Antioxidative Reactions of Caffeine: Formation of 8-Oxocaffeine (1,3,7-Trimethyluric Acid) in Coffee Subjected to Oxidative Stress

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Coffee was subjected to oxidation by addition of iron/hydrogen peroxide, resulting in the formation of 8-oxocaffeine (1,3,7-trimethyluric acid), which was quantified directly in coffee by analytical HPLC with electrochemical detection (ECD) and by liquid chromatography–tandem mass spectrometry techniques. 8-Oxocaffeine was found to be present in the range of 4–35 ppm in roasted and ground and in instant coffees, but not in fresh green coffee beans. This C-8 hydroxylated purine alkaloid reflects the antioxidative activity of caffeine in the beverage, and its formation is dependent on oxygen tension, hydrogen peroxide, and transition metal availability.

Keywords: *Caffeine; coffee; antioxidant; 1,3,7-trimethyluric acid*

INTRODUCTION

Foods and beverages that contain purine alkaloids, such as coffee, tea, soft drinks, cocoa, and chocolate, enjoy immense popularity worldwide. The presence of methylxanthines, such as caffeine, theobromine, and theophylline, makes an important contribution to the organoleptic quality of these products. For instance, in the case of coffee, caffeine can account for 10–30% of the bitter taste (Viani, 1988). Because of its wide use in foods, beverages, and medicinal preparations, caffeine represents one of the most intensely investigated chemicals for toxicological and physiological effects, with positive behavioral characteristics that include alertness, well-being, motivation, and increased concentration (Barone and Grice, 1994).

It has been reported that caffeine induces chromosomal aberrations in mammalian assay systems if submitted at very high doses (Nehlig and Debry, 1994). However, there is no evidence that caffeine shows mutagenic or carcinogenic effects either in animal models such as the rat exposed to elevated doses of 0.1 and 0.2% in the diet (Takayama and Kuwabara, 1982) or in humans at the levels of consumption in caffeine-containing beverages and medicinal preparations (D'Ambrosio, 1994). Caffeine also shows beneficial *in vitro* and *in vivo* protective effects, some of which include radioprotection properties (Farooqi and Kesavan, 1992), inhibition of tumorigenesis of certain chemicals (Welsch *et al.*, 1988), modulation of antitumor activity of antitumor agents (Sadzuka *et al.*, 1993), and enhancement of P-450 and phase II detoxification enzymes (Gandhi and Khanduja, 1992).

Coffee and tea have been the subject of much debate in recent years due to the observed *in vitro* mutagenicity and genotoxicity in bacterial test systems (Aeschbacher, 1991; Alejandre-Duran *et al.*, 1987; Nakasato *et al.*, 1984; Nagao *et al.*, 1986). A major part of the mutagenicity caused by these beverages is due to the adventitious production of hydrogen peroxide, which reaches levels of between 20 and 160 μM in freshly prepared coffee (Nagao *et al.*, 1986; Tsuji *et al.*, 1991; Stadler *et al.*, 1994) and of *ca.* 50 μM in black tea (Ariza *et al.*, 1988). Strong evidence has also accumulated revealing that coffee and tea may indeed exert antimutagenic and anticarcinogenic effects both *in vitro* (Obana *et al.*, 1986; Shiraki *et al.*, 1994; Stadler *et al.*, 1994) and *in vivo* (Stalder *et al.*, 1990; Conney *et al.*, 1992; Abraham, 1989). The beneficial antioxidant properties of these beverages are apparently linked to their polyphenolic constituents (Stich *et al.*, 1991; Shiraki *et al.*, 1994) and in the case of coffee also to products that arise during the roasting (pyrolysis) process (Kroyer *et al.*, 1989; Macku and Shibamoto, 1991). It is feasible that the oxidation of polyphenolics and the accompanied generation of hydrogen peroxide in coffee and tea can give rise to organic free radicals. In fact, the presence of stable organic free radicals in roasted coffee beans and in coffee solutions has been demonstrated by electron paramagnetic resonance (EPR) techniques (Santanilla *et al.*, 1981; Troup *et al.*, 1988, 1989). These free radicals are generally attributed to organic compounds and are relatively stable and unreactive, and their formation may be associated with the charring of polysaccharide coffee components during roasting (Morrice *et al.*, 1993).

