JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY

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pubs.acs.org/JAFC

Chemical Interaction between Polyphenols and a Cysteinyl Thiol under Radical Oxidation Conditions

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ABSTRACT: Chemical interaction between polyphenols and thiols was investigated under radical oxidation conditions using a model cysteinyl thiol derivative, *N*-benzoylcysteine methyl ester. The radical oxidation was carried out with a stoichiometric amount of 2,2-diphenyl-1-picrylhydrazyl (DPPH), and the decreases in the amounts of polyphenols and the thiol were measured by HPLC analysis. Cross-coupling products between various polyphenols and the thiol were examined by LC-MS in reactions that showed decreases in both the polyphenols and the thiol. The LC-MS results indicated that three phenolic acid esters (methyl caffeate, methyl dihydrocaffeate, and methyl protocatechuate) and six flavonoids (kaempferol, myricetin, luteolin, morin, taxifolin, and catechin) gave corresponding thiol adducts, whereas three polyphenols (methyl ferulate, methyl sinapate, and quercetin) gave only dimers or simple oxidation products without thiol substituents. Thiol adducts of the structurally related compounds methyl caffeate and methyl dihydrocaffeate were isolated, and their chemical structures were determined by NMR analysis. The mechanism for the thiol addition was discussed on the basis of the structures of the products.

KEYWORDS: *polyphenol, thiol adduct, radical oxidation, cross coupling, cysteine derivative*

INTRODUCTION

Polyphenols have been recognized as useful functional constituents of plant-derived foods because of their potent antioxidant activity. Recently, some polyphenols have been found to possess various regulatory activities on signaling pathways in living cells in addition to the antioxidant activity.[1] Therefore, scientific interest in polyphenols continues to increase, not only in food science but also in medical science. It should be noted that antioxidant activity, a major function of polyphenols, is based on sequential radical reactions including radical trapping from radical species generated from biomolecules.[2] More efficient antioxidative polyphenols trap radicals more rapidly and then get converted to radicals themselves. The polyphenol radicals are not very stable, so they subsequently react with surrounding molecules. Foods and living cells consume the polyphenols they contain via a similar radical reaction process when they are subjected to oxidative stress. Polyphenol radicals produced react with food or cell constituents to give new stable products. These products accumulate in foods or living cells and show both beneficial and nonbeneficial functions.[3,4] During lipid oxidation, some polyphenol radicals have been reported to react with lipid peroxyl radicals by a radical−radical coupling, and several coupling products have been identified.[5−8] Such radical-coupling reactions are very fast and constitute an important termination process for the oxidative deterioration of foods by phenolic antioxidants. It is well-known that the thiol group (sulfhydryl group) is easily converted to the thiyl radical and that the thiyl radical has potent ability to react with other radical species.[9] Among various food constituents, a major thiol-bearing compound is cysteine, an important constitutional unit of peptides and proteins. Under radical oxidation conditions, polyphenol radicals can react with the thiyl radical to produce sulfur-bearing polyphenols,[10,11] which are expected to show various functions in foods or living cells. In addition, covalent bond formation between proteins and polyphenols via thioether linkages has been found recently.[12−15] This protein modification is believed to be a result of a prooxidant effect of polyphenols. The modification affected the functionality of the proteins in food and living systems.[16−19]

In this investigation, we carried out 2,2-diphenyl-1-picrylhydrazyl (DPPH)-induced radical reaction of polyphenols in the presence of a cysteinyl thiol derivative (*N*-benzoylcysteine methyl ester). DPPH is a stable radical reagent and has been frequently employed to evaluate the antioxidant capacity of polyphenols.[20] As a radical reagent, DPPH has the advantage of allowing easy regulation of the amount of radicals in the reaction. This is an advantage because an excess amount of reactive radicals damages reaction products. The reaction mixture produced by equimolecular DPPH radicals was then analyzed by LC-TOFMS to determine the coupling products between polyphenols and *N*-benzoylcysteine methyl ester. Next, the chemical structures of the coupling products were estimated from high-resolution MS results, and then the structures were determined by NMR after the products had been isolated. The results of this comprehensive study should provide fundamental information about the chemical interaction between polyphenols and cysteine-bearing biomolecules such as proteins and peptides in foods and living cells.

MATERIALS AND METHODS

Chemicals, Instruments, and Software. Methyl caffeate, methyl ferulate, methyl sinapate, methyl dihydrocaffeate, methyl protocatechuate, methyl vanillate, and methyl syringate (each purity > 95%) were synthesized from corresponding carboxylic acids. Briefly, to a methanol solution of 100 mg of each carboxylc acid were added 10 drops of

Received: February 29, 2012
Revised: May 2, 2012
Accepted: May 3, 2012
Published: May 3, 2012

dx.doi.org/10.1021/jf3008822 | J. Agric. Food Chem. 2012, 60, 5142−5151

concentrated sulfuric acid. After 2 h at 40 °C, the solution was concentrated to ca. 2 mL, ethyl acetate (50 mL) was added, and the mixture was washed twice with saturated NaCl aqueous solution and then saturated $NaHCO_3$ aqueous solution, subsequently. The washed ethyl acetate layer was dried over anhydrous Na_2SO_4 and evaporated to dryness to give each methyl ester. When purification was required, silica gel column chromatography was carried out to obtain pure sample. Kaempferol (purity ≥ 98%), morin (purity ≥ 90% as hydrate), myricetin (purity ≥ 97%), and propyl gallate (purity ≥ 98%) were purchased from Tokyo Kasei (Tokyo, Japan). Luteolin (purity not specified) was purchased from Kanto Chemicals (Tokyo, Japan). Taxifolin (purity ≥ 90% as hydrate) was purchased from Sigma-Aldrich (St. Louis, MO, USA). DPPH was obtained from Wako Pure Chemicals (Osaka, Japan). Quercetin (purity not specified, but no other peak was observed by our HPLC analysis), catechin [(+)-form, purity = 98% as hydrate], and all solvents (extra pure grade or HPLC grade) were obtained from Nacalai Tesque (Kyoto, Japan). NMR spectra were measured with an ECX-400 spectrometer (JEOL, Tokyo, Japan) using the manufacturer-supplied pulse sequences [^{1}H, ^{13}C, proton−proton correlated spectroscopy (HH−COSY), nuclear Overhauser enhancement spectroscopy (NOESY), heteronuclear multiple quantum coherence (HMQC), and heteronuclear multiple bond correlation (HMBC)]. Mass spectra were obtained with a XEVO QtofMS spectrometer (Waters Japan, Tokyo, Japan) in ESI mode. A LC-20AD low-pressure gradient system (Shimadzu, Kyoto, Japan) equipped with an SPD-M20A photodiode array detector and a DGU-20A3 degasser was employed for the analytical HPLC. PDA data were analyzed with LC solution (ver. 6.10, Shimadzu). An LC-6AD system (Shimadzu) equipped with a UV-970 detector (JASCO, Tokyo, Japan) was used for preparative HPLC. Molecular orbital calculation was performed by MOPAC2009 software using PM3 parameter via Chem & Bio 3D ver. 12.0 (PerkinElmer, Boston, MA, USA) as interface software.

