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Quantitation of Volatiles and Nonvolatile Acids in an Extract from Coffee Beverages: Correlation with Antioxidant Activity

KAZUTOSHI FUJIOKA AND TAKAYUKI SHIBAMOTO*

Department of Environmental Toxicology, University of California, 1 Shields Avenue, Davis, California 95616

The antioxidant activities of a commercial brewed coffee were investigated by measuring malonaldehyde (MA) formation from oxidized cod liver oil using a gas chromatographic method (MA-GC assay) and a thiobarbituric acid method (TBA assay). The highest antioxidant activity obtained by the MA-GC assay was from regular whole brewed coffee (97.8%) at a level of 20%, and the highest antioxidant activity obtained by the TBA assay was from decaffeinated whole brewed coffee (96.6%) at a level of 5%. Among 31 chemicals identified in a dichloromethane extract, guaiacol, ethylguaiacol, and vinylguaiacol exhibited antioxidant activities, which were comparable to that of α-tocopherol. Among nine chlorogenic acids (three caffeoylquinic acids, three feruloylquinic acids, and three dicaffeoylquinic acids) identified, 5-caffeoylquinic acid contained the greatest amount both in regular (883.5 μg/mL) and in decaffeinated (1032.6 μg/mL) coffees; it exhibited 24.5% activity by the MA-GC assay and 45.3% activity by the TBA assay at a level of 10 μg/mL. Caffeic and ferulic acids showed moderate antioxidant activities in both assays.

KEYWORDS: Antioxidant activity; brewed coffee; chlorogenic acid; heterocyclic compounds

INTRODUCTION

There have been numerous reports on diseases associated with coffee consumption (*1*). Epidemiological studies on the relationship between coffee drinking and risks of cancer have been conducted extensively over the last four decades (*2*). A possible relationship between coffee consumption and different diseases, including bladder cancer (*3*), pancreatic cancer (*4*), and coronary heart disease (*5*), has been reported. There is, however, still no strong evidence to confirm that coffee consumption actually causes diseases, such as various cancers (*6*).

Not all of the reported effects of coffee drinking are harmful. A recent review article stated that epidemiological and experimental studies have shown the positive effect of regular coffee drinking on various aspects of health, such as psychoactive responses (alertness), metabolic rate, and gonad and liver function (*7*). The beneficial effects of coffee may be due to the presence of antioxidants, which prevent the oxidative damage associated with many diseases, such as cancer (*8*) and arteriosclerosis (*9*).

There are many reports on the antioxidant activities of coffee. The first constituents of coffee reported to possess antioxidant activity were high molecular weight substances such as melanoidins (*10*). Later, low molecular weight volatile compounds, particularly heterocyclic flavor compounds, were found in coffee aroma essences, which exhibited potent antioxidant activities (*11*).

In the present study, the antioxidant activities of model beverage samples prepared with a commercial brewed coffee were investigated in order to examine the health benefits, which might be obtained from coffee drinking.

MATERIALS AND METHODS

Coffee and Chemicals. A commercial ground, roasted coffee was bought from a local market. Cod liver oil, malonaldehyde (MA) tetrabutylammonium salt, 1-methylpyrazole (1-MP), 2-methylpyrazine (2-MP), *N*-methylhydrazine, tris (hydroxymethyl) aminomethane (Tris), tris (hydroxymethyl) aminomethane hydrochloride, sodium dodecyl sulfate (SDS), thiobarbituric acid (TBA), 10% trichloroacetic acid (TCA), and 1,1,3,3-tetramethoxypropane (TMP) were purchased from Sigma-Aldrich Chemical Co. (St. Louis, MO). 5-Caffeoylquinic acid was bought from Cayman Chemical Co. (Ann Arbor, MI). All authentic volatile chemicals were obtained from Takata Koryo Co., Ltd. (Osaka, Japan) as a gift. A 500 μM TMP stock solution was prepared by dissolving 20.5 mg of TMP into deionized water in a 250 mL volumetric flask.

Preparation of Brewed Coffee Samples. Ground, roasted regular, or decaffeinated coffee (50 g) was brewed with 600 mL of deionized water using a Mr. Coffee NCX-20 model coffee maker (Sunbeam Products Inc., Boca Raton, FL) equipped with a paper filter. Brewed coffee was immediately cooled to room temperature in an ice bath.