Recently, caffeine was reported to be an efficacious scavenger of the highly reactive hydroxyl radical, but without mention of the specific reaction products (Shi *et al.*, 1991). Studies addressing the antimutagenic and free radical scavenging activities of coffee have demonstrated that this beverage can exert potent antioxidative and antimutagenic effects (Morrice *et al.*, 1993; Stadler *et al.*, 1994; Abraham, 1994) analogous to those already observed for green and black tea and their catechin and tannin-type polyphenolics.

In this study we demonstrate that caffeine, present as a natural constituent in coffee, is an efficient scavenger of the deleterious hydroxyl radical *in situ*, forming the major reaction product 8-oxocaffeine (1,3,7-trimethyluric acid), which can be monitored in coffee by analytical HPLC with ECD or LC–MS/MS techniques.

EXPERIMENTAL PROCEDURES

Chemicals. All reagents were prepared fresh before use. Hydrogen peroxide (3% wt), $FeCl_3 \cdot 6H_2O$, ammonium acetate, citric acid monohydrate, and caffeine were purchased from Merck (Darmstadt, Germany). Bovine liver catalase (EC 1.11.1.6), superoxide dismutase (SOD, horseradish type, EC 1.15.1.1), horseradish peroxidase (HRPase, type II, EC 1.11.1.7), 8-oxocaffeine (1,3,7-trimethyluric acid), and ethylenediaminetetraacetate disodium salt (EDTA) were from Sigma (Buchs,

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Switzerland). Chromabond (500 mg) C_{18} EC cartridges were from Macherey & Nagel (Düren, Germany). $MnCl_2$ and $CuCl_2$ were from Aldrich (Buchs, Switzerland).

Coffee Preparation. Instant coffee solutions (European shelf brands) were prepared by dissolving 2.5 g of instant coffee powder in 150 mL of boiling Millipore grade water. For t_0 samples, solutions were placed immediately on ice and aliquots (each 2 mL) acidified with 1 M HCl (50 μL) in Eppendorf "Safe-lock" tubes. After acidification, the tubes were kept on ice for 10 min to promote precipitation and then centrifuged (2 min, 14 000 rpm, Eppendorf systems). Aliquots (1 mL) of the clear supernatants were removed and applied to preconditioned (2 × bed volumes methanol; 2 × bed volumes water; 2 × bed volumes 10 mM HCl) C_{18} Chromabond (500 mg) columns. After penetration of the coffee solution by gravity-induced flow, the column was washed with 0.5 mL of water and the aqueous effluent discarded. The columns were then eluted with 2 mL of a solvent mixture A + B (7 + 3), comprised of A = 95% water, 5% methanol, and 2 mM acetic acid and B = 95% methanol, 5% water, and 2 mM acetic acid. The clear yellow effluent was dried under speed vacuum and the residue redissolved in 0.3 mL of the same solvent mix. This solution was filtered through 0.2 μm inorganic membrane filters (Anotop 10 plus, Whatman) and stored (4 °C) in 1 mL tightly closed Eppendorf tubes until analyzed. Prior to HPLC, the samples were diluted 1:3 (v/v) with Millipore grade water and injected (20 μL) manually.

Roasted and ground (R&G) coffee was prepared from ground beans (European shelf brands). The coffee was brewed in an automatic drip coffee maker (Bosch TKA 17) equipped with a gold filter. Typically, 46 g of ground coffee was placed into the filter and 850 mL of Millipore grade water poured into the water reservoir. After the hot water had passed through the coffee, the brew was collected in a pot kept at 75 °C on an integrated hot plate. For t_0 samples, the filtered coffee solution was immediately placed on ice and workup commenced as described above for the instant coffee procedure. After C_{18} treatment, the column effluent was dried (speed vacuum) and redissolved in 0.6 mL of solvent mix A + B (7 + 3; A = 95% water, 5% methanol, 2 mM acetic acid; B = 95% methanol, 5% water, 2 mM acetic acid) and 0.3 mL of water, giving a total volume of 0.9 mL. This solution was filtered (0.2 μm) and injected directly onto HPLC without further dilution.

HPLC Electrochemical Detection. Analytical reversed-phase HPLC was carried out using a Hewlett-Packard Model 1050 Ti series apparatus equipped with a HP 1050 UV–vis detector and an Antec Decade electrochemical detector with an integrated pulse dampener and combined electrode and column thermostated oven chamber. The typical separation conditions employed a Supelcosil LC_{18} DB column (25 cm × 4.6 mm; 5 μm; column temperature, 31 °C; solvents A = 50 mM citric acid/NaOH at pH 5.25; solvent B = 100% methanol). Flow was initially isocratic at 10% B for 40 min, then going to 80% B over 10 min, and returning to 10% B over 10 min, eluting 8-oxocaffeine at t_R = 37.2 min. Electrochemical detection was with a glassy carbon electrode operated at 31 °C, potential set at 0.85 vs the AgCl/KCl reference electrode. Absorbance (UV) was monitored simultaneously at 290 nm.