Preparation of *N,N′*-Dibenzoylcystine Dimethyl Ester and *N*-Benzoylcysteine Methyl Ester. To a solution of L-cystine (10 g) in 4.2% (w/w) NaOH aqueous solution (50 mL) were dropwise added benzoyl chloride (9.8 mL) and 4.2% (w/w) NaOH aqueous solution (50 mL) with stirring in a water bath (25 °C). The reaction continued for 3 h, and 6 N HCl (22 mL) was added to the reaction mixture. The produced precipitate was filtered and dried over P_2O_5 to give *N,N′*-dibenzoylcystine. The dibenzoylcystine (11 g) was dissolved in methanol (300 mL), and concentrated sulfuric acid (0.3 mL) was added to the solution. After the solution had been kept overnight at 23 °C, it was poured into chloroform (300 mL), washed with saturated NaCl aqueous solution, then washed with saturated $NaHCO_3$ aqueous solution, and partitioned. The chloroform layer was dried over anhydrous Na_2SO_4 and evaporated to give *N,N′*-dibenzoylcystine dimethyl ester (**19**, 8.0 g): ESI-MS (*m/z*) $[M + Na]^+$, calcd for $C_{22}H_{24}N_2O_6S_2Na$, 499.0974; found, 499.0968; ^{1}H NMR (400 MHz, acetone-d_6) δ 8.15 (2H, br d, J = 7.6 Hz, 2- and 2′-NH), 7.90 (4H, AA′BB′C, two benzoyl-H2 and H6), 7.54 (2H, AA′BB′C, two benzoyl-H4), 7.45 (4H, AA′BB′C, two benzoyl-H3 and H5), 5.00 (m, H2 and H2′), 3.71 (6H, s, 1-OCH_3 and 1′-OCH_3), 3.40 (2H, dd, J = 14.0 and 4.8 Hz, H3a and H3′a), 3.27 (2H, dd, J = 14.0 and 9.2 Hz, H3b and H3′b).

To a methanol solution (570 mL) of the dibenzoylcystine dimethyl ester (**19**, 7.05 g) was added dithiothreitol (4.56 g). After the solution had been kept overnight at 23 °C, it was evaporated to dryness, and the residue was purified by silica gel column chromatography eluted with ethyl acetate/hexane (2:5, v/v) to give 5.5 g of *N*-benzoylcysteine methyl ester (**18**): ESI-MS (*m/z*) $[M + H]^+$, calcd for $C_{11}H_{14}NO_3S$, 240.0694; found, 240.0672; ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.84 (2H, AA′BB′C, benzoyl-H2 and H6), 7.54 (1H, AA′BB′C, benzoyl-H4), 7.47 (2H, AA′BB′C, benzyol-H3 and H5), 7.06 (1H, br d, J = 2.0 Hz, 2-NH), 5.10 (1H, m, H2), 3.84 (6H, s, 1-OCH_3), 3.15 (2H, m, H3), 1.40 (1H, t, J = 4.5 Hz, 3-SH).

HPLC Analysis of Radical Reactions of Polyphenols in the Presence of Thiol Derivative *N*-Benzoylcysteine Methyl Ester. To a mixture of 5 mmol/L polyphenol solution (acetonitrile, 1 mL) and 5 mmol/L *N*-benzoylcysteine methyl ester (**18**) solution (acetonitrile, 1 mL) was added 5 mmol/L DPPH solution (acetonitrile, 2 mL), and the solution was kept at 23 °C. At 1 and 2 h after the addition, aliquots of 50 μL each were taken from the solution and diluted twice with acetonitrile. Five microliters of the diluted solution was analyzed by HPLC under the following conditions: column, 250 × 4.6 mm i.d., 5 μm, Cosmosil 5C_{18}-AR-II (Nacalai Tesque); flow rate, 1.0 mL/min; solvent A, 1% acetic acid in water; solvent B, acetonitrile; linear gradient from 5% solvent B (0 min) to 100% solvent B (40 min) and then 100% solvent B (until 50 min); detection, absorbance at 245 and 280 nm; column temperature, 23 °C. Two control experiments, which were the DPPH reaction of polyphenol only and the reaction of *N*-benzoylcysteine methyl ester only at the same concentration, were carried out, and the products were analyzed under the same conditions. The concentrations of each polyphenol, *N*-benzoylcysteine methyl ester, and produced dimer (*N,N′*-dibenzoylcystine) were calculated by calibration curves obtained by HPLC using pure compounds.

LC-MS Analysis of Reaction Products from Polyphenols and *N*-Benzoylcysteine Methyl Ester. Ten microliters of the acetonitrile solution (1.25 mmol/L as starting polyphenol) of the DPPH oxidation product was injected into an LC-MS instrument, Acquity UPLC and Xevo QTofMS (Waters Japan, Tokyo, Japan), through a sample injector, Acquity sample manager (Waters). The LC-MS analysis was carried out under the following conditions: (separation conditions) column, 250 × 4.6 mm i.d., 5 μm, Cosmosil 5C_{18}-AR-II (Nacalai Tesque); flow rate, 0.5 mL/min; solvent A, ultrapure water; solvent B, acetonitrile (LC-MS grade, Merck, Darmstadt, Germany); (gradient conditions) linear gradient from 5% solvent B (0 min) to 100% solvent B (80 min) and then 100% solvent B until 100 min; UV absorbance detection, 280 nm; column temperature, 23 °C; (MS conditions) mode, ESI negative; capillary voltage, 2.4 kV; cone voltage, 40 V; source temperature, 150 °C; desolvation temperature., 500 °C; cone gas flow rate, 50 L/h; desolvation gas flow rate, 1000 L/h; MS^E low collision energy, 6 V; MS^E high collision energy, from 20 to 30 V. The elemental composition of each peak compound was calculated from the high-resolution MS data of the protonated or ion-adducted molecular ion by MassLynx software (V. 4.1, Waters).

Isolation and Identification of Reaction Products from Methyl Caffeate and *N*-Benzoylcysteine Methyl Ester. To an acetonitrile solution (40 mL) of methyl caffeate (**1**, 21 mg, 1.25 mmol/L) and *N*-benzoylcysteine methyl ester (**18**, 25 mg, 1.25 mmol/L) was added DPPH (81 mg, 2.5 mmol/L) in acetonitrile (40 mL). After the mixture had been kept for 2 h at 23 °C, it was evaporated to give a brown residue (118 mg). Part of the residue (80 mg) was purified by preparative silica gel TLC (Merck) developed with ethyl acetate/hexane (1:1, v/v) to afford 7.3 mg of a thiol adduct (**20**): ESI-MS (*m/z*) $[M + H]^+$, calcd for $C_{21}H_{20}NO_7S$, 430.0960; found, 430.0956;^{1}H NMR (400 MHz, $CDCl_3$) δ 8.24 (1H, d, J = 16.0 Hz, H3), 7.77 [2H, m (AA′BB′C), H2 and H6 of the benzoyl group of *N*-benzoylcysteine methyl ester moiety], 7.55 [1H, m (AA′BB′C), H4 of the benzoyl group of *N*-benzoylcysteine methyl ester moiety], 7.44 [2H, m (AA′BB′C), H3 and H5 of the benzoyl group of *N*-benzoylcysteine methyl ester moiety], 7.20 (1H, d, J = 8.2 Hz, H6′), 7.04 (1H, d, J = 7.6 Hz, 2″-NH), 6.91 (1H, d, J = 8.2 Hz, H5′), 6.34 (1H, d, J = 16.0 Hz, H2), 4.96 (1H, ddd, J = 7.6, 7.6, and 3.6 Hz, H2 of *N*-benzoylcysteine methyl ester moiety), 3.80 (3H, s, 1-OCH_3 or 1-OCH_3 of *N*-benzoylcysteine methyl ester moiety), 3.74 (3H, s, 1-OCH_3 of *N*-benzoylcysteine methyl ester moiety or 1-OCH_3), 3.23 (1H, dd, J = 14.2 and 3.6 Hz, H3a of *N*-benzoylcysteine methyl ester moiety), 3.16 (1H, dd, J = 14.2 and 7.6 Hz, H3b of *N*-benzoylcysteine methyl ester moiety). ^{13}C NMR (100 MHz, $CDCl_3$) δ 170.8 (C1″), 168.1 (C1 or carbonyl of the benzoyl group of *N*-benzoylcysteine methyl ester moiety), 167.7 (carbonyl of the benzoyl group of *N*-benzoylcysteine methyl ester moiety or C1), 146.6 (C4′), 145.9 (C3′), 142.6 (C3), 133.0 (C1 of the benzoyl group of *N*-benzoylcysteine methyl ester moiety), 132.5 (C4 of the benzoyl group of *N*-benzoylcysteine methyl ester), 130.1 (C1′), 128.8 (C3 and C5 of the benzoyl group of *N*-benzoylcysteine methyl ester), 127.3 (C2 and C6 of the benzoyl group of *N*-benzoylcysteine methyl ester moiety), 120.2 (C6′), 119.6 (C2′), 118.1 (C2), 116.5 (C5′), 53.7 (C2 of *N*-benzoylcysteine methyl ester), 53.3 (1-OCH_3 or 1-OCH_3 of *N*-benzoylcysteine methyl ester moiety), 51.9