Brewed coffee (500 mL) was then extracted with 150 mL of dichloromethane at room temperature using a liquid−liquid continuous extractor for 6 h. The extract was dried over anhydrous sodium sulfate overnight. After the sodium sulfate was removed, the extract was condensed using a rotary evaporator and then the volume was reduced to 4 mL under a purified nitrogen stream. The extract was used for volatile analysis and antioxidant tests. The residual aqueous sample was used for antioxidant tests. The brewed coffee samples were stored at 5 °C until used.

Analysis of Dichloromethane Extract of Brewed Coffee. A dichloromethane extract was analyzed by gas chromatography (GC)

* To whom correspondence should be addressed. Tel: +1 530-752-4523. Fax: +1 530-752-3394. E-mail: tshibamoto@ucdavis.edu.

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and GC/mass spectrometry (MS) using the newly developed headspace-solid-phase microextraction-gas chromatographic (HS-SPME-GC) method (*12*). Both the GC retention index (*13*) and the MS fragmentation pattern of each GC component were compared with those of the authentic compounds for qualitative analysis. The concentration (μg/g of ground coffee) of chemicals identified was calculated using the following equation (*14*):

$$\text{concentration} = \frac{\text{weight of extract (without solvent)} \times \text{GC peak area \%}/100}{\text{weight of ground coffee}}$$

A HP model 6890 GC interfaced to a HP 5791A mass selective detector (GC/MS) was used for mass spectral identification of the GC components, at a MS ionization voltage of 70 eV. A 60 m $\times$ 0.25 mm i.d. (d_f = 0.25 μm) DB-WAX bonded-phase fused-silica capillary column (J & W Scientific, Folsom, CA) was used for GC analysis. The linear velocity of the helium carrier gas was 30 cm/s. The injector and the detector temperatures were 250 °C. The oven temperature was programmed from 50 to 200 °C at 2 °C/min and held at 200 °C for 90 min. The linear velocity of the helium carrier gas was 30 cm/s with a split ratio of 1:20. The identification of GC components was also conducted by comparing their mass spectra with those contained within the NIST AMDIS software (version 2.1).

Analysis of Chlorogenic Acids (CGAs), Caffeic Acid, and Ferulic Acid in Whole Brewed Coffee. The regular and decaffeinated brewed coffees were treated with Carrez reagents I and II (*15*) to eliminate polymeric components according to a previously reported method (*16*). A sample (3 mL) containing 0.1 mL of Carrez reagents I and II and 0.8 mL of methanol was vortex-mixed in a centrifuge tube and allowed to stand for 10 min. The precipitate was separated by centrifuging at 5000 rpm for 10 min. The solution was then decanted and filtered with an Acrodisc Syringe Filter fitted with a 0.2 μm HT Tuffryn membrane (Pall Corp., Ann Arbor, MI). Citric acid (10 mM) was used to suppress ionization of acidic compounds and to achieve separation using the reverse-phase high-performance liquid chromatography (HPLC).

Quantitative analyses of CGAs (3-caffeoylquinic acid, 4-caffeoylquinic acid, 5-caffeoylquinic acid, 3-ferulolylquinic acid, 4-ferulolylquinic acid, 5-ferulolylquinic acid, 3,4-dicaffeoylquinic acid, 3,5-dicaffeoylquinic acid, and 4,5-dicaffeoylquinic acid), caffeic acid, and ferulic acid in the whole brewed coffee were performed using an Agilent 1100 model HPLC system equipped with a Zorbax Eclipse XDB C-18 column (5 μm, 150 mm $\times$ 4.6 mm) and a multiple wavelength detector. Mobile phase A was 10 mM citric acid, and mobile phase B was methanol. The gradient mode was initially set at A/B ratio of 85/15 from 0 to 5 min, then linearly increased to 60/40 at 40−45 min. The flow rate was 1.0 mL/min. The detector was set at 325 nm for CGA and at 276 nm for caffeine; the injection volume was 20 μL.

