Gallic Acid Metabolites Are Markers of Black Tea Intake in Humans

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Gallic acid is one of the main phenolic components of black tea. The objective of this study was to identify urinary gallic acid metabolites with potential for use as markers of black tea intake. In an initial study, nine compounds, assessed by using gas chromatography−mass spectrometry, were found to increase in concentration in urine after 3 cups of black tea over 3 h. A subsequent study employed a controlled crossover design in which 10 subjects consumed 5 cups per day of black tea or water for 4 weeks in random order. Twenty-four hour urine samples were collected at the end of each period. Of the 9 candidate compounds identified in the initial study, only 3 were present at higher concentrations in urine of all 10 subjects during tea-drinking in comparison to water-drinking periods. These compounds were identified as 4-*O*-methylgallic acid, 3-*O*-methylgallic acid, and 3,4-*O*-dimethylgallic acid, all methyl ether derivatives of gallic acid. It is suggested that these compounds have the potential to be used as markers of black tea intake.

Keywords: *Black tea; gallic acid; 4-O-methylgallic acid; 3-O-methylgallic acid; 3,4-O-dimethylgallic acid*

INTRODUCTION

The potential health benefits of drinking tea are suggested to be chiefly due to polyphenolic components of tea (Hertog et al., 1993; Jick et al., 1973; Keli et al., 1996). Results of epidemiological studies show that black tea (Hertog et al., 1993; Jick et al., 1973; Keli et al., 1996) and polyphenols derived from black tea (Hertog et al., 1993, 1995; Keli et al., 1996; Knekt et al., 1996) are associated with reduced risk of cardiovascular disease. Tea can be a major dietary source of polyphenolic compounds: polyphenols make up ∼30−40% of the weight of the tea leaf (Harbowy and Ballentine, 1997).

Compounds that provide an indication of tea polyphenol intake and exposure may be useful as markers of tea intake. Markers of tea intake will be useful in epidemiological and intervention studies focusing on tea and disease-related endpoints. Identification of such compounds has proved to be troublesome, due largely to the complex nature of many of the polyphenols present in black tea and lack of understanding of the metabolism of these compounds. Methods for the measurement of a particular class of polyphenolic compounds (van het Hof et al., 1998) or total polyphenolic concentrations (He and Kies, 1994) may be useful in providing an indication of black tea intake, but these methods are nonspecific.

Gallic acid is one of the main phenolic components of tea. Gallic acid occurs in black tea in free and esterified forms and is estimated to be present at ∼5% of the weight of the tea leaf (Harbowy and Ballentine, 1997). We propose that gallic acid metabolites may be useful as markers of black tea intake. The methyl ether derivative of gallic acid, 4-*O*-methylgallic acid (4OMGA), has previously been identified as a major metabolite of gallic acid in humans (Shahrzad and Bitsch, 1998). The possibility that gallic acid is metabolized to other methyl ether derivatives and/or other phenolic acids has been investigated in the present study.

The major objectives of this study were therefore (1) to identify phenolic acid metabolites of gallic acid that increase in concentration in human urine following black tea ingestion and (2) to establish whether concentrations of these compounds are consistently raised during regular black tea ingestion in comparison to regular water ingestion. Compounds present at consistently higher concentrations in urine of volunteers taking their usual diet during regular black tea ingestion can be considered to be of possible use as markers of black tea ingestion.

MATERIALS AND METHODS

Two studies were performed to identify and describe urinary gallic acid metabolites with potential for use as markers of black tea intake. In the first study phenolic acids (lower molecular weight compounds in the acid fraction of urine) that increased significantly in concentration after 3 cups of tea over 3 h in comparison to a baseline fasting urine sample were assessed. The methyl ether derivatives of gallic acid were specifically targeted as likely metabolites of gallic acid. Compounds identified as having increased acutely were then assessed in 24-h urine samples during 4 weeks of regular black tea or water (5 cups per day) consumption.

Subjects. Ten healthy subjects (eight men, two women) were recruited from the general population in response to newspaper advertisements. Volunteers were excluded after initial screening if they reported the use of any medication or dietary supplements; a history of major illness including heart disease, diabetes, liver disease, renal disease, and gastrointestinal conditions; current smoking or smoking cessation for <6 months; body mass index > 35 kg/m^2; an alcohol intake

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averaging >40 g per day; or regular tea intake averaging <1 cup per day. The Royal Perth Hospital Ethics Committee approved the project, and all participants gave informed written consent.