(1-OCH_3 of *N*-benzoylcysteine methyl ester moiety or 1-OCH_3), 40.4 (C3 of *N*-benzoylcysteine methyl ester moiety).

Isolation and Identification of Reaction Products from Methyl Dihydrocaffeate and *N*-Benzoylcysteine Methyl Ester. To an acetonitrile solution (200 mL) of methyl dihydrocaffeate (**4**, 106 mg, 1.25 mmol/L) and *N*-benzoylcysteine methyl ester (**18**, 121 mg, 1.25 mmol/L) was added DPPH (404 mg, 2.5 mmol/L) in acetonitrile (207 mL). Two hours after the addition, the mixture was concentrated to afford a product mixture (622 mg). Part of the product (216 mg) was subjected to silica gel column chromatography [silica gel, high-purity silica gel, Cosmosil 75SL-II-Prep 15 g, high-purity silica gel elution solvent, ethyl acetate/hexane (1:1, v/v, 260 mL), ethyl acetate/hexane (3:2, v/v, 80 mL), and then ethyl acetate/hexane (2:1, v/v, 250 mL)] to give three cysteine adducts, **21** (8.5 mg), **22** (10 mg), and **23** (17 mg).

21 (monothiol adduct): ESI-MS (*m*/*z*) $[M - H]^-$, calcd for $C_{21}H_{22}NO_7S$, 432.1096; found, 432.1117; ^1H NMR (400 MHz, $CDCl_3$) δ 7.70 (2H, m, H2 and H6 of the benzoyl group of *N*-benzoylcysteine methyl ester moiety), 7.51 (1H, m, H4 of benzoyl group of *N*-benzoylcysteine methyl ester moiety), 7.32 (1H, m, H3 and H5 of the benzoyl group of *N*-benzoylcysteine methyl ester moiety), 6.97 (1H, d, *J* = 7.6 Hz, 2-NH of *N*-benzoylcysteine methyl ester moiety), 6.85 (1H, d, *J* = 2.0 Hz, H6′), 6.70 (1H, d, *J* = 2.0 Hz, H2′), 5.04 (1H, dt, *J* = 7.6 and 3.6 Hz, H2″), 3.73 (3H, s, 1-OCH_3 of *N*-benzoylcysteine methyl ester moiety), 3.65 (3H, s, 1-OCH_3), 3.39 (1H, dd, 14.8 and 3.6 Hz, H3″a), 3.18 (1H, dd, 14.8, 7.2 Hz, H3″b), 2.77 (2H, t, *J* = 7.2 Hz, H3), 2.53 (2H, t, *J* = 7.2 Hz, H2).

22 (dithiol adduct): ESI-MS (*m*/*z*) $[M - H]^-$, calcd for $C_{32}H_{33}N_2O_{10}S_2$, 669.1577; found, 669.1597; ^1H NMR (400 MHz, $CDCl_3$) δ 7.75 (2H, m, H2 and H6 of the benzoyl group of one of *N*-benzoylcysteine methyl ester moieties), 7.70 (2H, m, H2 and H6 of the benzoyl group of one of *N*-benzoylcysteine methyl ester moieties), 7.53 (1H, m, H4 of the benzoyl group of one of *N*-benzoylcysteine methyl ester moieties), 7.51 (1H, m, H4 of the benzoyl group of one of *N*-benzoylcysteine methyl ester moieties), 7.44 (2H, m, H3 and H5 of the benzoyl group of one of *N*-benzoylcysteine methyl ester moieties), 7.13 (1H, d, *J* = 7.2 Hz, 2-NH of one of *N*-benzoylcysteine methyl ester moieties), 7.04 (1H, d, *J* = 8.0 Hz, 2-NH of one of *N*-benzoylcysteine methyl ester moieties), 6.87 (1H, s, H6′), 4.99 (2H, complex, H2 of the two *N*-benzoylcysteine methyl ester moieties), 3.70 (3H, s, 1-OCH_3 of one of *N*-benzoylcysteine methyl ester moieties), 3.69 (3H, s, 1-OCH_3 of one of *N*-benzoylcysteine methyl ester moieties), 3.65 (3H, s, 1-OCH_3), 3.44 (1H, dd, *J* = 14.4 and 4.4 Hz, H3b of 5′-thiol), 3.33 (1H, dd, *J* = 14.0 and 6.0 Hz, H3a of 5′- thiol), 3.27 (2H, d, *J* = 5.2 Hz, H3 of 2′-thiol), 3.00 (2H, m, H3), 2.51 (2H, m, H2).

23 (trithiol adduct): ESI-MS (*m*/*z*) $[M - H]^-$, calcd for $C_{43}H_{44}N_3O_{13}S_3$, 906.2036; found, 906.2058; ^1H NMR (400 MHz, $CDCl_3$) δ 7.93 (1H, br, OH), 7.78 (2H, m, H2 and H6 of the benzoyl group of one of *N*-benzoylcysteine methyl ester moieties), 7.77 (2H, m, H2 and H6 of the benzoyl group of one of *N*-benzoylcysteine methyl ester moieties), 7.72 (2H, m, H2 and H6 of the benzoyl group of one of *N*-benzoylcysteine methyl ester moieties), 7.58−7.38 (9H, complex, H3, H4, and H5 of the benzoyl groups of three *N*-benzoylcysteine methyl ester moieties), 7.32 [1H, d, *J* = 7.2 Hz, 2-NH of one of *N*-benzoylcysteine methyl ester moieties (I)], 7.25 [1H, d, *J* = 7.2 Hz, 2-NH of one of *N*-benzoylcysteine methyl ester moieties (II)], 7.14 [1H, d, *J* = 8.0 Hz, 2-NH of one of *N*-benzoylcysteine methyl ester moieties (III)], 4.96 [1H, m, H2 of one of *N*-benzoylcysteine methyl ester moieties (I)], 4.92 [1H, m, H2 of one of *N*-benzoylcysteine methyl ester moieties (II)], 4.85 [1H, ddd, *J* = 8.0, 6.4, and 4.4 Hz, H2 of one of *N*-benzoylcysteine methyl ester moieties (III)], 3.71 (3H, s, 1-OCH_3 of one of *N*-benzoylcysteine methyl ester moieties), 3.65 (3H, s, 1-OCH_3), 3.60 (6H, s, 1-OCH_3 of two *N*-benzoylcysteine methyl ester moieties), 3.53 (1H, m, H3a), 3.47 [1H, dd, *J* = 13.2 and 6.4 Hz, H3a of one of *N*-benzoylcysteine methyl ester moieties (III)], 3.44 [2H, m, H3 of one of *N*-benzoylcysteine methyl ester moieties (I)], 3.36 (1H, m, H3b), 3.27 [1H, dd, *J* = 13.2 and 4.4 Hz, H3b of one of *N*-benzoylcysteine methyl ester moieties (III)], 3.23 [2H, m, H3 of one of *N*-benzoylcysteine methyl ester moieties (II)], 2.46−2.28 (2H, m, H2ab).