Concentrations of components were calculated using the regression equation of their concentration and peak area. The limits of quantification were calculated as 10 times the standard deviation of the lowest concentration of the standard solution (50 μM). Recovery efficiency was determined with a coffee sample spiked with 250 μM each of 5-caffeoylquinic acid, caffeic acid, and ferulic acid. Measurements were carried out in triplicate.

Identification of components in the brewed coffee was confirmed by a Hewlett-Packard 1100 liquid chromatograph interfaced to an Applied Biosystems API 2000 MS/MS via an atmospheric pressure chemical ionization (APCI) source operating in the positive ion mode at 400 °C.

Antioxidant Activities of Brewed Coffee Samples Measured by MA-GC Assay. The antioxidant activities of whole brewed coffee, brewed coffee extract, water fraction, and the major compounds found in brewed coffee were determined by analyzing the percentage inhibition (E) of MA formation from the oxidized cod liver oil using the following equation (*17*):

$$E\,(\%) = \left(1 - \frac{\text{amount of MA in the sample oxidized without antioxidant} - \text{amount of MA in the sample oxidized with antioxidant}}{\text{amount of MA in the sample oxidized without antioxidant}}\right) \times 100$$

An aqueous solution (2 mL) containing 4 μL of cod liver oil, 0.05 M Tris buffer (pH 7.4), 0.2 μmol of H_2O_2, 0.4 μmol of $FeCl_2$, 0.30 mmol of KCl, 0.2% SDS, and 20 μL of solution of a coffee constituent (10, 50, or 200 μg/mL) in an 8 mL scintillation vial with a screw cap was incubated for 16 h at 37 °C in a water bath equipped with a shaker (Precision Scientific, Inc., Chicago, IL). Known antioxidants, α-tocopherol and BHT, were used to validate the method. The sample tubes were covered with aluminum foil during incubation to avoid photooxidation induced by light. After incubation, 20 μL of 4% BHT solution in ethanol was added to stop the oxidation. The MA in the samples was derivatized to 1-MP by adding *N*-methylhydrazine (4 μL). The tube was stirred magnetically for 1 h at room temperature. 2-MP aqueous solution (20 μL, 4 mg/mL) was added to the reaction solution as a GC internal standard.

The MA formed in the oxidized cod liver oil was analyzed as 1-MP with a HS-SPME-GC method previously reported (*12*). The headspace of a sample vial was collected on poly(dimethylsiloxane)/divinylbenzene fiber (65 μm thickness, Supelco, Inc., Bellefonte, PA) and then directly injected into an Agilent Technologies model 6890N GC equipped with a 30 m $\times$ 0.25 mm (d_f = 25 μm) ZB-WAX fused-silica capillary column (Phenomenex) and a nitrogen−phosphorus detector. The GC oven temperature was held at 60 °C for 1 min and then programmed to 180 °C at 12 °C/min and held for 1 min. The injector and detector temperatures were 200 and 300 °C, respectively. The linear velocity of the helium carrier gas was 30 cm/s. The desorption time was set at 5 min.

Antioxidant Activities of Brewed Coffee Samples Measured by TBA Assay. A TBA assay for the identical samples tested by the MA-GC assay was performed according to the procedure described previously (*18*), with minor modifications. A TCA−TBA reagent was prepared by mixing 20 mL of 10% TCA solution, 20 mL of 0.65% TBA solution, and 2 mL of 1% SDS. One hundred microliters of samples or standard TMP solutions (0, 10, 25, 50, and 100 μM) and 2.0 mL of the TCA−TBA reagent were placed into a 15 mL disposable centrifuge tube. After the centrifuge tube was vortex-mixed for 2 s, the mixture was heated at 80 °C for 1 h in a water bath. The mixture was cooled in an ice bath for 10 min, and then, the absorbance at 532 nm (A_{532}) was measured using a Hewlett-Packard 8452A diode array spectrophotometer in triplicate.