LC–MS/MS. The experiments were carried out using a Finnigan TSQ 700 mass spectrometer connected via a thermospray interface to a Waters 600-MS pump and a Waters 717 autosampler. The column was a Hewlett-Packard LiChrospher 100 RP-18 (125 × 4 mm, 5 μm) operated at room temperature at a flow rate of 1 mL/min. The mobile phase was as follows: solvent A, 50 mM ammonium acetate, pH 5.5; solvent B, methanol. Flow was initially 100% A for 10 min, then going to 100% B over 15 min, and returning to 100% A over 5 min. Detection was achieved by tandem mass spectrometry after collision-induced dissociation of the protonated molecular ion of 8-oxocaffeine (m/z 211). The daughter ion $[M + H - CH_3]^+$ was selectively recorded. A collision energy of 17 eV in the laboratory frame was used. Argon, the collision gas, was set to 1.3 mTorr.

Isolation of 8-Oxocaffeine. Instant coffee powder (2 g) was suspended in 50 mL of a solvent mixture of chloroform/methanol/aqueous ammonia (140:60:1.5 v/v) in a stoppered Erlenmeyer flask, and the mixture was stirred for 16 h at room temperature. The suspension was filtered and concentrated to dryness *in vacuo* (45 °C). The residue was taken up in 4 mL of 10 mM HCl, and nonsoluble material was removed by centrifugation (14 000 rpm, 2 min, Eppendorf systems). The supernatant was charged onto a total of four C_{18} Chromabond cartridges and solid-phase extraction performed as described above for the instant coffee workup. The organic column effluents were combined and concentrated under vacuum (50 °C). The residue was taken up in methanol and stirred magnetically for 1 h. The coffee extract was again centrifuged (14 000 rpm, 2 min) and the clear supernatant applied to three TLC plates (silica gel, $60F_{254}$, 20 × 20 cm; 0.5 mm plate thickness, Merck) developed in the solvent mixture chloroform/acetone/1-butanol/aqueous ammonia (3:3:4:1 v/v). The zone on the plates corresponding to the R_f of 8-oxocaffeine (0.1) was scraped off and eluted with methanol, concentrated *in vacuo* (40 °C), and rechromatographed on a silica gel plate (0.5 mm) in the solvent system chloroform/methanol/aqueous ammonia (140:60:1.5 v/v). The zone corresponding to 8-oxocaffeine R_f (0.58) was scraped off and eluted with methanol. After concentration under vacuum (40 °C), the fraction was redissolved in solvent mix A + B (7 + 3; A = 95% water, 5% methanol, 2 mM acetic acid; B = 95% methanol, 5% water, 2 mM acetic acid) and further purified by HPLC. Conditions employed a Hewlett-Packard 1090 chromatograph equipped with a photodiode array detector and a Supelcosil LC_{18} DB column (25 × 4.6 mm). The gradient commenced (0.8 mL/min) with 100% solvent A (50 mM ammonium acetate, pH 5.5), going to 50% B (B = 100% methanol) over 20 min, and then to 100% B over 5 min, resting for 5 min at 100% B and then returning to 100% A within 10 min. The peak corresponding to 8-oxocaffeine (identification by retention time, t_R = 15.2, and comparison of the on-line UV spectrum with that of an authentic standard) was collected and concentrated *in vacuo* (50 °C). Excess ammonium acetate was removed by lyophilization, and the purified compound was subjected to high-resolution EI-MS. Calculated for $C_8H_{10}N_4O_3$: m/z 210.07529; found m/z 210.07525.