Experimental Design: Study 1. Urinary phenolic acids were measured in two volunteers before and after consumption of 3 cups of black tea. Subjects had fasted for at least 12 h and had not consumed tea for at least 2 weeks prior to measurements. Black tea was prepared by allowing 2 g of tea leaves to infuse in boiled water for 1 min. The tea was ingested without additives such as milk and sugar. A spot urine sample was taken for baseline measurements. The subjects then consumed 3 cups of black tea at hourly intervals over the following 3 h (at 1, 2, and 3 h after the baseline sample). A spot urine sample was taken again 1 h after the last drink of tea. Urine samples were frozen at −80 °C until analysis.

Experimental Design: Study 2. Compounds identified as having increased acutely were measured in 24-h urine samples collected during 4-week periods of regular black tea or water consumption. During the study subjects avoided all coffee, chocolate drinks, herbal infusions, and all teas—apart from that supplied. After a 4-week wash-out period during which subjects consumed 5 cups per day of hot water, subjects were randomly assigned to drink either black tea or hot water for 4 weeks. Subjects then crossed over to the alternate drink for a further 4 weeks. Black tea or hot water intake was 5 cups per day without additives such as milk and sugar. The tea was prepared by allowing 2 g of tea leaves to infuse in 250 mL of boiled water for 1 min. Twenty-four-hour urine samples were collected during the last week of each of the 4-week intervention periods. The volume of urine was recorded, and then an aliquot was frozen at −80 °C for subsequent analysis.

Assessment of Phenolic Compounds by Gas Chromatography—Mass Spectrometry (GC-MS). Urine (2 mL) and 1-hydroxy-2-naphthoic acid (1 μg, internal standard) were acidified to pH 4.8 with 1 M HCl. β-Glucuronidase (30 μL, with 3000 units of activity) (Sigma catalog no. G707) was added, mixed, and incubated at 37 °C for 24 h with occasional mixing. The samples were acidified (pH 2, 1 M HCl) and extracted with ethyl acetate (1 × 2 mL), and the aqueous phase was discarded. The organic phase was extracted with sodium bicarbonate (5% w/w, 1 × 2 mL), and the organic phase was discarded. The aqueous phase was acidified (pH 2, 5 M HCl) and extracted with ethyl acetate. The organic phase was retained, and the solvent was removed under a stream of nitrogen to yield the acid fraction of the urine samples. The trimethylsilyl derivative was made using BSTFA (50% v/v) in pyridine.

Phenolic acids and other compounds in the acid fraction were analyzed on a Hewlett-Packard HP 5890 gas chromatograph coupled to an HP 5970 mass spectrometer. Larger molecular weight polyphenols such as flavonoids were not specifically targeted. An HP-5MS column (25 m × 0.20 mm, 0.33 μm film thickness, Hewlett-Packard) was used with helium as the carrier gas and an inlet pressure of 30 kPa. Injections were made in a splitless mode. The initial column temperature of 120 °C was held for 0.5 min and then increased at 15 °C/min to 280 °C, at which it was held for 5 min. The mass spectrometer was operated in the electron impact mode (70 eV). All ions were monitored in the scan mode. Analyte abundances were compared using extracted ion chromatograms.

Compound Identification. Those compounds that were present at a higher concentration during regular black tea drinking, compared to the water period, in all 10 subjects were identified. Identification of the compounds was based on retention times and mass spectra compared with authentic standards.

4-O-Methylgallic Acid. The authentic standard of 4-*O*-methylgallic acid was prepared according to literature procedures (Luz Cardona et al., 1986). Briefly, gallic acid (1 mol equiv) was methylated with methyl sulfate (2 mol equiv) and potassium carbonate (2 mol equiv) in acetone at room temperature for 12 h. The methyl ester was hydrolyzed with 1 mol/L methanolic potassium hydroxide under nitrogen to give 4-*O*-methylgallic acid, which was purified by preparative thin-layer chromatography on silica gel.

