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In Vitro Antioxidant Activity of Coffee Compounds and Their Metabolites

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In this paper we report the antioxidant activity of different compounds which are present in coffee or are produced as a result of the metabolism of this beverage. In vitro methods such as the ABTS$^{\bullet+}$ [ABTS = 2,2′-azinobis(3-ethylbenzothiazoline-6-sulfonic acid)] decolorization assay and the oxygen radical absorbance capacity assay (ORAC) were used to assess the capacity of coffee compounds to scavenge free radicals. The importance of caffeine metabolites and colonic metabolites in the overall antioxidant activity associated with coffee consumption is shown. Colonic metabolites such as *m*-coumaric acid and dihydroferulic acid showed high antioxidant activity. The ability of these compounds to protect human low-density lipoprotein (LDL) oxidation by copper and 2,2′-azobis(2-amidinopropane) dihydrochloride was also explored. 1-Methyluric acid was particularly effective at inhibiting LDL oxidative modification. Different experiments showed that this caffeine metabolite is not incorporated into LDL particles. However, at physiologically relevant concentrations, it was able to delay for more than 13 h LDL oxidation by copper.

KEYWORDS: Coffee; antioxidant activity; LDL; ORAC; ABTS; coffee metabolites

INTRODUCTION

Coffee was discovered around the sixth century by Abyssinian/Ethiopian shepherds in the province of Keffa (Ethiopia). Since then, coffee has become one of the most widely consumed psychoactive beverages. In recent years, due to the increasing interest in finding physiologically functional foodstuffs, the relationship between coffee and health has been extensively studied (*1*). Antioxidant activity in foods and beverages is one of the properties that has generated much interest within the scientific community (*2*−*5*). In coffee, antioxidant activity is generally associated with its content of indigenous phenolic compounds as well as Maillard reaction products, the latter being generated during roasting (*2*, *6*, *7*). Among the different phenolic compounds in coffee, the most abundant are hydroxycinnamic acids which exist mainly in the esterified form. The best example is chlorogenic acid (5-caffeoylquinic acid) (CGA) with an average content of 100 mg per cup of coffee (*8*). Few free phenolic acids are present in coffee, although small quantities of caffeic, ferulic, and vanillic acids have been detected (*9*).

Different studies have reported the antioxidant activity of CGA and caffeic acid, mainly by using in vitro studies but also as a result of ex vivo and in vivo investigations (*10*−*13*). Data have been published regarding the ability of CGA metabolites, such as ferulic, isoferulic, or vanillic acids, to exert radical scavenging activity (*13*, *14*). However, minor attention has been directed to the potential antioxidant activity of some other compounds that are also related to coffee consumption.

Since only one-third of ingested CGA is absorbed, large amounts reach the colon, where it is hydrolyzed by the microflora to caffeic acid and quinic acid (*15*, *16*). These compounds are extensively metabolized to a range of products including *m*-coumaric, dehydroferulic, 3-hydroxyphenylpropionic, and hippuric acids (*15*−*17*). Furthermore, another major compound in coffee is caffeine, which may account for as much as 2.2% of the dry matter in Robusta coffee (*18*). Unlike CGA, caffeine is rapidly and completely absorbed in humans and later metabolized by the liver to produce dimethylxanthines, 1-methyluric acid (1-U), and other related metabolites (*19*). Considering the relatively high amount of caffeine in coffee, significant quantities of its metabolites are anticipated in blood and might be involved in the physiological effects related to coffee consumption.

Therefore, the purpose of this study was to evaluate the contribution of metabolites of CGA and caffeine to the antioxidant activity of coffee. Two representative in vitro methods, the ABTS$^{\bullet+}$ [ABTS = 2,2′-azinobis(3-ethylbenzothiazoline-6-sulfonic acid)] decolorization assay and the oxygen radical absorbance capacity (ORAC) assay, were used. As oxidized low-density lipoprotein (LDL) is widely believed to

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be involved in the pathogenesis of atherosclerosis (*20*), the ability of coffee-related compounds to inhibit LDL oxidative modification initiated by 2,2′-azobis(2-amidinopropane) dihydrochloride (AAPH) or catalyzed by Cu(II) was also studied. Finally, to better understand the mechanisms involved in protection against LDL oxidation, the possible incorporation of physiologically relevant concentrations of some compounds into the LDL particles was evaluated.

MATERIALS AND METHODS

Chemicals. Caffeic acid, ferulic acid, hippuric acid, vanillic acid, CGA, *m*-coumaric acid, caffeine, 1-methyl uric acis (1-U), 1,3,7-trimethyluric acid (1,3,7-TU), 1-methylxanthine (1-X), paraxanthine (PX), 3-hydroxyphenylpropionic acid, and (±)-6-hydroxy-2,5,7,8-tetramethylchromane-2-carboxylic acid (Trolox) were obtained from Sigma (Gillingham, U.K.). Isoferulic acid was purchased from Extrasynthese (Genay, France), and dihydroferulic acid was obtained from Lancaster Synthesis Ltd. (Morcambe, U.K.). For the ORAC analysis, AAPH and fluorescein were purchased from Sigma. For the $ABTS^{\bullet+}$ assay, ABTS and potassium persulfate were obtained from Sigma.