RESULTS AND DISCUSSION

Radical Reaction of Polyphenols in the Presence of *N*-Benzoylcysteine Methyl Ester. Six phenolic acids (in ester form), seven flavonoids, and two phenolic diterpenoids (Figure 1) were subjected to radical oxidation reaction in the presence of 1 equiv of a thiol molecule. DPPH was employed as a radical inducer. DPPH is a stable radical species and can be handled stoichiometrically. As the thiol compound, we used *N*-benzoylcysteine methyl ester (**18**). It is well-known that the thiol group is highly nucleophilic and can be converted easily to a reactive thiyl radical, so thiol compounds are possible reagents for both nucleophilic and radical reactions. It should be noted that the radical reactivity of thiols is influenced by the surrounding functional groups. An amino group at the β-position enhances reactivity,[9] whereas an amide group at the same position reduces reactivity. In food or living systems, thiol-bearing molecules are mainly proteins and peptides containing cysteine. Therefore, an *N*-benzoylated cysteine derivative (**18**) is a suitable model for cysteine-containing peptides and proteins, and it has the advantage of being detectable with the UV detector of an HPLC instrument.

The radical reaction of polyphenols with equimolecular *N*-benzoylcysteine methyl ester in acetonitrile was started by the addition of 2 equiv of DPPH at 23 °C. After 2 h, the constituents of the reaction mixture were analyzed by HPLC. Quantitative analysis results for each polyphenol, *N*-benzoylcysteine methyl ester, and the disulfide dimer of *N*-benzoylcysteine methyl ester are summarized in Figure 2.

DPPH produces polyphenol radicals as well as thiyl radicals. These radicals may react with each other to afford a coupling product. In addition, polyphenol radicals have the ability to form oxidation products such as quinone derivatives. The quinone derivatives may also react with thiols by a nucleophilic reaction.[21] Therefore, the chemical interaction of polyphenols and thiols is complicated and requires intensive analysis of not only polyphenols and their products but also thiols and their products.

By HPLC analysis, we examined the decrease in the amount of each polyphenol and *N*-benzoylcysteine methyl ester from each concentration of 1.25 mmol/L and the increase in the amount of the disulfide derivative of *N*-benzoylcysteine methyl ester (*N*,*N*′-dibenzoylcystine dimethyl ester), which is an expected oxidative product from *N*-benzoylcysteine methyl ester, in DPPH reactions. The amounts of methyl vanillate (**6**) and methyl syringate (**7**) did not decrease during their reactions with *N*-benzoylcysteine methyl ester,[22] but the amount of *N*-benzoylcysteine methyl ester decreased during those reactions. On the other hand, the amounts of propyl gallate (**8**) and carnosic acid (**16**) decreased during their reaction with *N*-benzoylcysteine methyl ester, whereas the decrease in the amount of *N*-benzoylcysteine methyl ester was negligible during those reactions. These results indicate that methyl vanillate (**6**) and methyl syringate (**7**) are too stable to react with DPPH and that propyl gallate (**8**) and carnosic acid (**17**) show probably potent antioxidant activity against thiol oxidation by DPPH. In the polyphenol chemistry, strong electron-withdrawing substituents at the phenolic part weaken the activity for radicals, whereas some electron-donating groups enhance the radical trapping activity.[22] Much reactivity difference between these polyphenols and the thiol probably

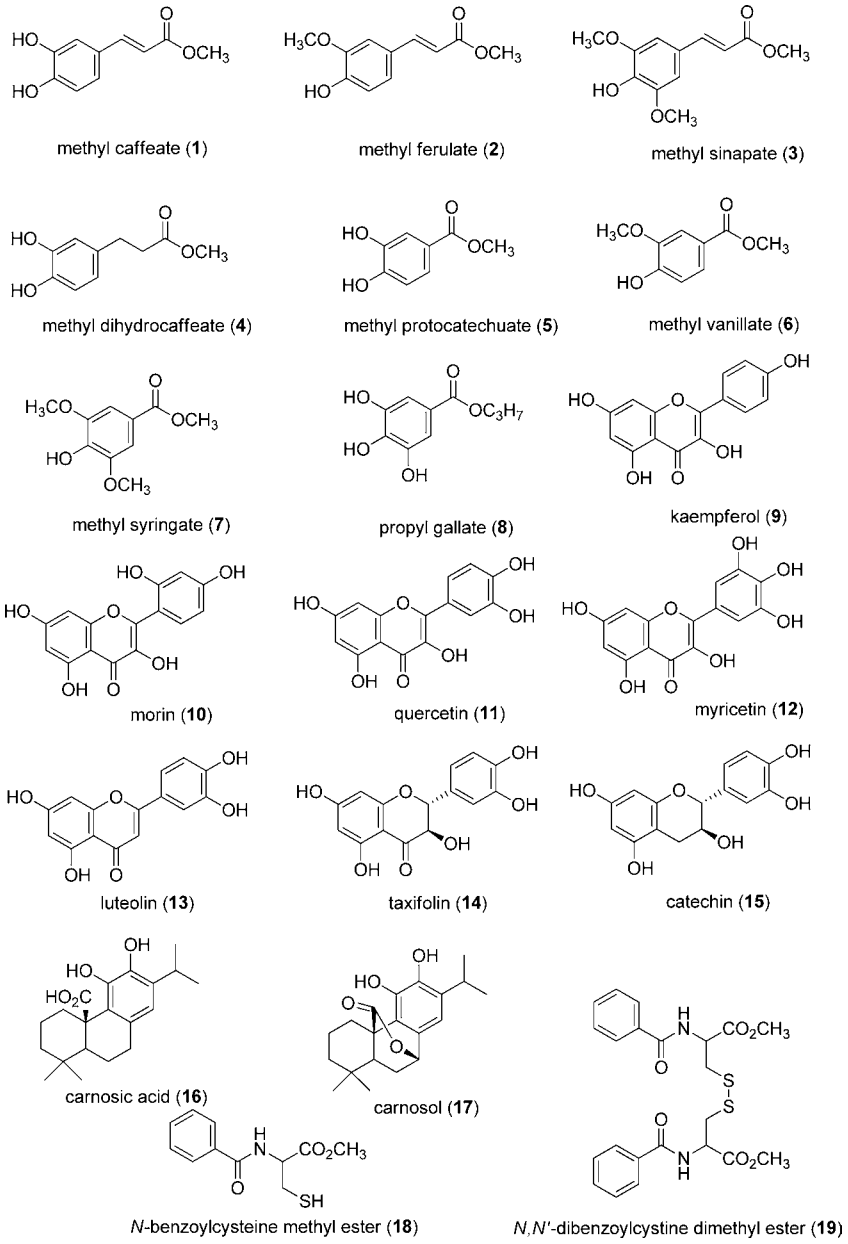

Figure 1. Chemical structures of the investigated polyphenols and cysteinyl thiol derivatives.

made the cross-reaction impossible. Therefore, any chemical interaction between these compounds and thiol molecules including peptides and proteins is not expected. Very interestingly, carnosol (**16**), a diterpenoid antioxidant, showed a slight decrease of its concentration but very high disulfide production during the reaction, when compared with the control experiment (DPPH plus *N*-benzoylcysteine methyl ester only). These results indicated that carnosol (**16**) has the ability to catalyze thiol dimerization to form a disulfide. Disulfide formation is important not only for the tertiary structure formation of proteins but also for protein−protein interaction in foods, for example, in wheat dough formation. It is well-known that dehydroascorbic acid affects disulfide formation and interchange of protein−glutathione disulfide in wheat constituents to result in good-quality wheat flour dough.[23,24] The radical oxidation in the presence of carnosol (**16**) may have similar possibilities for dough formation. The other polyphenols showed decreases of both polyphenol and *N*-benzoylcysteine methyl ester, which prompted us to seek cross-coupling products of polyphenols and *N*-benzoylcysteine methyl ester by using the LC-MS technique.