RESULTS AND DISCUSSION

Antioxidant Activities of Brewed Coffee Samples. Figure 1 shows the results of the antioxidant test on brewed coffee samples measured by the MA-GC assay (**A**) and the TBA assay (**B**). The values are means ± SD (n = 3). The quantification limit of 1-MP for the MA-GC assay was 10.3 μmol/mL in the present study. A synthetic antioxidant BHT was simultaneously examined at a concentration of 10 μg/mL as a positive standard. Whole-brewed coffee samples, both regular and decaffeinated, exhibited dose-dependent activities by the MA-GC assay. Regular whole-brewed coffee showed the highest antioxidant activity at a level of 20% (97.8 ± 04%) by the MA-GC assay, whereas decaffeinated whole-brewed coffee exhibited the highest activity at the level of 5% (96.6 ± 7.3%) by the TBA assay. The level of inhibition of MA production was generally higher for the TBA assay than for the MA-GC assay. For example, in the case of 1% whole regular-brewed coffee, the result from the TBA assay (16.7 ± 4.7%) was much higher than that from the MA-GC assay (7.1 ± 4.8%). This may be an overestimation of the amount of MA by the TBA assay because it measures the total TBA-carbonyl adducts (*19*).

In the case of the water fraction, a sample from decaffeinated coffee exhibited a much higher activity (94.4% by the MA-GC assay and 100% by the TBA assay) than that from regular coffee

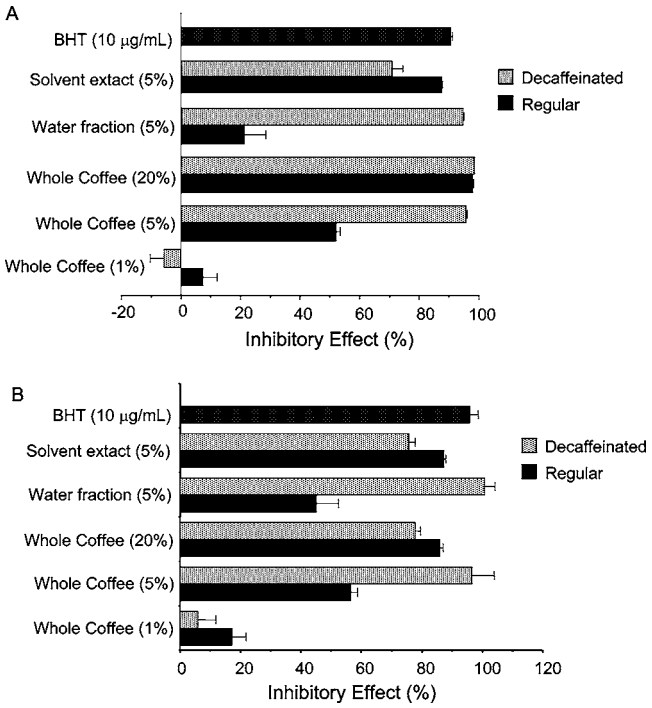

Figure 1. Results of the antioxidant test on brewed coffee samples measured by the MA-GC assay (**A**) the TBA assay (**B**).

(21.0% by the MA-GC assay and 44.5% by the TBA assay). On the other hand, a dichloromethane extract from a regular coffee showed higher activities (87.1% by the MA-GC assay and 96.8% by the TBA assay) than those from decaffeinated coffee (70.8% by the MA-GC assay and 75.6% by the TBA assay) in the present study. The potent antioxidant activities of dichloromethane extracts from brewed coffee have also been reported previously (*11*). These results suggest that the antioxidant activity of coffee samples depends on the chemical composition of the samples.

Major Volatile Compounds Found in Dichloromethane Extracts of Brewed Coffees. There are numerous reports on the volatile compounds identified in brewed coffees (*20*). **Table 1** shows the major volatile compounds identified positively in dichloromethane fractions in the present study. Many heterocyclic compounds, which have been known as major volatile flavor compounds in coffee (*21*), were identified as main components of whole-brewed coffees. These included eight pyrazines, seven furans, four pyrroles, one pyridine, and one thiophene. Furfuryl alcohol (200.3 μg/g in regular, 196.6 μg/g in decaffeinated) was found in the greatest amount followed by furfural (22.6 μg/g in regular, 31.4 μg/g in decaffeinated), pyridine (17.5 μg/g in regular, 19.2 μg/g in decaffeinated), 2-MP (12.0 μg/g in regular, 14.4 μg/g in decaffeinated), 5-methylfuran-2-carboxaldehyde (10.4 μg/g in regular, 14.9 μg/g in decaffeinated), pyrrole-2-carboxaldehyde, and 2,5-dimethylpyrazine (10.6 μg/g in regular, 16.1 μg/g in decaffeinated). In addition, five phenolic compounds (guaiacol and its analogues) were found as possible antioxidants (*22*).