$ABTS^{\bullet+}$ Decolorization Assay. The experiment was carried out according to Re et al. (*21*). The $ABTS^{\bullet+}$ chromophore was produced by the oxidation of 7 mM ABTS with potassium persulfate (2.45 mM) in water (final concentration). Before the experiments were performed, the radical was diluted with phosphate-buffered saline (PBS) at pH 7.4 to give an absorbance of 0.7 (±0.02) at 734 nm. The compounds were dissolved either in ethanol or in a solution of NaOH (0.1 M) and diluted with PBS to obtain appropriate concentrations. Then 20 μL aliquots were mixed with 2 mL of $ABTS^{\bullet+}$ reagent, and the absorbance was monitored for 10 min at 30 °C using a Lambda Bio 20 UV/vis spectrometer (Perkin-Elmer, Cambridge, U.K.). Readings taken after 5 min were used to calculate the antioxidant activity. Trolox, the water-soluble analogue of vitamin E, was used as a reference standard. A standard curve was prepared by measuring the percent inhibition values at different concentrations of Trolox. Inhibition values were calculated as follows:

$$\text{inhibition (\%)} = 100[(A_{t=0(\text{sample})}) - (A_{t=5(\text{sample})})/(A_{t=0(\text{sample})})] - [(A_{t=0(\text{solvent})}) - (A_{t=5(\text{solvent})})/(A_{t=0(\text{solvent})})]$$

The Trolox equivalent antioxidant capacity (TEAC) of each compound represents the concentration of Trolox with the same antioxidant capacity as the compound. Triplicate solutions of each compound were prepared, and the activity of each was measured in duplicate.

ORAC Assay. The method of Ou et al. (*22*) was used with minor modifications as described below. The reaction was carried out in 75 mM phosphate buffer (pH 7.4), and the final reaction volume was 3 mL. The compounds were dissolved either in ethanol or in a solution of NaOH (0.1 M) and diluted with 75 mM phosphate buffer to obtain appropriate concentrations.

In a cuvette were placed 2.25 mL of fluorescein (100 μM in 75 mM phosphate buffer), 375 μL of each compound at the appropriate concentration, and 375 μL of AAPH (150 mM in 75 mM phosphate buffer) to start the reaction. The mixture of test compound and fluorescein was incubated at 37 °C for 10 min before addition of the AAPH. The fluorescence was recorded every 5 min until the final value was less than 10% of the initial fluorescence value. During the experiments, the solutions were kept at 37 °C in a water bath and only removed to perform the measurements. Phosphate buffer (75 mM) was used as a blank. To calculate ORAC values, the area under the fluorescence decay curve (AUC) was determined for Trolox at different concentrations to create a calibration curve. The AUC values were calculated as follows:

$$\text{AUC} = (0.5 + f_1/f_0 + f_2/f_0 + f_3/f_0 + f_4/f_0 + f_5/f_0 + f_6/f_0 + f_7/f_0 + ... + f_i/f_0) \times 5$$

where f_0 is the initial fluorescence reading at 0 min and f_i is the fluorescence reading at time *i*. For each batch of samples, a standard of Trolox (30 μM) was analyzed as a quality control.

The relative ORAC activity (Trolox equivalents) of each compound was calculated as

$$\text{ORAC} = [(\text{AUC}_{\text{compd}} - \text{AUC}_{\text{blank}})/(\text{AUC}_{\text{Trolox}} - \text{AUC}_{\text{blank}})] \text{ (concentration of Trolox/concentration of the sample)}$$

Triplicate solutions of each compound were prepared, and the activity of each was measured in duplicate.

Rapid LDL Isolation. LDL (1.019−1.063 g/mL) was isolated by sequential density ultracentrifugation from normal human blood in the presence of EDTA (*23*). Briefly, after the plasma was obtained by centrifugation (800*g* for 30 min), the density was adjusted to 1.21 g/mL by the addition of KBr (1.006 g/mL). A first ultracentrifugation step was carried out using a Beckman NVT 65-near vertical rotor at 402000*g* for 50 min at 4 °C. The visible orange band was collected and the density adjusted to 1.15 g/mL with a high-density solution (2.62 M NaCl, 2.98 M KBr, and 297 μM EDTA). After this band was overlaid with a second KBr solution (1.063 g/mL), a second ultracentrifugation step was carried out at 402000*g* for 3 h at 4 °C. The aims of this second step were to remove possible residual albumin and to concentrate the LDL. Finally, the LDL was collected from the top of the tubes and dialyzed overnight against a low phosphate dialysis buffer (140 mM NaCl, 8.1 mM Na_2HPO_4, 1.9 mM NaH_2PO_4, and 100 μM EDTA) to remove KBr. LDL protein was measured according to the method of Lowry et al. (*24*) using bovine serum albumin as a standard.

LDL Oxidation. LDL (50 μg of protein/mL) was oxidized in PBS (Gibco, Paisley, Scotland) at 37 °C with either $CuSO_4$ (usually 5 μM net above the concentration of EDTA carried over from the storage buffer, which was below 1 μM) or AAPH (final concentration 1 mM). The oxidation of LDL was performed in the presence or absence of the different compounds tested in this study. The compounds were first prepared in ethanol and then diluted in PBS to reach the desired concentrations with the exception of 1-U, 1-X, PX, 1,3,7-TU, and uric acid, which were prepared in 0.1 M NaOH and then diluted with PBS. The kinetics of LDL oxidation was determined by monitoring the change of absorbance at 234 nm with a Lambda Bio 20 UV/vis spectrometer (Perkin-Elmer, Cambridge, U.K.) equipped with an eight-position automated sample changer.