LC-MS Analysis of Reaction Products of DPPH Radical Reaction of Polyphenols and *N*-Benzoylcysteine Methyl Ester. The LC-MS analytical results of the DPPH radical reactions of polyphenols and *N*-benzoylcysteine methyl ester (**18**) are summarized in Table 1. The high-resolution ESI-MS (negative ion mode) data of the peaks observed in HPLC of these reaction mixtures clarified that methyl caffeate (**1**), methyl dihydrocaffeate (**4**), methyl protocatechuate (**5**), kaempferol (**9**), myricetin (**12**), luteolin (**13**), morin (**10**), taxifolin (**14**), and catechin (**15**) underwent coupling reactions with *N*-benzoylcysteine methyl ester, whereas methyl ferulate (**2**), methyl sinapate (**3**), and quercetin (**11**) did not afford any coupling products under the conditions employed. LC-MS analysis of the reactions of methyl ferulate (**2**) and methyl sinapate (**3**) revealed that corresponding polyphenol dimers

dx.doi.org/10.1021/jf3008822 | J. Agric. Food Chem. 2012, 60, 5142−5151

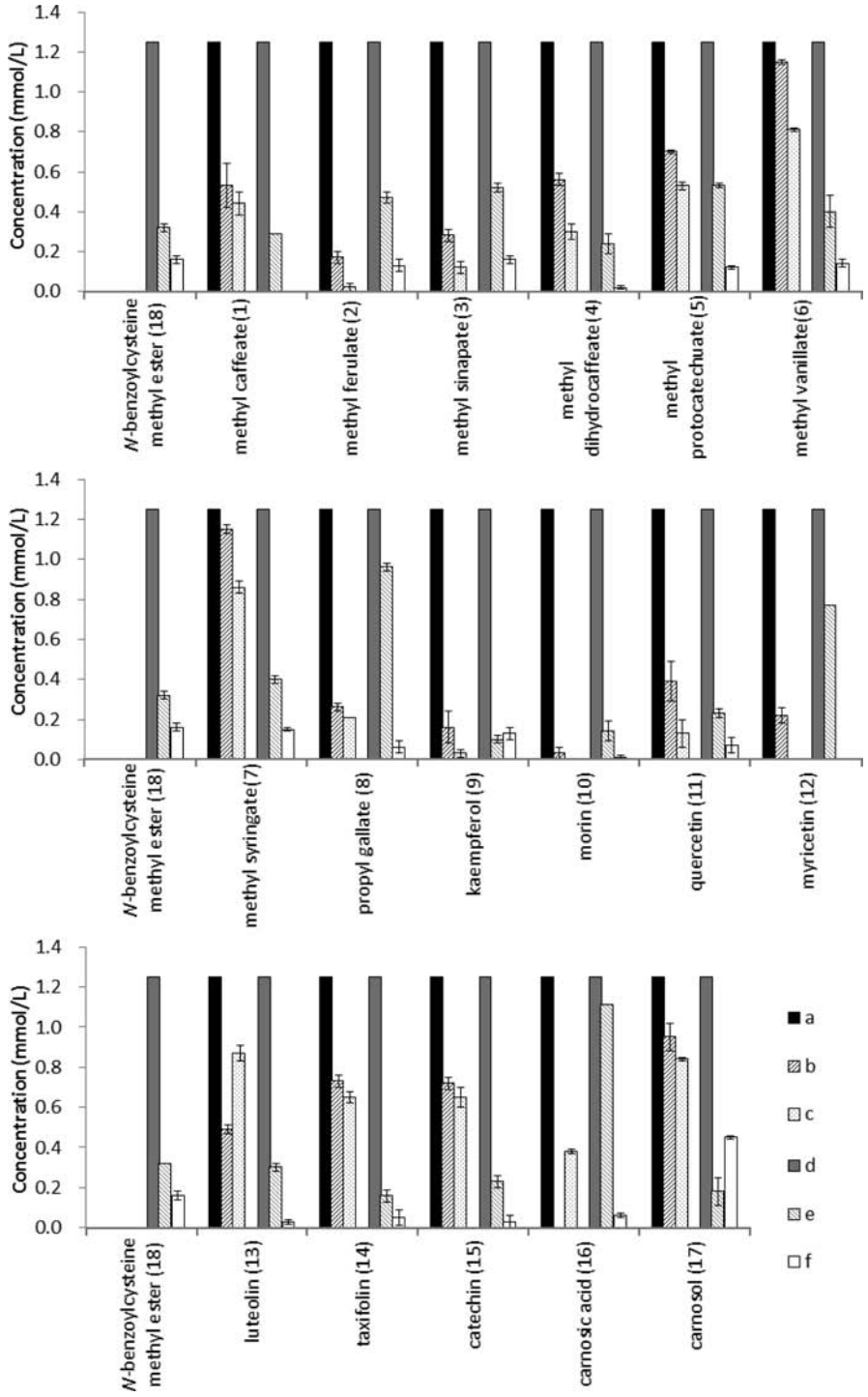

Figure 2. Concentrations of polyphenols, cysteinyl thiol (*N*-benzoylcysteine methyl ester) and its disulfide dimer in DPPH radical oxidation reaction: (a) initial concentration of each polyphenol; (b) final concentration of each polyphenol in the presence of *N*-benzoylcysteine methyl ester; (c) final concentration of each polyphenol after DPPH radical oxidation without *N*-benzoylcysteine methyl ester; (d) initial concentration of *N*-benzoylcysteine methyl ester; (e) final concentration of *N*-benzoylcysteine methyl ester after DPPH radical oxidation with each polyphenol; (f) concentration of disulfide dimer of *N*-benzoylcysteine methyl ester (*N*,*N*′-dibenzoylcystine dimethyl ester) produced after DPPH radical oxidation reaction with each polyphenol. The bar graph set in the left side of each line, which is indicated as data for the *N*-benzoylcysteine methyl ester, shows the results of DPPH oxidation of *N*-benzoylcysteine methyl ester without any polyphenol. All data are presented as the mean ± SD (n = 3).

