) is thermally liberated from chlorogenic acids upon coffee roasting. Right after preparation of the coffee brew, hydroxyhydroquinone (**2**) is converted into its highly reactive quinone (**a**) by a transition metal mediated oxidation with air oxygen. Odorous thiols such as 2-furfurylthiol (**b**), generated upon roasting of coffee beans, are then rapidly trapped upon nucleophilic attachment to the quinone (**a**), giving rise to the conjugate **9**. As our quantitative studies showed that high amounts of **9** were generated in coffee brew before the concentration of **10** increased, it can be assumed that **10** is formed from **9** by another oxidative coupling with 2-furfurylthiol. Because some amounts of compound **9** could be already detected in freshly prepared coffee, to a minor extent the same reaction might lead to that conjugate during roasting of coffee beans. As **9** and **10** were found to be highly unstable (data not shown), and, after attaining a maximum, the concentrations of the conjugates decreased in coffee with increasing storage time, conjugates **9** and **10** are supposed to be either further converted into oligomeric materials of unknown structure or bound to phenols as part of macromolecules. This is well in line with the findings of an earlier study showing that [²H]₂-furfurylthiol was covalently bound to the high molecular weight coffee melanoidins as confirmed by means of ²H NMR spectroscopy (*2*).

Formation Pathway of Conjugate 8. It is interesting to note that the concentration of conjugate **8** was increased with increasing incubation time as well as increasing thiol concentration (**Table 3**), but its putative precursor 4-ethylcatechol (**4**) was not affected in these experiments (**Table 2**). Whereas the initial amount of conjugate **8** in the freshly prepared coffee might be derived from the oxidative coupling of 4-ethylcatechol (**4**) and 2-furfurylthiol during coffee roasting, as already proposed for the catechol analogue **7**, another chemical pathway needs to be considered for the increase in the concentration of **8** during coffee storage.

The most likely mechanism for the formation of **8** during storage might be that 2-furfurylthiol reacts not with 4-ethylcatechol in an oxidative coupling reaction but in a nonoxidative nucleophilic addition to 4-vinylcatechol, a well-known degradation product of the caffeic acid moiety of chlorogenic acid (*19*). As shown in **Figure 5**, thermal decomposition of chlorogenic acid (**c**) generates 4-vinylcatechol (**d**) as well as 4-ethylcatechol (**4**) upon reduction. Whereas **4** has to be oxidized to form the quinone intermediate (**e**) to which thiols can add to give the conjugate **8**, 4-vinylcatechol (**d**) can easily form **e** by a proton-catalyzed keto−enol tautomerism running via intermediate **f**. As this acid-catalyzed reaction pathway should be favored in the acidic coffee brew, the reaction between 4-vinylcatechol and 2-furfurylthiol is suggested as the key mechanism leading to the formation of conjugate **8** during coffee storage.

Influence of the Roasting Degree on the Concentrations of Conjugates 7−10 in Coffee. As the data obtained so far
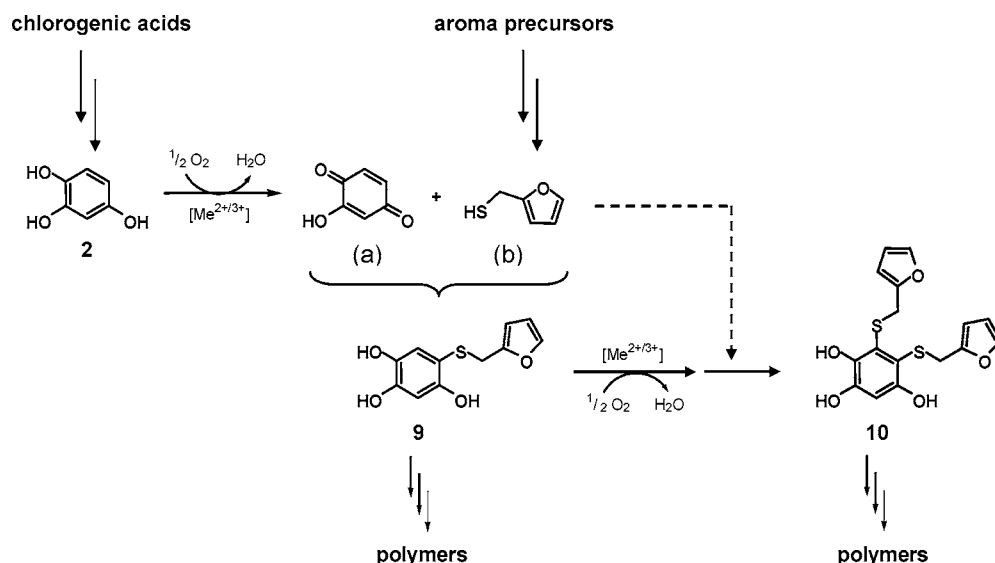
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Figure 4. Reaction sequence showing the oxidative coupling of 2-furfurylthiol to hydroxyhydroquinone (**2**) upon coffee storage.
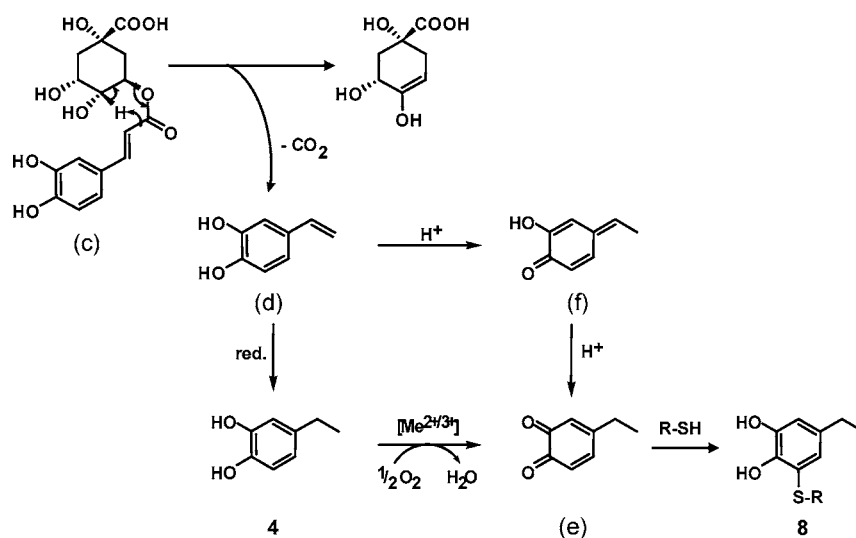