When the kinetics of LDL oxidation was the same in the presence or absence of the studied compounds, the duration of the lag phase was measured, defined as the interval between the intercept of the propagation phase of the curve with the initial absorbance axis. In the case of different kinetics, the time needed to reach 50% of the maximal amount of conjugated dienes was used (for instance, for LDL oxidation mediated by AAPH).

Plasma Incubation. Pooled plasma (4 mL) from healthy individuals was incubated at 37 °C for 3 h with caffeic acid, 1,3,7-TU, or 1-U (0.5, 3, and 30 μM, respectively). The compounds were dissolved in ethanol (caffeic acid) or in a solution of 0.1 M NaOH (1-U or 1,3,7-TU) and diluted with PBS to obtain appropriate concentrations.

The experiments were performed in triplicate, and PBS was used as a control. LDL was isolated and oxidized by AAPH or $CuSO_4$ at 37 °C as described above.

Statistical Analysis. Statistical analysis was performed by one-way analysis of variance (ANOVA) to compare the means of more than two groups using Statgraphics plus for Windows, version 5.1 (Manugistics, Maryland).

RESULTS AND DISCUSSION

In this study, the antioxidant activity of the most important coffee-related compounds was evaluated. A total of 14 chemical compounds were selected on the basis of their natural presence in coffee or due to their importance as main metabolites found following coffee consumption. Naturally occurring phenolic compounds include CGA, caffeic acid, vanillic acid, and caffeine. Metabolites include those formed from caffeine (1-U,

Table 1. Antioxidant Activity of Coffee-Related Compounds Obtained Using the ABTS$^{\bullet+}$ and ORAC Methods[a]

	ABTS$^{\bullet+}$ method (TEAC[b])	ORAC method
CGA	1.15 ± 0.05 e	3.53 ± 0.14 bc
caffeic acid	1.27 ± 0.12 d	4.52 ± 0.14 a
ferulic acid	2.32 ± 0.09 a	3.88 ± 0.18 b
isoferulic acid	1.57 ± 0.02 c	4.20 ± 0.21 a
vanillic acid	1.52 ± 0.01 c	3.21 ± 0.14 c
dihydroferulic acid	1.62 ± 0.06 b	2.60 ± 0.16 d
m-coumaric acid	1.07 ± 0.07 f	3.14 ± 0.17 c
hippuric acid	nd	nd
3-(hydroxyphenyl)propionic acid	1.04 ± 0.07 f	1.67 ± 0.23 e
caffeine	nd	nd
1,3,7-TU	0.93 ± 0.04 f	0.71 ± 0.01 f
1-U	1.00 ± 0.14 f	0.58 ± 0.04 g
1-X	0.24 ± 0.04 g	1.33 ± 0.16 e
PX	nd	nd

[a] Values (μM Trolox equivalents) are the mean ± SD obtained for triplicate solutions measured in duplicate. Values followed by a different letter within a column are significantly different. nd = not detected. [b] Trolox equivalent antioxidant capacity.

1,3,7-TU, PX, and 1-X) and CGA (isoferulic, dihydroferulic, *m*-coumaric, hippuric, 3-(hydroxyphenyl)propionic, and ferulic acids), the latter compound also being detected in small quantities in coffee. Among the compounds naturally found in coffee, caffeine and CGA are by far the most abundant (100 mg/200 mL cup of coffee), although this concentration depends very much on how the beverage is prepared (*25*, *26*).

ABTS$^{\bullet+}$ Analysis. The antioxidant activity of the different compounds tested in this study is shown in **Table 1**. The compound with the highest antioxidant activity was ferulic acid. The TEAC value of CGA, the most abundant phenolic compound in coffee, was slightly lower than that of caffeic acid. Isoferulic acid possessed less antioxidant activity than ferulic acid. Isoferulic acid has been proposed as one of the markers of caffeoylquinic acid metabolism (*27*) together with dihydroferulic acid, which showed significantly stronger antioxidant activity than both CGA and caffeic acid. It is important to note the high antioxidant activity detected in two of the most important colonic metabolites of CGA, i.e., *m*-coumaric and 3-(hydroxyphenyl)propionic acids. Both compounds showed antioxidant values only slightly lower than that of CGA. On the contrary, hippuric acid showed no activity.

Regarding caffeine and its metabolites, the antioxidant activity was very different among them. Caffeine and PX showed no activity. In contrast, 1-U and 1,3,7-TU possessed TEAC values only slightly lower than those detected for caffeic acid and CGA, and the values were not significantly different from those for *m*-coumaric and 3-(hydroxyphenyl)propionic acids.