were produced along with a disulfide from *N*-benzoylcysteine methyl ester. These data indicated that oxidation of the phenolic acids was independent of thiol oxidation. In the case of quercetin (**11**), Awad et al.[25] have reported several cysteine adducts of enzymatically oxidized quercetin; however, our MS data showed the formation of three oxidation products and no thiol adducts. The instability of cysteine adducts of quercetin (**11**) was also mentioned by Awad et al.[26] Hence, quercetin (**11**) adducts might be too unstable to be detected by HPLC or did not form under our conditions. Other flavonoids

dx.doi.org/10.1021/jf3008822 | J. Agric. Food Chem. 2012, 60, 5142−5151

Table 1. MS Analytical Results of Major Product Peaks from the Reaction of Polyphenols and *N*-Benzoylcysteine Methyl Ester

polyphenol	retention time (min) on HPLC	observed deprotonated molecular ion (m/z)	expected molecular formula for the ion	observed typical fragment ions (m/z) in MS and MS^E and their molecular formulas	estimated product
methyl caffeate (**1**)	23.2	430.0956	$C_{21}H_{20}NO_7S$	225.0219 ($C_7H_9O_4S$) 165.0005 ($C_9H_5O_2S$) 133.0282 ($C_8H_5O_2$)	monothiol adduct
methyl ferulate (**2**)	27.1	413.1201	$C_{22}H_{21}O_8$		dimer
methyl sinapate (**3**)	22.6	473.1470	$C_{24}H_{25}O_{10}$		dimer
	22.9	473.1446	$C_{24}H_{25}O_{10}$		dimer
	23.5	473.1447	$C_{24}H_{25}O_{10}$		dimer
methyl dihydrocaffeate (**4**)	23.6	432.1139	$C_{21}H_{22}NO_7S$	153.0002 ($C_7H_5O_2S$)	monothiol adduct
	26.6	669.1597	$C_{32}H_{33}N_2O_{10}S_2$	464.0833 ($C_{21}H_{22}NO_7S_2$) 259.0099 ($C_{10}H_{11}O_4S_2$) 225.0228 ($C_{10}H_9O_4S$)	dithiol adduct
	28.0	906.2058	$C_{43}H_{44}N_3O_{13}S_3$	701.1314 ($C_{32}H_{33}N_2O_{10}S_3$) 496.0565 ($C_{21}H_{22}NO_7S_3$)	trithiol adduct
methyl protocatechuate (**5**)	21.8	404.0811	$C_{19}H_{18}NO_7S$	199.0053 ($C_8H_7O_4S$)	monothiol adduct
kaempferol (**9**)	20.3	540.0923	$C_{26}H_{22}NO_{10}S$	522.0828 ($C_{26}H_{20}NO_9S$) 283.0222 ($C_{15}H_7O_6$) 255.0287 ($C_{14}H_7O_5$)	monothiol and water adduct
morin (**10**)	23.6	538.0776	$C_{26}H_{20}NO_{10}S$	299.0167 ($C_{15}H_7O_7$) 271.0220 ($C_{14}H_7O_6$) 151.0022 ($C_7H_3O_4$)	monothiol adduct
quercetin (**11**)	20.2	317.0286	$C_{15}H_9O_8$	299.0183 ($C_{15}H_7O_7$) 271.0226 ($C_{14}H_7O_6$) 151.0025 ($C_7H_3O_4$)	oxidative water adduct
	20.3	317.0283	$C_{15}H_9O_8$	299.0177 ($C_{15}H_7O_7$) 271.0247 ($C_{14}H_7O_6$) 151.0025 ($C_7H_3O_4$)	oxidative water adduct
	24.1	299.0189	$C_{15}H_7O_7$	271.0223 ($C_{14}H_7O_6$)	oxidation product
myricetin (**12**)	19.1	554.0761	$C_{26}H_{20}NO_{11}S$	349.0006 ($C_{15}H_9O_8S$) 315.0148 ($C_{15}H_7O_8$) 287.0192 ($C_{14}H_7O_7$) 151.0025 ($C_7H_3O_4$)	monothiol adduct
	22.1	791.1218	$C_{37}H_{31}N_2O_{14}S_2$	586.0458 ($C_{26}H_{20}NO_{11}S_2$) 380.9734 ($C_{15}H_9O_8S_2$) 346.9846 ($C_{15}H_7O_8S$) 328.9749 ($C_{15}H_5O_7S$)	dithiol adduct
luteolin (**13**)	21.7	522.0859	$C_{26}H_{20}NO_9S$	317.0103 ($C_{15}H_9O_6S$) 165.0004 ($C_8H_5O_2S$) 151.0022 ($C_7H_3O_4$)	monothiol adduct
	25.3	759.1382	$C_{37}H_{31}N_2O_{12}S_2$	554.0580 ($C_{26}H_{20}NO_9S_2$) 348.9864 ($C_{15}H_9O_6S_2$)	dithiol adduct
taxifolin (**14**)	20.1	540.0942	$C_{26}H_{22}NO_{10}S$	522.0846 ($C_{26}H_{20}NO_9S$) 335.0225 ($C_{15}H_{11}O_7S$) 317.0139 ($C_{15}H_{10}O_6S$) 208.9904 ($C_9H_5O_4S$)	monothiol adduct
	20.6	540.0964	$C_{26}H_{22}NO_{10}S$	522.0843 ($C_{26}H_{20}NO_9S$) 335.0287 ($C_{15}H_{11}O_7S$) 317.0147 ($C_{15}H_9O_6S$) 208.9931 ($C_9H_5O_4S$)	monothiol adduct

dx.doi.org/10.1021/jf3008822 | *J. Agric. Food Chem.* 2012, 60, 5142−5151

Table 1. continued

polyphenol	retention time (min) on HPLC	observed deprotonated molecular ion (m/z)	expected molecular formula for the ion	observed typical fragment ions (m/z) in MS and MS^E and their molecular formulas	estimated product
	24.2	778.1449	$C_{37}H_{33}N_2O_{13}S_2$	759.1334 ($C_{37}H_{31}N_2O_{12}S_2$) 572.0678 ($C_{26}H_{22}NO_{10}S_2$) 446.0390 ($C_{20}H_{16}NO_7S_2$) 240.9625 ($C_9H_5O_4S_2$)	dithiol adduct
	25.8	1014.1944	$C_{48}H_{44}N_3O_{16}S_3$	809.1183 ($C_{37}H_{33}N_2O_{13}S_3$) 775.1334 ($C_{37}H_{31}N_2O_{13}S_2$) 683.0851 ($C_{31}H_{27}N_2O_{10}S_3$) 570.0552 ($C_{26}H_{20}NO_{11}S_2$)	trithiol adduct
catechin (**15**)	17.3	526.1191	$C_{26}H_{24}NO_9S$	321.0418 ($C_{15}H_{13}O_6S$) 180.9972 ($C_8H_5O_3S$) 165.0029 ($C_8H_5O_2S$)	monothiol adduct
	18.1	526.1173	$C_{26}H_{24}NO_9S$	321.0433 ($C_{15}H_{13}O_6S$) 183.0127 ($C_8H_7O_3S$) 141.0004 ($C_6H_5O_2S$)	monothiol adduct
	21.7	763.1630	$C_{37}H_{35}N_2O_{12}S_2$	558.0881 ($C_{26}H_{24}NO_9S_2$) 353.0147 ($C_{15}H_{13}O_6S_2$) 214.9837 ($C_8H_7O_3S_2$)	dithiol adduct
	23.8	1000.2159	$C_{48}H_{46}N_3O_{15}S_3$	795.1360 ($C_{37}H_{35}N_2O_{12}S_3$) 590.0613 ($C_{26}H_{24}NO_9S_3$)	trithiol adduct