3-O-Methylgallic Acid. The method of Jurd (1959) was modified for the synthesis of 3-*O*-methylgallic acid (3OMGA). Methyl gallate was first prepared according to a standard Fischer−Speier esterification using gallic acid and 1% methanolic HCl. Methyl gallate (0.5 g, 2.72 mmol) was mixed with dichlorodiphenylmethane (0.695 g, 2.93 mmol) and potassium carbonate (1.62 g, 11.7 mmol) and heated at 170−180 °C for 10 min until it turned brown and no further gas (HCl) was evolved. The mixture was extracted with ethyl acetate (3 × 20 mL), and the combined extracts were filtered, washed with water (2 × 20 mL), and dried ($MgSO_4$); the solvent was removed under reduced pressure and with warming to yield a brown oil, which formed brown amorphous crystals with cooling. The solid was dissolved in the minimum of ether, and an equal volume of hexane was added. Colorless crystals of methyl 3-hydroxy-4,5-diphenylmethylenedioxybenzoate formed (660 mg, 70%, mp 162−164 °C, lit. 164 °C; Jurd, 1959).

Dimethyl sulfate (180 mg, 1.4 mmol) was dissolved in ether (2 mL) and added to a solution of methyl 3-hydroxy-4,5-diphenylmethylenedioxybenzoate (395 mg, 1.1 mmol) in ether (10 mL) and a slurry of potassium carbonate (160 mg, 1.2 mmol) in acetone (3 mL). The mixture was stirred at room temperature overnight. Water (5 mL) and saturated sodium chloride solution/hydrochloric acid (20 mL, pH 3) were added, the organic phase was separated, and the aqueous phase was extracted with ethyl acetate (2 × 20 mL). The combined organic phase was dried ($MgSO_4$), and the solvent was removed under reduced pressure with warming to yield a white solid. This was recrystallized from ether/hexane to yield methyl 3-methoxy-4,5-diphenylmethylenedioxybenzoate [378 mg, 1.0 mmol, 92%, mp 135 °C (sharp), lit. 135 °C; Jurd, 1959].

Methyl 3-methoxy-4,5-diphenylmethylenedioxybenzoate was treated with 80% acetic acid (108 °C, 5 h), diluted with 4 volumes of water, washed with hexane (2 × 10 mL, discarded), and extracted with ethyl acetate (2 × 20 mL). The organic extracts were combined, and the solvent was evaporated under reduced pressure to yield a white solid, which was dissolved in equal volumes of methanolic potassium hydroxide (1 M) and water and heated at 55 °C for 36 h under nitrogen. The solution was diluted with water, acidified (pH 1.5, 5 M HCl), extracted with ethyl acetate (5 × 10 mL), and purified by silica preparative thin-layer chromatography plates. The plates were developed with ethyl acetate/hexane/glacial acetic acid = 50:50:1. The lowest band was removed, extracted with ethyl acetate, and shown to be 3OMGA by a combination of 200 MHz ^{1}H NMR and GC-MS.

3,4-O-Dimethylgallic Acid. The authentic standard of 3,4-*O*-dimethylgallic acid (3,4OdiMGA) was purchased from Sigma (catalog no. H-7009, St. Louis, MO).

For quantification, response factors were established by measuring peak areas versus response in comparison to the internal standard.

Statistics. All statistical analyses were performed using SPSS (Chicago, IL). Results are presented as means ± SEM, and $P < 0.05$ was used as the level of significance. The paired samples *t* test was used to compare urinary concentrations of compounds during the water-drinking period with the urinary concentrations during the tea-drinking period.

RESULTS

The subjects recruited for this study were between 43 and 75 years with a mean age of 61.8 ± 2.7 years. They were healthy nonsmokers with a body mass index at baseline of between 23.5 and 35.7 kg/m^2 (mean = 28.0 ± 1.2 kg/m^2).