ORAC Analysis. Table 1 shows the ORAC values of the different compounds evaluated in this study. Naturally occurring compounds in coffee such as CGA, caffeic acid, vanillic acid, and ferulic acid showed the highest activity, with the coffee metabolite isoferulic acid also included in the most active compounds. Among them, caffeic and isoferulic acids possessed the highest antioxidant capacity against the peroxyl radical. Colonic metabolite *m*-coumaric acid also showed high antioxidant activity. Another microbial metabolite, 3-(hydroxyphenyl)-propionic acid, possessed an ORAC value significantly lower than those of the other phenolic acids. However, its role as an antioxidant might be important since it is found in plasma at levels of 15 μM (*15*). Hippuric acid, one of the most abundant colonic metabolites, was not active against the peroxyl radical. Finally, caffeine exhibited nondetectable activity, and its metabolites showed low antioxidant activity, with 1-X having the greatest effect.

The ABTS$^{\bullet+}$ and ORAC results both showed that caffeine and its metabolites possessed less antioxidant activity compared to phenolic acids. The antioxidant activity of methylxanthines is influenced by two main factors. One is the presence of an intact imidazole ring, and the second one is the existence of an electron donor at the C_8 position of the imidazole group (*28*, *29*). Both characteristics are present in 1,3,7-TU and 1-U, which showed the highest antioxidant activity among caffeine and its metabolites when the ABTS$^{\bullet+}$ method was applied. In fact, these two compounds possessed only slightly lower values than those of caffeic acid and CGA, two compounds with recognized antioxidant activity. To appreciate the importance of these results, it is necessary to underline that, after coffee consumption, 1,3,7-TU and 1-U have been described in blood at much higher concentrations than those of caffeic acid or CGA (**Table 2**).

On the other hand, 1-X is not methylated at N7, nor does it possess a hydroxyl group at C8. This could explain its higher antioxidant activity compared to those of PX and caffeine and its lower antioxidant activity compared to that of 1,3,7-TU by the ABTS$^{\bullet+}$ method.

The above-mentioned structural characteristics of methylxanthines do not seem to be so important for scavenging of peroxyl radicals as studied by the ORAC method. In this case, 1-X was the most active caffeine metabolite and 1,3,7-TU and 1-U showed low activity. Our ORAC results contrast with those of Lee (*28*), who observed higher antioxidant activity for 1-U than for 1-X. A plausible explanation of this discrepancy could be that Lee (*28*) used as a substrate B-phycoerythrin instead of fluorescein. B-phycoerythrin has been reported to vary from batch to batch and to interact with polyphenols (*22*), reasons that have led to the disuse of this compound in favor of fluorescein. A similar interaction between B-phycoerythrin and some of the caffeine metabolites can be expected to be present and, therefore, to influence the results.

From the results presented in **Table 1** and bearing in mind the chemical structures of the compounds (**Figure 1**), it could be inferred that an increase in the number of hydroxyl groups leads to an increase in the peroxyl scavenging activity. It has been described that the double bond in the chain of the hydroxycinnamic acid derivatives participates in stabilizing the radical by resonance (*11*). This would explain the higher activities of ferulic and isoferulic acids compared to vanillic and dihydroferulic acids. Nevertheless, this structure−activity relationship is not followed when the ABTS$^{\bullet+}$ results are considered. Ferulic acid, which possesses one hydroxyl group compared to two for caffeic acid, was the most active compound. In addition, isoferulic and ferulic acids behaved differently. This may be attributed to the mechanism of reaction of antioxidants with the ABTS$^{\bullet+}$ radical, which is unclear (*13*).

Among the colonic metabolites, 3-(hydroxyphenyl)propionic and *m*-coumaric acids possessed high antioxidant activity by the ABTS$^{\bullet+}$ method with values similar to those found for caffeic acid and CGA. The ORAC method also showed *m*-coumaric acid to be among the most active compounds, with 3-(hydroxyphenyl)propionic acid exerting only moderate antioxidant activity. Traditionally, little attention has been paid to colonic metabolites and their implication in antioxidant activity. The large amount of CGA reaching the intestine (only one-third is absorbed in the proximal part of the gut; see ref *15*) permits its metabolism by the microflora and the generation of

Table 2. Plasma Concentrations Described in the Literature for Coffee-Related Compounds after Coffee, CGA, Caffeic Acid, or Caffeine Intake and the Concentrations Used in the LDL Study

	concn used in the LDL study (mM)	published plasma concn (μM)	source	ref
caffeic acid	0.5	0.082–0.146[a,e]	200 mL of coffee	*25*
		0.24–0.83[b,e]		
		41.3 ± 6.1[b,f]	caffeic acid	*15*
			CGA	*15*
		1.2[b,f]	caffeic acid	*33*
		0.3[b,f]	CGA	*33*
chlorogenic acid			200 mL of coffee	*25*
			CGA	*15*
			CGA	*33*
vanillic acid	0.5			
isoferulic acid	0.5		200 mL of coffee	*25*
		4.5[b,f]	caffeic acid	*15*
			CGA	*15*
ferulic acid	0.5		200 mL of coffee	*25*
		7.3[b,f]	caffeic acid	*15*
		0.4[b,f]	CGA	*15*
dihydroferulic acid	0.5, 15[c]			
m-coumaric acid	0.5, 2	1.9 ± 0.3[b,f]	CGA	*15*
			caffeic acid	*15*
hippuric acid	0.5, 100	98.2 ± 15.8[b,f]	CGA	*15*
3-(hydroxyphenyl)propionic acid	0.5, 15	12.9 ± 2.9[b,f]	CGA	*15*
		1.4 ± 0.6[b,f]	caffeic acid	*15*
caffeine	0.5, 40	39–46[e]	caffeine	*29*
paraxanthine	0.5, 30[d]			
1-methyluric acid	0.5, 30[d]			
1-methlylxanthine	0.5, 30[d]			
1,3,7-trimethyluric acid	0.5, 3[d]			