[(myricetin (**12**), luteolin (**13**), morin (**10**), taxifolin (**14**), kaempferol (**9**), and catechin (**15**)] gave corresponding thiol adducts, consisting of mono-, di-, and/or a small amount of trithiol adducts. Mono- and diglutathione adducts of taxifolin (**14**) and luteolin (**13**) have been reported.[25] They determined from NMR analysis that substitutions occurred at the 2- and/or 5-positions of the B ring. Similar to their results, in our monothiol adducts of myricetin (**12**), luteolin (**13**), and morin (**10**), the substitution of *N*-benzoylcysteine methyl ester might have occurred on the B ring, because MS^E fragment analysis of each monothiol adduct showed a typical fragment at m/z 151($C_7H_4O_4$) due to the unreacted A ring.[27] Sang and co-workers identified cysteine adducts of enzymatically oxidized epigallocatechin, both at the 2-position of the B ring and at the 2-position of the 3-galloyl moiety by NMR analysis.[28] Similar adducts of epigallocatechin with a thiol-bearing protein were also observed by Mori et al.; however, they observed a protein−thiol adduct in only the galloyl part of gallocatechins and did not observe any in catechin (**15**).[29] A cysteine adduct of catechin (**15**) at the 4-position has been found as a thiolysis product from catechin polymer.[30] Our MS results concerning observation of MS^E fragments from the thiol-substituted B ring ($C_8H_7O_3S$ from a monothiol adduct and $C_8H_7O_3S_2$ from a dithiol adduct) strongly indicated that *N*-benzoylcysteine methyl ester reacted at the B ring (2′-, 5′-, and/or 6′-position) under our radical conditions, although catechin (**15**) has no galloyl moiety. The reaction of kaempferol (**9**) showed a predominant peak in the HPLC data, which had a molecular-related ion at m/z 540.0923 $(M - H)^-$ (estimated molecular formula, $C_{26}H_{23}NO_{10}S$). This MS unit indicated that the peak compound was an adduct of both *N*-benzoylcysteine methyl ester and a water molecule with kaempferol (**9**). Two major fragment ions ($C_{26}H_{20}NO_9S$ and $C_{15}H_7O_6$) in the MS^E of the peak compound, which correspond to a dehydrated ion and an oxidized kaempferol ion, respectively, also suggested that the compound was possibly formed by oxidative thiol addition and subsequent water addition.

For phenolic acid esters, methyl ferulate (**2**), methyl sinapate (**3**), and propyl gallate (**8**) did not yield any thiol adducts. LC-MS analysis of their reaction products revealed several dimers and their oxidation products. On the other hand, methyl caffeate (**1**), methyl dihydrocaffeate (**4**), and methyl protocatechuate (**5**) yielded the corresponding thiol adducts, which were also detected by LC-MS. Interestingly, methyl caffeate (**1**) and methyl protocatechuate (**5**) gave only monothiol adducts, whereas methyl dihydrocaffeate (**4**) afforded mono-, di-, and trithiol adducts. Bassil et al. have tentatively identified a 2-cysteine adduct of caffeic acid in the reaction of cysteine and caffeic acid by periodate oxidation.[31] Saito and Kawabata have also reported that several thiols reacted first at the 2-position of a quinone from protocatechuic acid.[32] Our thiol adduct might be similar to the product reported by Saito and Kawabata in the reaction position of *N*-benzoylcysteine methyl ester. Although methyl dihydrocaffeate (**4**) is structurally related to methyl caffeate (**1**), without a double bond at the 2-position, three kinds of dihydrocaffeate thiol adducts were observed in its reaction, in contrast with the reaction of methyl caffeate (**1**). LC-MS analytical results of the reaction mixture revealed that these adducts were mono-, di-, and trithiol adducts. Caffeates and dihydrocaffeates have structures similar to the B−C ring systems of flavones and flavanones, respectively. Detailed analysis of the structures of the thiol derivatives of both caffeates and dihydrocaffeates would afford valuable information for the chemical interaction of thiols with not only phenolic acids but also flavonoids.

Structure Identification of the Products of the Reaction of Methyl Caffeate and Methyl Dihydrocaffeate with *N*-Benzoylcysteine Methyl Ester. As shown in the panel A of Figure 3, methyl caffeate afforded one product, whereas methyl dihydrocaffeate gave three products during DPPH oxidation in the presence of *N*-benzoylcysteine methyl ester (panel B of Figure 3). The DPPH reaction of methyl caffeate (**1**) was carried out again, and the reaction mixture was then purified by silica gel TLC to give a monothiol adduct (**20**) [m/z 430.0956 $(M - H)^-$]. The structure of the adduct, especially the substituted position of the thiol, was determined by NMR analysis of isolated **20**. The ^1H NMR data of **20** showed signals due to a benzoylcysteine moiety without a thiol

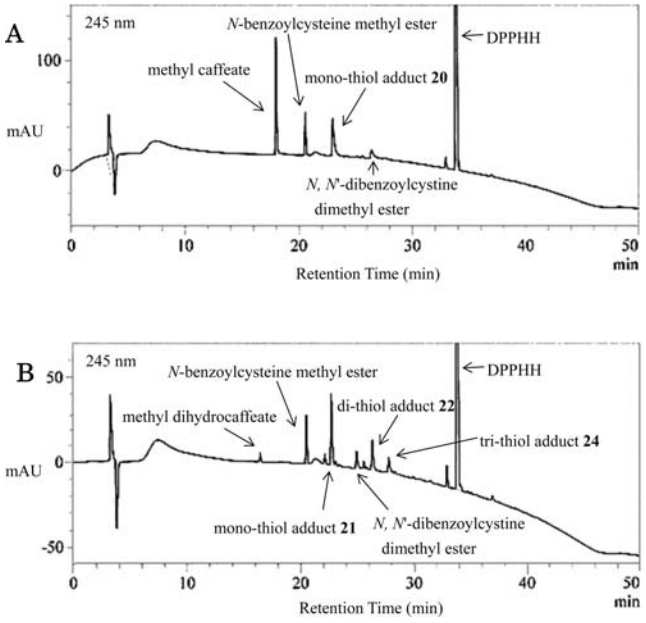

Figure 3. HPLC analytical profile for DPPH reaction (2 h) products of methyl caffeate (A) and methyl dihydrocaffeate (B) in the presence of *N*-benzoylcysteine methyl ester.

hydrogen signal, and the data also showed signals due to methyl caffeate (**1**) lacking one proton signal of the benzene moiety. These results revealed that the thiol reacted at the benzene moiety of methyl caffeate (**1**). The remaining two hydrogen signals (δ 7.20 and 6.91) for the methyl caffeate (**1**) benzene part showed an ortho relationship from their coupling constant (J = 8.2 Hz). Therefore, it was concluded that the *N*-benzoylcysteine methyl ester attaches at the 2′-position of methyl caffeate (**1**). The structure was also confirmed by a C−H long-range correlation between H3 of the thiol and C2′, which was observed in the HMBC spectrum of **20**.

A large-scale DPPH reaction in the presence of *N*-benzoylcysteine methyl ester was also performed with dihydrocaffeic acid methyl ester. The reaction mixture was purified by silica gel column chromatography to give a monothiol adduct (**21**), a dithiol adduct (**22**), and a trithiol adduct (**23**) as major products. The ^{1}H NMR analytical data of the monoadduct **21** showed a signal set due to a benzoylcysteine moiety and a signal set due to a methyl dihydrocaffeate moiety. It was determined that the thiol is attached at the 5′-position of the dihydrocaffeate moiety on the basis of the meta coupling constant (J = 2.0 Hz) between H2′ and H6′ (δ 6.85 and 6.70) on the benzene ring. The structure of the dithiol adduct, **22**, was also analyzed by ^{1}H NMR. The NMR data showed the presence of two *N*-benzoylcysteine methyl ester moieties and the lack of two protons on the dihydrocaffeate benzene moiety, indicating that the two thiols were attached to the benzene moiety. The remaining benzene ring proton was determined to be at the 6′-position, because NOE correlations from the 6′-proton (δ 6.87) to the methylene protons at the 3-position of the dihydrocaffeate (δ 3.00) and to the 3-methylene protons of the 5′-thiol moiety (δ 3.44 and 3.33) were observed in the NOESY spectrum. Therefore, the dithiol adduct **22** was determined to be the 2′,5′-dithiol adduct of methyl dihydrocaffeate. The structure of the trithiol adduct, **23**, was also determined by ^{1}H NMR. The data showed only two methylene signals [δ 3.53 (1H), 3.36 (1H), 2.37 (2H)] and a methyl signal [δ 3.65 (3H, s)] as signals of the protons due to methyl dihydrocaffeate. Therefore, three *N*-benzoylcysteine