Following 3 cups of black tea (study 1), gallic acid was not detected in urine. A total of nine compounds was found, using GC-MS, to have increased in concentration following black tea ingestion. The retention times, major ions, and compound names (if known) of these nine compounds are presented in Table 1. Each of these

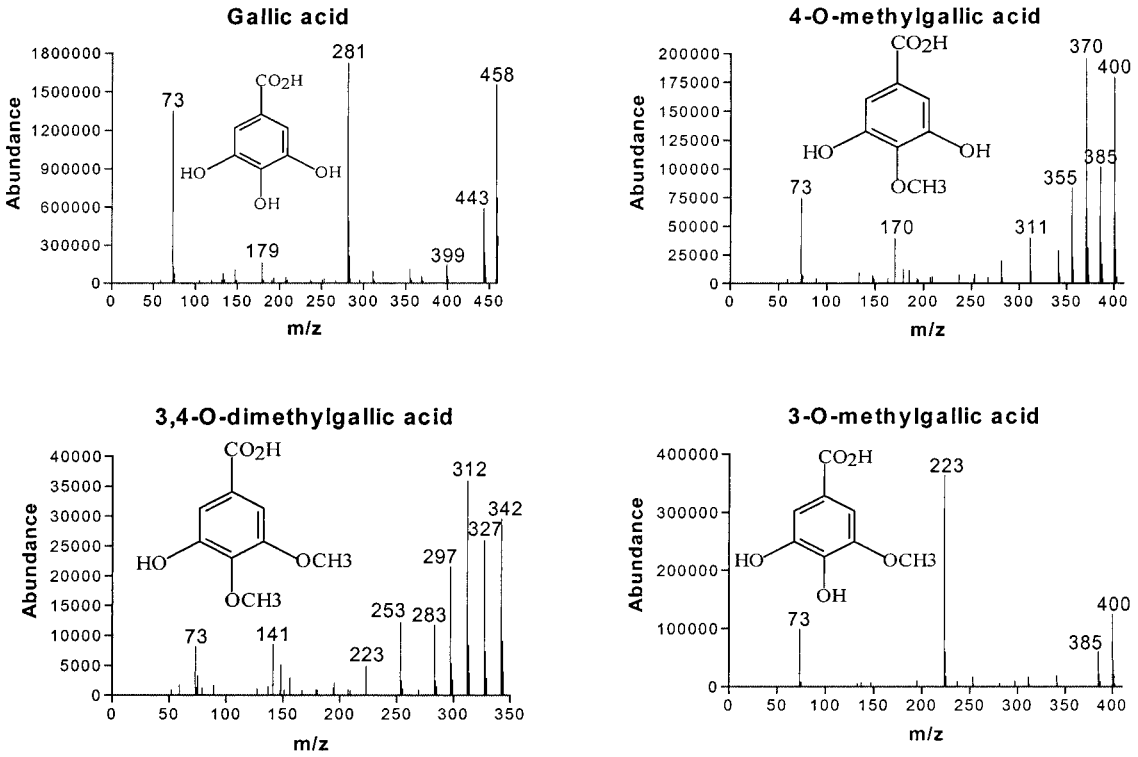

Figure 1. Structures and mass spectra for gallic acid and its major methylated metabolites identified in human urine (spectra presented are trimethylsilyl derivatives).

Table 1. Retention Times, Major Ions, and Compound Names (If Known) of the Nine Compounds Found To Have Increased Acutely in Concentration following Black Tea Ingestion

compound no.	retention time (min)	major ions	compound name
1	8.43	342, 312	3,4-*O*-dimethylgallic acid
2	8.61	400, 370	4-*O*-methylgallic acid
3	8.64	331, 215	
4	9.06	400, 223	3-*O*-methylgallic acid
5	9.40	317	
6	10.00	372, 156	
7	10.06	378, 338	
8	10.08	338, 308	ferulic acid[a]
9	10.72	391, 274	

[a] Tentative identification.

compounds was considered to be a candidate for investigation during periods of regular black tea and water consumption in study 2.

Concentrations of the nine candidate compounds were assessed in 24-h urine samples taken during 4-week periods of regular black tea or water consumption (study 2). Compounds that were present at higher concentration during the tea-drinking period in comparison to the water-drinking period in all 10 subjects were considered to be potentially useful as markers of black tea intake. Compounds present at higher concentrations during the water-drinking period were deemed to be derived from dietary sources other than tea. Of the nine candidate compounds only three fulfilled these criteria.