[a] Before treatment with glucuronidase/sulfatase enzymes. [b] After treatment with glucuronidase/sulfatase enzymes. [c] Value based on the concentration found in urine (*27*). [d] Values based on caffeine metabolism (*19*). [e] In humans. [f] In rats.

important amounts of different compounds including 3-(hydroxyphenyl)propionic and *m*-coumaric acids. Therefore, even 3-(hydroxyphenyl)propionic acid could have an important role in the overall activity of phenolic-rich foods, such as coffee, despite its moderate ability to scavenge peroxyl radicals.

As stated above, particularly important is the activity shown by dihydroferulic acid. Although the formation of dihydroferulic acid is not completely clear, several studies support the idea that this compound is mainly produced by the gut microflora (*27*, *30*, *31*). Consequently, this compound could be present at high concentration in blood compare to other coffee compounds such as CGA and caffeic acid and therefore play an important role as an antioxidant. Unlike other coffee metabolites, this compound has been detected in the nonconjugated form in urine (*27*). To our knowledge, this is the first time that the antioxidant activity of this compound has been reported.

Oxidative Modification of LDL. To obtain more physiologically relevant information concerning the antioxidant effect of these coffee-related compounds and their possible protection against atherosclerosis, their ability to protect LDL oxidation by copper or AAPH was assessed.

Our intention was to study the impact of the compounds at concentrations that are described in vivo after coffee is drunk or after the intake of the pure compound in an amount similar to that found in a coffee beverage (physiologically relevant concentrations). The concentrations used for each compound are given in **Table 2**.

First, to compare their activity, a common concentration was used for all compounds. The selected concentration (0.5 μM) was chosen because it is representative of the concentrations at which all the studied compounds may be detected in blood. The maximum concentration of phenolic compounds in plasma rarely exceeds 1 μM after the consumption of 10−100 mg of a single phenolic compound (*32*). In coffee, among the naturally occurring phenolic compounds, the only one present in such an amount is CGA. However, due to its poor absorption it is usually not detected in blood after coffee consumption (*25*). CGA yields important amounts of caffeic acid in the large intestine, which is extensively metabolized by the microflora. Thus, only concentrations of caffeic acid lower than 1 μM are usually found in plasma (*25*). Other naturally occurring phenolic compounds in coffee, such as ferulic or vanillic acids, are expected to be detected below 1 μM. Accordingly, the common concentration was fixed at 0.5 μM.

A second concentration for some of the studied compounds was selected since some coffee metabolites have been detected in plasma at much higher concentration than 0.5 μM. This higher level was based on those values described in the literature after the intake of coffee or the chemical compound separately (**Table 2**). For example, values of up to 15 μM 3-(hydroxyphenyl)-propionic acid or 100 μM hippuric acid have been detected in plasma throughout coffee-related studies with rats and humans (*25*, *33*). No data were available concerning the plasma concentration of dihydroferulic acid following coffee consumption. Nevertheless, this compound was selected for study due to its high urinary concentration following coffee consumption (*27*). On the other hand, previous studies have shown that CGA is only poorly absorbed in the small intestine (*15*), and no intact CGA has been detected in plasma following coffee consumption (*25*). Therefore, it was decided to omit this compound from the LDL study.

Phenolic Compounds. Caffeic, ferulic, isoferulic, and vanillic acids were tested for their potential to protect against LDL oxidation. **Figure 2** shows that only caffeic acid protected LDL from oxidation by copper. Similar results were found when the prooxidant was AAPH. Caffeic acid was less efficient at inhibiting LDL oxidation by AAPH compared to oxidation mediated by copper (**Figure 3**). By measuring the time needed