Figure 5. Formation pathways proposed for the generation of phenol/thiol conjugate **8**.

Table 4. Concentrations of Phenol/Thiol Conjugates **7**–**10** in Coffee Brews Varying in the Roasting Degree as Well as in Commercial Coffee Samples

	concentration (μg/L) of conjugate			
sample	7	8	9	10
filtered coffee, light roast[a]	0.14	0.22	1.16	0.05
filtered coffee, medium roast[a]	0.39	0.84	11.56	0.36
filtered coffee, dark roast[a]	0.54	0.99	13.57	0.39
soluble coffee[b]	1.18	1.07	2.07	<0.01
espresso-type coffee[c]	2.25	1.90	24.84	0.41
soluble espresso[d]	2.70	1.61	8.75	<0.01

[a] Freshly prepared coffee brews (100 mL, 54 g/L) made from coffees of different roasting degrees were used for quantitative analysis. [b] An aliquot (100 mL) of a coffee beverage prepared of soluble coffee powder (1.5 g/100 mL) was used for analysis. [c] An aliquot (100 mL) of a coffee beverage freshly prepared from espresso beans by means of a fully automatic coffee machine was used for analysis. [d] An aliquot (100 mL) of a coffee beverage prepared of soluble espresso powder (1.5 g/100 mL) was used for analysis.

indicated that phenol/thiol conjugates are already generated to a minor extent upon roasting of coffee beans, the influence of the roasting degree on the roasting induced production of compounds **7**–**10** was investigated. To achieve this, the phenol/thiol conjugates **7**–**10** were quantitatively determined in brews freshly prepared from light (CTN value 150), medium (CTN value 86), and dark roasted coffee beans (CTN value 56), respectively. The data given in **Table 4** show that the concentration of all four conjugates increased with increasing roasting degree. In particular, increasing of the roasting degree from light to medium resulted in an up to 10-fold concentration of **9** and **10**, a 4-fold concentration of **8**, and a 2-fold concentration of **7**. These data clearly confirm that the conjugates are already generated to some amount upon bean roasting. However, whereas the major amounts of conjugates **8**–**10** are generated during storage of the coffee beverage, the formation of conjugate **7** seems to be only influenced by the roasting degree.

Concentrations of Phenol/Thiol Conjugates 7–10 in Commercial Coffees. To gain insight into the influence of processing on the concentration of phenol/thiol conjugates in coffee beverages, the amounts of compounds **7**–**10** were quantitatively determined in beverages freshly prepared from a soluble coffee, an espresso-type coffee, and a soluble espresso-type coffee. As shown in **Table 4**, compound **9** was the quantitatively predominant conjugate in all commercial coffees with by far the highest

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concentrations of 24.8 μg/L in the espresso beverage. Amounts 2−10 times lower were found for compounds **7** and **8**, whereas conjugate **10** was detectable in trace amounts just in the espresso coffee.

On the basis of these results it can be concluded that the oxidative coupling of the odorous 2-furfurylthiol to hydroxy-hydroquinone (**2**) giving rise to the conjugates **9** and **10** is responsible for the decrease in the sulfury-roasty odor quality detected shortly after preparation of the coffee brew. Studies on how this thiol-trapping reaction can be counteracted are ongoing and will help to find possible means to increase the aroma shelf life of coffee beverages.

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