The three compounds were identified on the basis of retention time and mass spectral data in comparison with authentic standards as 4OMGA, 3OMGA, and 3,4OdiMGA. All compounds were methyl ether derivatives of gallic acid. The chemical structures and mass spectra of gallic acid and these methyl ether derivatives are presented in Figure 1. The 24-h urinary excretion

Table 2. 24-h Urinary Excretion of 4OMGA, 3OMGA, and 3,4-OdiMGA for Each Subject and Group Means during the Water- and Tea-Drinking Periods[a]

	4OMGA, μg/day		3OMGA, μg/day		3,4OdiMGA, μg/day	
subject	water	tea	water	tea	water	tea
1	0	414	0	155	5	67
2	0	503	13	124	25	107
3	33	706	13	122	57	174
4	20	934	16	351	17	151
5	32	220	25	183	41	140
6	81	1618	24	83	77	125
7	0	2580	0	381	0	183
8	0	1303	0	212	0	129
9	263	1376	92	305	75	177
10	118	1404	53	297	0	171
mean	55 ± 26	1106 ± 222[b]	24 ± 9	221 ± 33[b]	30 ± 10	142 ± 11[b]

[a] Mean values are presented with SEM, $n = 10$. [b] $P < 0.001$ for comparison with water period.

of these compounds during the water- and tea-drinking periods for each subject is presented in Table 2. The mean total excretions of the methyl ether metabolites of gallic acid were 108 ± 41 μg/day during the water-drinking period and 1469 ± 249 μg/day during the tea-drinking period ($P < 0.001$ for difference). Examples of extracted ion chromatograms showing urinary gallic acid metabolites from an individual drinking tea and water for 4 weeks each are presented in Figure 2.

We have also investigated whether other methyl ether derivatives of gallic acid were present in human urine following black tea ingestion. Other possible methyl ether derivatives of gallic acid, including 3,5-*O*-dimethylgallic acid (also known as syringic acid) and 3,4,5-*O*-trimethylgallic acid, were not present at increased concentrations following black tea ingestion.

DISCUSSION

We have identified three compounds with potential for use as markers of black tea polyphenol intake. These

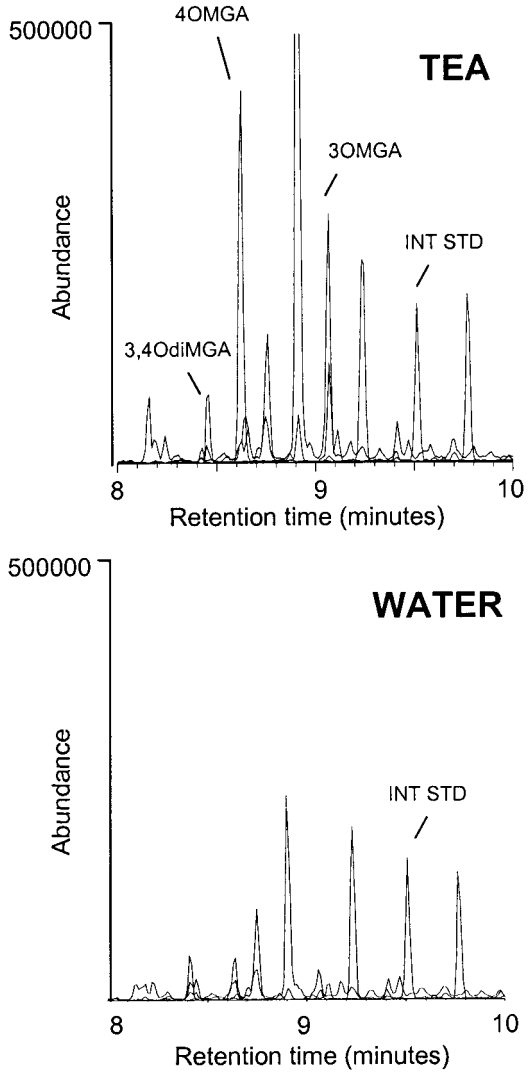

Figure 2. Extracted ion chromatograms (*m*/*z* 223, 317, 342, and 400) showing urinary gallic acid metabolites, 3,4-*O*-dimethylgallic acid (3,4OdiMGA), 4-*O*-methylgallic acid (4OMGA), and 3-*O*-methylgallic acid (3OMGA), from an individual drinking tea or water for 4 weeks each. The unidentified peak at 8.9 min was not consistently elevated during tea drinking.

compounds, all methyl ether derivatives of gallic acid, were 4OMGA, 3OMGA, and 3,4OdiMGA (Figure 1). The three compounds were present at increased concentrations following ingestion of 3 cups of black tea and present at significantly higher concentrations in all 10 subjects during regular black tea consumption in comparison to a water-drinking control period.