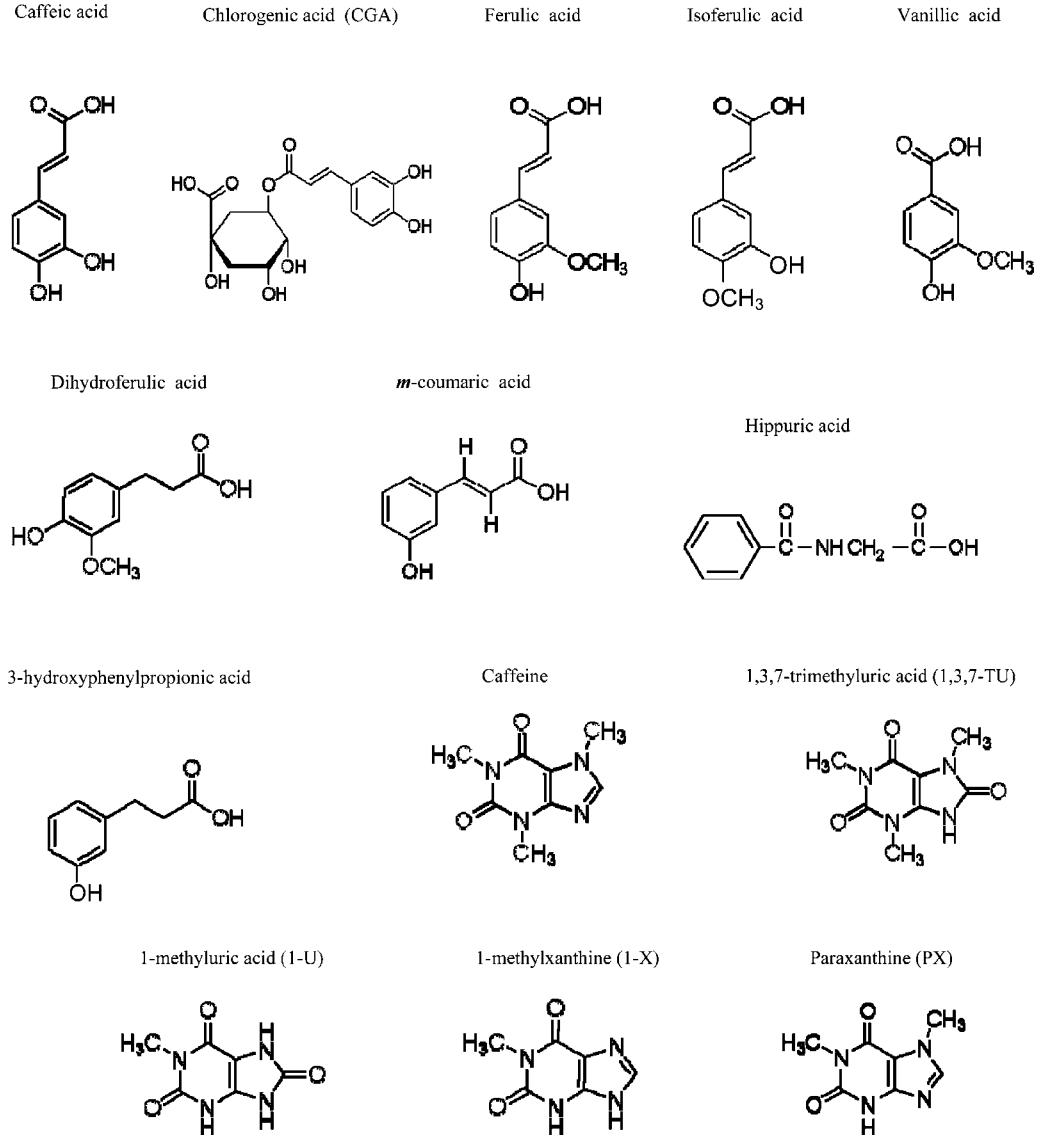

Figure 1. Chemical structures of the different compounds studied.

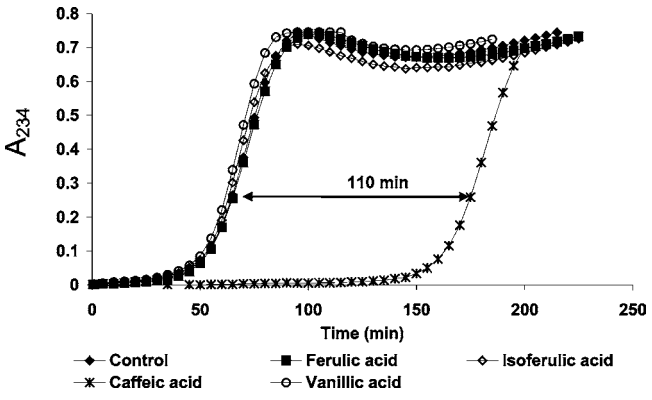

Figure 2. Oxidation of LDL particles by $CuSO_4$ (5 μM) in the absence (control) or presence of phenolic acids (0.5 μM).

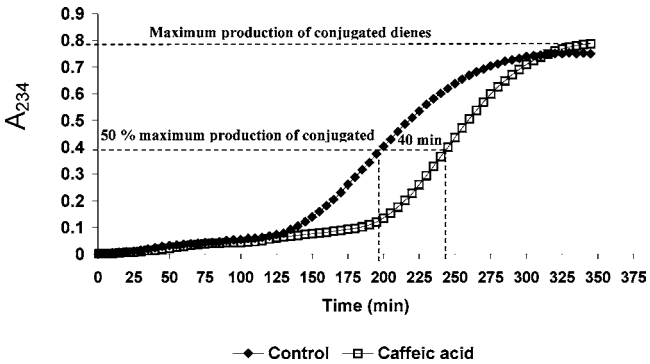

Figure 3. Oxidation of LDL particles by AAPH (1 mM) in the absence (control) or presence of caffeic acid (0.5 μM).

to reach 50% of the maximum concentration of conjugated dienes, it was observed that caffeic acid promoted a delay of about 40 min to reach this time. These results agree with those of Nardini et al. (*10*). By comparing the ORAC results with the data obtained for ferulic, isoferulic, and vanillic acids in the LDL study, it can be inferred that other factors apart from peroxyl radical scavenging are involved in LDL oxidation. It is very likely that the activity found for caffeic acid is due to its ability to bind copper and spare the β-carotene and α-tocopherol present in the LDL particle (*10*). Unlike caffeic acid, the same properties have not been described for ferulic, isoferulic, and vanillic acids.