Oxidation Experiments with Instant Coffee. Instant coffees (European shelf brands) were prepared at a concentration of 4.17 g/25 mL in hot Millipore grade water (*ca.* 95 °C) and placed immediately on ice. Typically, the assays were performed in a total reaction volume of 1 mL with an 80 mM potassium phosphate buffer (final concentration), pH 5.5, or as otherwise specified, with a final coffee concentration of 16.7 mg/mL (which corresponds to a standard cup of 2.5 g/150 mL in the final assay). Unless otherwise stated, the reaction mixtures (final concentrations) were comprised of EDTA (500 μM), $FeCl_3 \cdot 6H_2O$ (100 μM), and hydrogen peroxide (4.4 mM). Incubation was performed in closed Eppendorf "Safe-lock" tubes at 37 °C for 1 h. The reaction was terminated by acidification with 50 μL of 1 M HCl, short centrifugation (14 000 rpm, 0.5 min), and immediate C_{18} solid-phase extraction as described above for the analyses of instant coffee samples. The final dried (speed vacuum) column effluents were redissolved in 0.3 mL of solvent mix A + B (7 + 3; A = 95% water, 5% methanol, 2 mM acetic acid; B = 95% methanol, 5% water, 2 mM acetic acid) and diluted 1:3 (v/v) with water prior to injection onto HPLC, using the identical conditions as described for the analytical quantification of 8-oxocaffeine in instant coffee. To quantify 8-oxocaffeine, standard solutions of known concentrations were injected under identical conditions and the amounts in the coffee samples extrapolated from a standard curve.

High-Resolution Mass Spectra. High-resolution mass spectra were recorded on a Finnigan MAT 8430 instrument. Spectral data were collected on the molecular ion of 8-oxocaffeine using perfluorokerosene as the reference and at a resolution of 5000.

RESULTS

Quantification of 8-Oxocaffeine. The first aim of this study was to develop reliable and reproducible analytical techniques to accurately quantify 8-oxocaf-

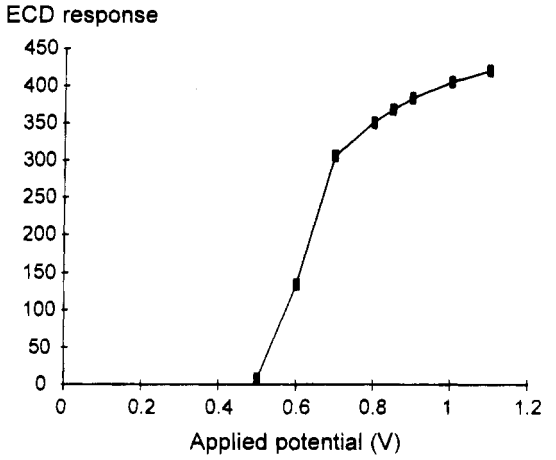

Figure 1. Hydrodynamic voltammogram of 8-oxocaffeine (applied voltage vs detector response).

feine in a complex mixture such as coffee. Uric acid derivatives are electrochemically oxidized at potentials between 0.3 and 0.5 V at neutral pH (Brajter-Toth *et al.*, 1981). Therefore, the electrochemical activity of 8-oxocaffeine prompted the usage of the generally more sensitive electrochemical detection (ECD) technique as a means of quantification. To determine the optimal ECD conditions for 8-oxocaffeine, a hydrodynamic voltammogram was recorded. As shown in Figure 1, oxidation of the molecule begins at 500 mV, and good sensitivity is achieved at potentials of 800 to 850 mV under the HPLC conditions employed (for details see Experimental Procedures). Prior to analysis, freshly prepared instant coffee solutions were subjected to a solid-phase cleanup procedure as described under Experimental Procedures. The recovery of 8-oxocaffeine after this step was determined by comparing the peak areas (HPLC-ECD) obtained by direct injection of a standard solution (4 ng injected) with those obtained following the solid-phase extraction procedure. Recoveries ($n = 6$) found were 97 ± 2.2% for 8-oxocaffeine. To quantify 8-oxocaffeine, the coffee solutions were spiked with different known amounts of standard compound (Figure 2) and the amount in the nonspiked sample extrapolated from a linear regression equation. In all cases, there was a good linear relationship between 8-oxocaffeine concentration in the coffee and peak area (HPLC-ECD or LC–MS/MS), giving correlation coefficients (r) of ≥0.996. The detection limits of 8-oxocaffeine were 0.5–1.0 ppm in coffee and gave a linear response range from 2.22 to 100 ng of spiked 8-oxocaffeine in the coffee solution.

A second quantification method, namely LC–MS/MS, was used to compare the results obtained by HPLC-ECD using the identical coffee samples. In addition, LC–MS/MS gives valuable structural confirmation and can therefore ideally complement the HPLC-ECD method. As depicted in Figure 3A, the characteristic fragmentation pattern of 8-oxocaffeine enabled detection (daughter ion at m/z 196) of levels of as low as 1–1.5 ppm in a coffee extract, revealing a chromatogram almost completely free of interferences from other coffee constituents (Figure 3B).