Gallic acid is a major phenolic component of tea. Most of the gallic acid in black tea will have derived originally from two flavonoids present at high concentrations (~15% of dry weight) in the green tea leaf. These compounds are epicatechin gallate (ECG) and epigallocatechin gallate (EGCG). About 35% of the mass of these compounds is gallic acid, present as gallate esters (Harbowy and Ballentine, 1997). It can therefore be estimated that ~5% of the weight of the green tea leaf will be gallic acid. The estimate is similar for black tea, but the form in which the gallic acid is present will differ from that in green tea given oxidative changes to the composition of catechins during black tea production. It is likely that free gallic acid is higher in the black tea—estimated at 1% of dry weight (Harbowy and Ballentine, 1997)—and possibly more available for uptake and metabolism. The total gallic acid intake may therefore be as high as 20−50 mg per cup of black tea, assuming 2 g of tea leaves infused in hot water gives 20−50% extraction.

The methyl ether derivatives of gallic acid identified in urine may have resulted from different sources in the black tea. They may have been derived from free gallic acid, hydrolysis of gallate esters, and/or metabolism of flavonoids after ring opening. More complex polyphenols can be metabolized to phenolic acids (Choudhury et al., 1999). It is therefore difficult to estimate bioavailablility. What is clear is that the total excretion of the gallic acid metabolites measured was ~1.5 mg/day when subjects were drinking 5 cups per day of black tea, which represents a small percentage of gallic acid intake. For example, assuming 20% extraction into hot water, the 1.5 mg/day of measured gallic acid metabolites would correspond to 7.5% of ingested free gallic acid, but only 1.5% of ingested total gallic acid.

The 4OMGA methyl ether derivative of gallic acid has previously been identified as a urinary metabolite of gallic acid in humans (Shahrzad and Bitsch, 1998) and rats (Zong et al., 1999). We have shown here that 4OMGA is the major metabolite of gallic acid, derived from black tea, in human urine. In addition, two other methyl ether derivatives have been identified as minor gallic acid metabolites. These compounds, 3OMGA and 3,4OdiMGA, have not previously been described as gallic acid metabolites.

The results of this study demonstrate that tea was the major dietary source of the gallic acid metabolites in 10 volunteers consuming their usual diet. However, it remains uncertain if any particular foods or beverages other than tea might contribute significantly to urinary excretion of these compounds and therefore potentially limit the use of these compounds as markers of tea ingestion. Contributions of other foods to urinary excretion of the methyl ether derivatives did not appear to be great in this study. However, the large variability in 4OMGA concentrations is suggestive of differences in uptake and/or metabolism between individuals. This may be a limiting factor in the usefulness of 4OMGA as a marker of black tea intake.

4OMGA has been measured in 24 h urine at ~1 mg/day following 375 mL per day of red wine ingestion (Abu-Amsha Caccetta, personal communication). This excretion is similar to the mean 1.1 mg/day following ingestion of 5 cups per day of black tea found in the present study. The 4OMGA present in urine following red wine ingestion may derive from gallic acid and/or tannic acid (Zhu et al., 1992), which are both present in red wine. We have not yet established whether 3OMGA and 3,4OdiMGA are also present in human urine following red wine ingestion and what the relative contribution of tea and red wine might be to these metabolites.

Another factor that may influence the value of gallic acid metabolites as markers of black tea is the kinetics of these compounds after ingestion. The location of gallic acid uptake and metabolism will have some bearing on the kinetics. The importance of the liver and of bacterial metabolism in the gut is not clear. To date, we have no data on the kinetics of gallic acid metabolites, except that plasma levels of 4OMGA peak at 2−4 h after red wine ingestion (Abu-Amsha Caccetta et al., 2000).