Caffeine and Caffeine-Related Compounds. At concentrations of 0.5 μM, both 1,3,7-TU and 1-U were able to inhibit LDL oxidation catalyzed by copper. The activity was greater for 1-U, which delayed the onset of the propagation phase by 50 min (**Figure 4A**). 1,3,7-TU altered the kinetics of the reaction

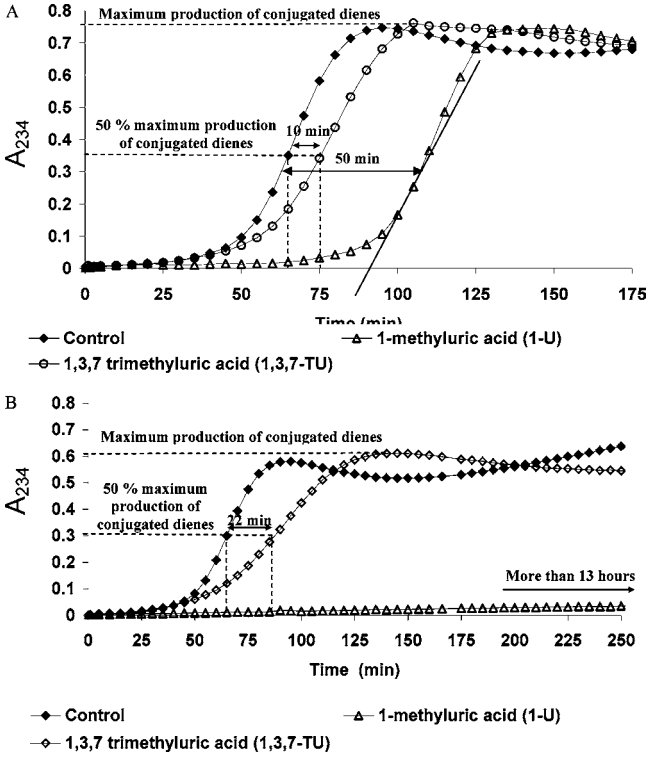

Figure 4. (**A**) Oxidation of LDL particles by $CuSO_4$ (5 μM) in the absence (control) or presence of 1-methyluric acid or 1,3,7-trimethyluric acid (both at 0.5 μM). (**B**) Oxidation of LDL particles by $CuSO_4$ (5 μM) in the absence (control) or presence of 1-methyluric acid (30 μM) or 1,3,7-trimethyluric acid (3 μM).

between LDL and copper. The time needed to reach 50% of the maximum absorbance was increased by 10 min compared to that of the control (**Figure 4A**). The ability to modify the kinetics is characteristic of compounds that are incorporated into the LDL particle, for example, probucol. The higher hydrophobicity of 1,3,7-TU compared to 1-U could possibly support this theory.

Caffeine is rapidly and almost completely absorbed in the stomach and small intestine (*28*). The human body converts 70−80% of caffeine into PX, which subsequently degrades to 1-X and 1-U. Considering that caffeine can reach a concentration higher than 40 μM in serum following the consumption of 300 mg of the pure compound (around four cups of coffee), 1-U levels of up to 30 μM could theoretically be detected. When 1-U was tested at this concentration, it produced a striking result, slowing the lag phase of conjugated diene formation by more than 13 h when LDL oxidation was promoted by copper (**Figure 4B**). When 1,3,7-TU was used at a concentration of 3 μM, the kinetics of the reaction was again altered and the time needed to reach 50% of the maximum absorbance was increased by more than 20 min compared to that of the control (**Figure 4B**).

1-U was also very effective against LDL oxidation initiated by the aqueous peroxyl radical generator AAPH. An LDL sample incubated with 1-U (30 μM) took 4 h more than the control to reach 50% of the maximum production of conjugated dienes (**Figure 5**). These results undoubtedly demonstrate the positive effect that other coffee-related compounds besides phenolic acids could have on human health. To understand the importance of the results reported here, it is necessary to remark that, following coffee consumption, 1,3,7-TU and 1-U are detected in blood at much higher concentrations than that of caffeic acid. By comparing each compound at the concentration at which they could be detected in plasma (30 μM for 1-U and

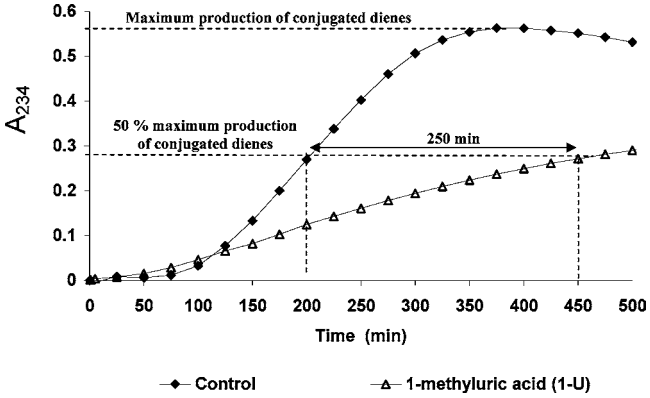

Figure 5. Oxidation of LDL particles by AAPH (1 mM) in the absence (control) or presence of 1-methyluric acid (30 μM).