Acidic Compounds Found in Brewed Coffee. Table 2 shows the acidic compounds found in brewed coffee. The standard curves for the acid quantification were satisfactory ($R^2 = 0.9998$). The recovery efficiencies of the acidic compounds from brewed coffee were 96.8 ± 1.4% for 5-caffeoylquinic acid, 102.0 ± 2.2% for caffeic acid, and 100.5 ± 2.1% for ferulic acid in the present study. The quantification limits in brewed coffee were 3.5 μg/mL for 5-caffeoylquinic acid, 1.7 μg/mL for caffeic acid, and 5.1 μg/mL for ferulic acid. The total concentration of CGA in brewed coffee was 2488.5 μg/mL in regular coffee and 2727.9 μg/mL in decaffeinated coffee, which was consistent with previous reports (*23*).

Among the CGAs identified in the present study, 5-caffeoylquinic acid was present at the highest level both in regular (883.5 ± 3.8 μg/mL) and in decaffeinated (1032.6 ± 28.2 μg/mL) coffees, followed by 4-caffeoylquinic acid (66.2 ± 2.5 in regular and 661.8 ± 17.0 μg/mL in decaffeinated) and 3-caffeoylquinic acid (636.0 ± 1.4 μg/mL in regular and 605.9 ± 10.5 μg/mL in decaffeinated). 5-Caffeoylquinic acid was also the major acid found in the Arabica and Robusta green beans, where it comprised 66 and 56% of the total acids, respectively (*24*). Therefore, 5-caffeoylquinic acid was tested for antioxidant activities as a representative CGA. Caffeic acid and ferulic acid

Table 1. Major Components Identified in Dichloromethane Extracts of Regular and Decaffeinated Brewed Coffees

compound	KI[b]	concentration (ppm)[a] regular	decaffeinated
pyridine	1169	17.5	19.2
pyrazine	1197	1.7	2.1
dihydro-2-methyl-3(2H)-furanone	1242	4.9	5.7
2-MP	1247	12.0	14.4
2,5-dimethylpyrazine	1302	10.6	16.1
2,6-dimethylpyrazine	1308	3.4	3.5
2-ethylpyrazine	1311	3.8	5.0
2,3-dimethylpyrazine	1322	3.9	5.4
2-ethyl-6-methylpyrazine	1357	2.6	2.6
2-ethyl-5-methylpyrazine	1362	4.1	5.1
furfural	1423	22.6	31.4
2-furanmethyl acetate	1495	6.8	3.8
5-methyl-2-furancarboxyaldehyde	1530	10.4	14.9
1-methylpyrrole-2-carboxyladehyde	1574	1.8	2.4
γ-butyrolactone	1581	45.3	45.7
1-methyl-2-acetylpyrrole	1609	1.4	1.5
furfurylalcohol	1619	200.3	196.6
2(5H)-furanone	1702	3.9	6.0
3-methyl-1,2-cyclopentanedione	1781	8.0	7.3
guaiacol (2-methoxyphenol)	1808	1.6	2.7
3-ethyl-2-hydro-2-cyclopenten-1-one	1845	1.0	0.4
2-thiophenemethanol	1890	0.6	0.9
maltol	1915	2.8	2.3
phenol	1928	1.2	0.7
pyrrole-2-carboxaldehyde	1950	12.3	0.7
ethylguiacol (4-ethyl-2-methoxyphenol)	1984	6.4	11.8
5-methylpyrrole-2-carboxaldehyde	2046	6.5	1.1
vinylguaiacol (4-vinyl-2-methoxyphenol)	2135	4.6	7.5
2.5-dihydroxy acetophenone	2162	0.5	0.5
o-catecol	2321	0.4	0.4
5-(hydroxymethyl)-2-furfural	2440	4.7	6.5

[a] In whole brewed coffee. [b] Kovat's index on DB-WAX.