In conclusion, we have identified three metabolites of gallic acid (4OMGA, 3OMGA, and 3,4OdiMGA) in

human urine following black tea ingestion. Black tea was the major dietary source of these compounds in volunteers consuming their usual diet. Therefore, we suggest that these compounds have potential to be used as markers of black tea ingestion.

ABBREVIATIONS USED

4OMGA, 4-*O*-methylgallic acid; 3OMGA, 3-*O*-methylgallic acid; 3,4OdiMGA, 3,4-*O*-dimethylgallic acid; NMR, nuclear magnetic resonance; GC-MS, gas chromatography−mass spetrometry; ECG, epicatechin gallate; EGCG, epigallocatechin gallate.

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LITERATURE CITED

Abu-Amsha Caccetta, R.; Croft, K. D.; Beilin, L. J.; Puddey, I. B. Ingestion of red wine significantly increases plasma phenolic acid concentrations but does not acutely effect ex vivo lipoprotein oxidizability. *Am. J. Clin. Nutr.* **2000**, *71*, 67−74.

Choudhury, R.; Srai, S. K.; Debnam, E.; Rice-Evans, C. A. Urinary excretion of hydroxycinnamates and flavonoids after oral and intravenous administration. *Free Radical Biol. Med.* **1999**, *27*, 278−286.

Harbowy, M. E.; Ballentine, D. A. Tea Chemistry. *Crit. Rev. Plant Sci.* **1997**, *16*, 415−480.

He, Y. H.; Kies, C. Green and black tea consumption by humans: impact on polyphenol concentrations in feces, blood and urine. *Plant Foods Hum. Nutr.* **1994**, *46*, 221−229.

Hertog, M. G.; Feskens, E. J.; Hollman, P. C.; Katan, M. B.; Kromhout, D. Dietary antioxidant flavonoids and risk of coronary heart disease. *Lancet* **1993**, *342*, 1007−1011.

Hertog, M. G. L.; Kromhout, D.; Aravanis, C., et al. Flavonoid intake and long-term risk of coronary heart disease and cancer in the Seven Countries Study. *Arch. Intern. Med.* **1995**, *155*, 381−386.

Jick, H.; Miettinen, O. S.; Neff, R. K.; Shapiro, S.; Heinonen, O. P.; Slone, D. Coffee and myocardial infarction. *N. Engl. J. Med.* **1973**, *289*, 63−67.

Jurd, L. Plant polyphenols: VI experiments on the synthesis of 3,3′- and 4,4′-di-*O*-methylgallic acid. *J. Am. Chem. Soc.* **1959**, *81*, 4606−4610.

Keli, S. O.; Hertog, M. G. L.; Feskens, E. J. M.; Kromhout, D. Dietary flavonoids, antioxidant vitamins, and incidence of stroke. *Arch. Intern. Med.* **1996**, *156*, 637−642.

Knekt, P.; Jarvinen, R.; Reunanen, A.; Maatela, J. Flavonoid intake and coronary mortality in Finland: a cohort study. *Br. Med. J.* **1996**, *312*, 478−481.

Luz Cardona, M.; Isabel Fernandez, M.; Begona Garcia, M.; Pedro, J. R. Synthesis of natural polyhydroxystilbenes. *Tetrahedron* **1986**, *42*, 2735−2740.

Shahrzad, S.; Bitsch, I. Determination of gallic acid and its metabolites in human plasma and urine by high-performance liquid chromatography. *J. Chromatogr. B, Biomed. Sci. Appl.* **1998**, *705*, 87−95.

van het Hof, K. H.; Kivits, G. A.; Weststrate, J. A.; Tijburg, L. B. Bioavailability of catechins from tea: the effect of milk. *Eur. J. Clin. Nutr.* **1998**, *52*, 356−359.

Zhu, J.; Ng, J.; Filippich, L. J. Determination of tannic acid and its phenolic metabolites in biological fluids by high performance liquid chromatography. *J. Chromatogr.* **1992**, *577*, 77−85.

Zong, L.; Inoue, M.; Nose, M.; Kojima, K.; Sakaguchi, N.; Isuzugawa, K.; Takeda, T.; Ogihara, Y. Metabolic fate of gallic acid orally administered to rats. *Biol. Pharm. Bull.* **1999**, *22*, 326−329.

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