0.5 μM for caffeic acid), 1-U is more than 7 times more efficient than caffeic acid in protecting LDL against oxidative modification by copper (**Figures 2** and **4B**). A similar result is observed with the oxidation mediated by AAPH, with 1-U being more than 10 times more effective than caffeic acid (**Figures 3** and **5**).

Caffeine, PX, and 1-X were ineffective against either AAPH- or copper-mediated oxidation of LDL at any of the concentrations tested in this study.

Colonic Compounds. None of the colonic metabolites protected LDL against oxidative modification at any of the concentrations tested in this study.

Interactions between Copper and Coffee-Related Compounds. 1,3,7-TU, 1-U, and caffeic acid were incubated with excess copper (100 μM) to evaluate the contribution of their possible ability to bind this metal to the inhibitory effects on LDL oxidation. Compounds can protect against LDL oxidative modification by different mechanisms. When a compound exerts a protective effect against LDL oxidation due uniquely to its ability to chelate copper, an increase in the concentration of the metal above a certain point will provoke the loss of the protective effect from the compound. As a result, the lag time will be the same as that observed for the control. On the other hand, when copper chelating activity is not the main mechanism involved in LDL defense, an increase in copper concentration will result in the same lag phase as observed using a lower concentration of the metal. In our study, the duration of the lag phase was the same as that observed when 5 μM copper was used. Therefore, these results show that their protective effects on LDL cannot be explained entirely by their binding to copper ions.

However, an additional experiment showed that caffeic acid is also able to chelate copper, although this is not the main mechanism involved in protecting against LDL oxidation. Adding copper to caffeic acid caused a dramatic change in the UV spectrum of caffeic acid, with restoration of the original spectrum by adding EDTA, indicating that caffeic acid binds copper (results not shown). This is in agreement with the findings of Nardini et al. (*10*).

While 1,3,7-TU showed no significant changes in its original spectrum after the incubation with copper, the spectrum for 1-U showed a slight change in its maximum at 237 nm after 1 h of incubation with copper. Unlike caffeic acid, the addition of EDTA did not recover the original spectrum. It could be that 1-U is able to reduce Cu^{2+} to Cu^{+}, as described for uric acid (*34*). The reduction of Cu^{2+} would prevent the reaction of the cuprous ions with α-tocopherol present in the LDL particle to generate α-tocopherol radicals, which are involved in the chain

reaction of lipid peroxidation (*34*). This mechanism may explain, at least in part, the ability of 1-U to protect LDL against oxidation as its capacity to scavenge peroxyl radicals as assessed by the ORAC assay is much lower than that shown by caffeic acid (**Table 1**). In addition, its ability to scavenge other radical species generated during LDL oxidation apart from peroxyl radicals should not be discarded.

Incubation of Plasma with Coffee-Related Compounds. Incubating plasma with antioxidants and then isolating LDL particles provided specific information on the possible incorporation of antioxidants into LDL, mimicking likely in vivo processes since antioxidants operate in plasma or interstitial fluid rather than with purified LDL. A delay in the onset of the propagation phase indicates that the compounds have been incorporated into the LDL particles during their incubation with plasma.

Although it has been reported previously that caffeic acid is not incorporated into the LDL particles (*35*), to our knowledge no similar experiments have been reported for caffeine metabolites. Despite the results obtained after their incubation with pure LDL, both 1,3,7-TU and 1-U failed to be incorporated into LDL since the propagation phase remained unaltered (results not shown). In principle, the hydrophobicity of 1,3,7-TU would make this compound a reasonable candidate to be incorporated into the LDL particles. However, the low concentration tested (3 μM), although physiologically relevant, probably prevents its incorporation, or it is insufficient to give an antioxidant effect. Therefore, it seems that 1,3,7-TU and 1-U are not incorporated into LDL particles and they exert their protective activity by scavenging radicals in plasma or interstitial fluids.

In conclusion, this study shows that other coffee compounds in addition to caffeic acid and CGA are responsible for the antioxidant activity associated with coffee consumption. Some phenolic compounds generated by microbial metabolism, i.e., *m*-coumaric acid and dihydroferulic acid, possess high antioxidant activity. Even though these compounds failed to protect against LDL oxidation, their role in the overall antioxidant activity of coffee may deserve further investigation. Particularly significant is the protective effect against LDL oxidation of some caffeine metabolites such us 1,3,7-TU and especially 1-U. 1-U showed 7 times higher activity than caffeic acid when both compounds were assessed at physiologically relevant concentrations. To understand the importance of the results reported here, it is necessary to remark that, following coffee consumption, 1-U is detected in blood at much higher concentrations than caffeic acid. We demonstrated that 1,3,7-TU and 1-U are not incorporated into LDL particles at physiologically relevant concentrations but 1-U might play a key role in protecting LDL against different prooxidants present in plasma or interstitial fluid. Further investigation is needed to clarify the different mechanisms involved in the protection of LDL oxidation by 1-U, in addition to those related to the reduction of Cu^{2+} to Cu^{+} and the prevention of α-tocopherol-mediated peroxidation. Additionally, in vivo studies relating the consumption of coffee to caffeine metabolites as well as phenolic acids in plasma would help to confirm the importance of these results.

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