Table 2. Acidic Compounds Found in Brewed Coffees[a]

compounds	concentration (μg/mL) in regular	decaffeinated
3-caffeoylquinic acid	636.0 ± 1.4	605.9 ± 10.5
4-caffeoylquinic acid	662.1 ± 2.5	661.8 ± 17.0
5-caffeoylquinic acid	883.5 ± 3.8	1032.6 ± 28.2
3-feruloylquinic acid	41.7 ± 0.5	27.4 ± 0.5
4-feruloylquinic acid	85.7 ± 1.2	115.3 ± 3.9
5-feruloylquinic acid	116.9 ± 2.0	194.4 ± 7.8
3,4-dicaffeoylquinic acid	26.6 ± 0.8	41.0 ± 1.1
3,5-dicaffeoylquinic acid	15.5 ± 1.1	20.2 ± 1.1
4,5-dicaffeoylquinic acid	20.5 ± 0.5	29.3 ± 0.7
caffeic acid	ND	ND
ferulic acid	ND	ND

[a] ND, not detected.

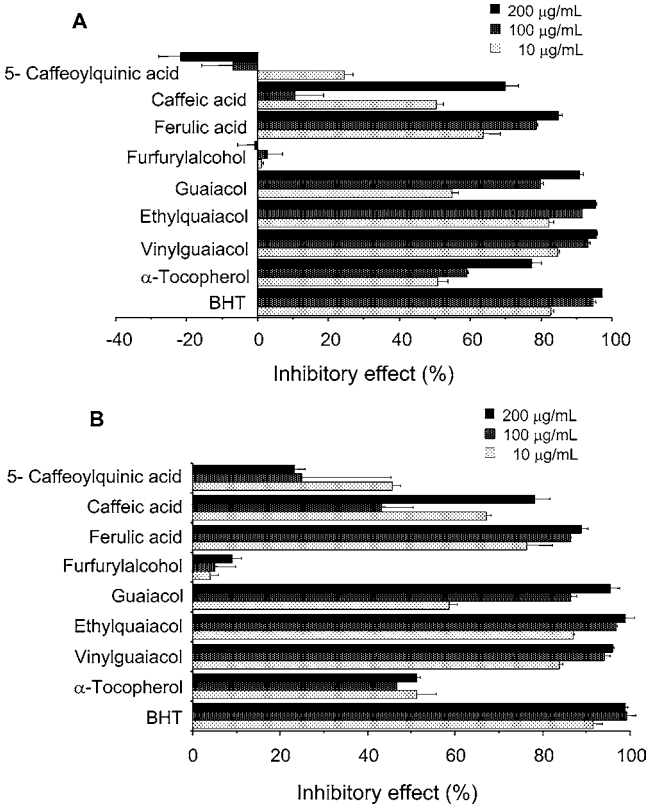

Figure 2. Results of an antioxidant test on chemicals found in brewed coffee measured by the MA-GC assay (**A**) and the TBA assay (**B**).

were not detected in the present study. However, the presence of caffeic acid and ferulic acid in brewed coffee has been reported (*25*). These compounds are metabolites of CGA, which are possibly distributed in the blood after the consumption of coffee (*26*). Therefore, the antioxidant activities of caffeic and ferulic acids were tested in addition to 5-caffeoylquinic acid.

Antioxidant Activities of Chemicals Present in Brewed Coffee. Figure 2 shows the results of an antioxidant test on chemicals found in brewed coffee measured by the MA-GC assay (**A**) and the TBA assay (**B**). Furfuryl alcohol, which was found in the dichloromethane extract at the highest level (200.3 μg/g in regular and 196.6 μg/g in decaffeinated), did not show any appreciable activity at any concentration in either of the assays in the present study, suggesting that furfuryl alcohol does not contribute to antioxidant activity in brewed coffee. It is reported that unsubstituted furan possessed a potent antioxidant activity, whereas 2-substituted furans, such as furfural and 2-acetylfuran, exhibited much less activity than that of unsubstituted furan (*27*). 5-Hydroxy furfural (4.7 μg/g in regular and 6.5 μg/g in decaffeinated) did not show significant activity (*28*).