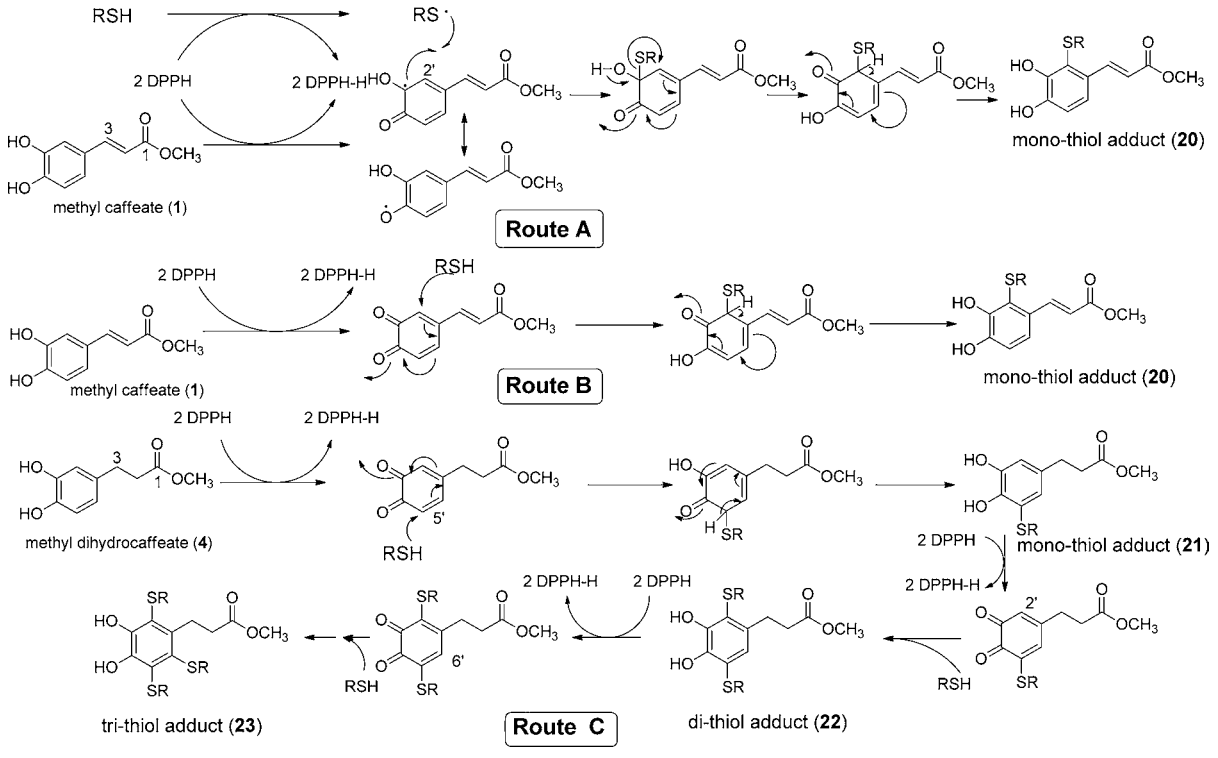

Figure 4. Proposed reaction mechanism for the formation of the monothiol adduct of methyl caffeate and the mono-, di-, and trithiol adducts of methyl dihydrocaffeate: route A, radical coupling mechanism for monothiol adduct **20**; route B, nucleophilic addition mechanism for monothiol adduct **20**; route C, nucleophilic addition mechanism for thiol adducts **21**, **22**, and **23**.

dx.doi.org/10.1021/jf3008822 | J. Agric. Food Chem. 2012, 60, 5142−5151

methyl ester moieties should be attached to the dihydrocaffeate benzene moiety. From these results, triadduct **23** was determined to be the 2′,5′,6′-trithiol adduct of dihydrocaffeic acid methyl ester.

From these NMR data, all structures of the thiol adducts of methyl caffeate (**1**) and methyl dihydrocaffeate were successfully identified. Interestingly, thiol substitution positions are different in monoadducts of caffeate and dihydrocaffeate. Monoadduct **20** from methyl caffeate (**1**) has the thiol at the 2′-position, whereas monoadduct **21** from methyl dihydrocaffeate (**4**) has it at the 5′-position. In addition to the substituted position, methyl caffeate (**1**) gave only the monoadduct under the reaction conditions; however, methyl dihydrocaffeate gave higher substituted thiol adducts under the same conditions. The thiol adducts from methyl dihydrocaffeate (**4**) all have the 5′-position thiol substituent, which strongly suggested that the first addition of the thiol to methyl dihydrocaffeate (**4**) occurs at the 5′-position. A thiol can be easily converted to an active thiyl radical in addition to it having potent nucleophilicity; therefore, both types of reactions (radical and ionic) should be considered in the production of these thiol adducts. We previously reported that a lipid peroxyl radical reacted at the 3′-position of methyl caffeate (**1**).[8] This position is a radical-stabilizing position by the captodative effect. When the thiyl radical is formed along with caffeate radicals, reaction should occur at the 3′-position. After the radical coupling, the thiol can migrate via a reaction such as a dienone−phenol rearrangement to the 2′-position to form adduct **20** (route A in Figure 4). On the other hand, when quinone formation (dehydration of the caffeate) is faster than thiyl radical formation, the thiol can react at any of the 2′-, 5′-, and 6′-positions by nucleophilic addition. MO calculation using MOPAC2007 software showed that the lowest unoccupied molecular orbital (LUMO) is distributed mainly on the 2′-position of the quinone derivative of methyl caffeate (**1**). Therefore, nucleophilic addition of the thiol can occur at the 2′-position on the quinone derivative (route B in Figure 4).

It should be noted that the quinone of methyl dihydrocaffeate is more stable than that of methyl caffeate (**1**) because the former has no electron-withdrawing group on the benzene ring, in contrast to the latter. Hence, the dihydrocaffeate quinone is probably produced much more rapidly than the caffeate quinone and possibly than thiyl radicals. The quinone produced reacts next with the thiol at the 5′-position by nucleophilic addition, because the 5′-position is the least sterically hindered position, despite there being no difference in the reactivity between the 2′-, 5′-, and 6′-positions as estimated from the MO calculation results. The monothiol adduct of the dihydrocaffeate probably has higher reactivity to radicals than the original dihydrocaffeate to form another quinone derivative. Therefore, the next thiol addition might take place to yield the dithiol adduct **22** and the trithiol adduct **23** (route C in Figure 4).

It is well recognized that foods are complex systems with various biomolecules. Polyphenols show potent antioxidant activity by their antiradical property in the food systems; therefore, the potent reactivity of polyphenols should have them react with other food components. Our results show the possibility of a cross-reaction of some polyphenols with thiol-bearing food components and indicate the accumulation of the products in oxidizing foods.

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Funding

Financial support from the Japan Society for the Promotion of Science (Kakenhi No. 23800929) and the Iijima Memorial Foundation for the Promotion of Food Science and Technology is gratefully acknowledged.

Notes

The authors declare no competing financial interest.

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