The antioxidant activities of many heterocyclic compounds reported in the present study (**Table 1**) have already been examined using aldehyde/carboxylic acid assay. Pyrroles, including pyrrole-2-carboxaldehyde (12.3 μg/g in regular and 0.7 μg/g in decaffeinated), inhibited hexanal oxidation by >80% at 10 μg/mL level over 40 days, but pyrazines exhibited no appreciable antioxidant activity (*28*). Maltol (2.8 μg/g in regular and 2.3 μg/g in decaffeinated) inhibited hexanal oxidation by 100% at a level of 250 μg/mL for 10 days (*29*). An analogue of dihydro-2-methyl-3(2H)-furanone (4.9 μg/g in regular, μg/g in decaffeinated), 2,5-dimethyl-4-hydroxy-3(2H)-furanone, exhibited moderate antioxidant activity in a MA-GC assay (*30*).

Three phenolic compounds (guaiacol, vinlyguaiacol, and ethylguaiacol) exhibited strong antioxidant activities in both testing systems in the present study. They inhibited MA formation by over 90% at a level of 200 μg/mL in both testing systems. Ethylguaiacol showed comparable activities to BHT at all levels tested. The lowest level tested, 10 μg/mL, is calculated as 10 mg/L in the actual brewed coffee. The combined levels of guaiacol, vinylguaiacol, and ethylguaiacol (total = 12.6 μg/g) are, in part, responsible for the antioxidant activities of coffee. The antioxidant activities of guaiacol and methylguaiacol have been previously reported (*31*).

Caffeic and ferulic acids exhibited moderate antioxidant activities in both testing systems. It has been reported that caffeic and ferulic acids moderately inhibited MA formation from ethyl arachidonate oxidized with Fenton's reagents (*30*). The antioxidant activities of caffeic acid derivatives, particularly the caffeic acid phenethyl ester, have been reported (*32*). Ferulic acid has been reported as a natural antioxidant (*30*) and is found in various natural plants, including rice bran (*33*).

5-Caffeonylquinic acid showed somewhat unusual activities in both testing systems. It exhibited reverse dose-dependent antioxidant activities. 5-Caffeonylquinic acid showed weak−moderate antioxidant activity at a level of 10 μg/mL (24.5 ± 2.5% by the MA-GC assay and 45.3 ± 2.0% by the TBA assay). In the case of the MA-GC assay, 5-caffeonylquinic acid exhibited pro-oxidant activity at levels of 100 μg/μL and 200 μg/mL (−21.7 ± 6.3%). These results may be explained by the fact that both assays were based on the measurement of MA present in oxidized substances. The MA-GC assay is strictly specific to MA. Therefore, it is influenced by a blank value at high levels of testing samples, which produce MA themselves. 5-Caffeonylquinic acid may produce MA from its sugar moiety because a deoxyribose moiety of 2″-deoxyribonucleosides produced 84−214 nmol of MA from 16 μmol of 2″-deoxyribonucleosides (*34*). MA values determined for 5-caffeonylquinic acid at the levels of 100 and 200 mg/mL might include the MA formed from the testing chemical. On the other hand, the TBA assay measures not only MA but also any carbonyl compounds that form a TBA adduct; consequently, values obtained by this method always overestimate MA (*35*). Therefore, positive values obtained at lower levels by the TBA assay, in the present study, might be due to the overestimation of MA.

The present study reports that brewed coffee contains both lipid-soluble (dichloromethane extracts) and water-soluble (CGA) compounds. The lipid-soluble components, including guaiacol and its analogues, are presumed to be absorbed into the blood and to act as antioxidants in the blood and liver. Antioxidant activities of these components are not as strong as other well-known natural antioxidants, such as α-tocopherol. However, the total sum of antioxidant activities of these components may significantly contribute to the antioxidant activity in brewed coffee. The water-soluble components are partially absorbed into the blood but mainly remain in the stomach. CGA especially is highly hydrophilic and stable under the gastric pH of 2.0. The coexistence of CGA and iron ions, which exist in many foods as free ions in the stomach, may increase its pro-oxidant properties and lead to the oxidation of lipids in the stomach (*26*). The benefits and risks of drinking coffee may be related to the bivalent properties of active compounds in brewed coffee.

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