Extraction of eight different instant coffees showed that 8-oxocaffeine was present in all of the brands that were analyzed (Table 1), ranging from 4 to 31 ppm (micrograms per gram of coffee powder). Also, there was a good correlation of the values of coffee samples analyzed by LC–MS/MS and the identical samples analyzed using HPLC with ECD. Closer inspection of

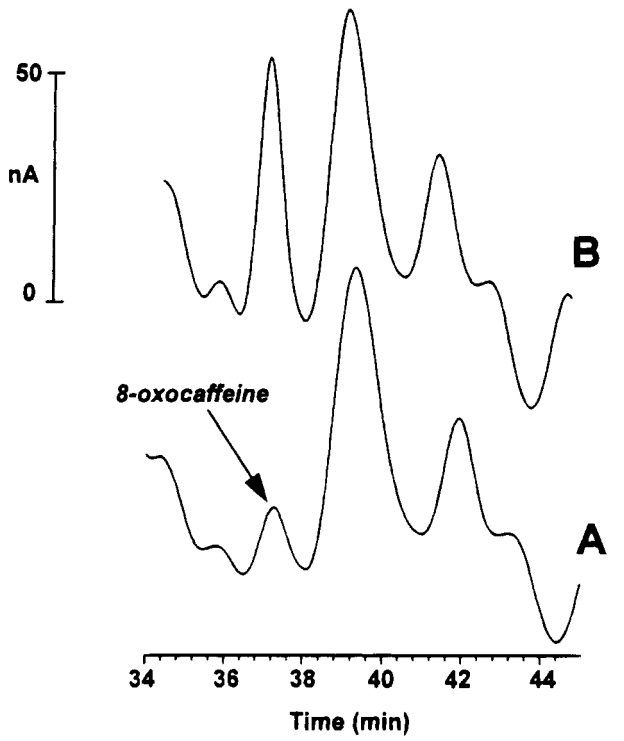

Figure 2. Excerpt of an HPLC profile (electrochemical detection) of solid-phase treated instant coffee: (A) nonspiked; (B) spiked with 35.5 ng of 8-oxocaffeine standard.

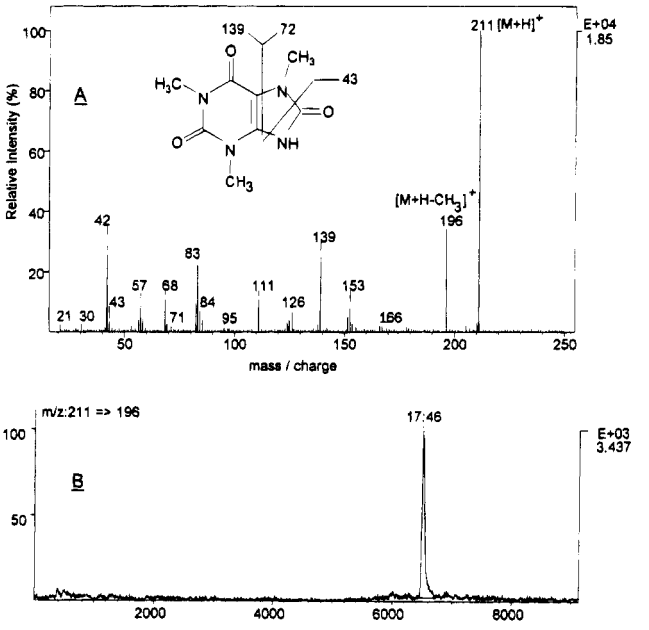

Figure 3. (A) Daughter mass spectrum of the protonated molecular ion (m/z 211) of 8-oxocaffeine; (B) LC–MS/MS detection of 8-oxocaffeine in a coffee extract.

the results shows that there is no significant difference in the 8-oxocaffeine content in the light-roasted brand compared to the medium- and dark-roasted coffees, which suggests that the degree of roasting does not necessarily reflect 8-oxocaffeine production. This was further corroborated by the strong variation in the 8-oxocaffeine content within the six different medium-roast brands analyzed. The levels of 8-oxocaffeine in a freshly prepared cup of coffee (instant or R&G) prepared with Millipore grade water and incubated at 37 °C did not increase over a period of 1 h, which demonstrates that hydroxyl radical production under these conditions is negligible. Comparison of the levels of caffeine (2.8–

Table 1. Quantification[a] of 8-Oxocaffeine in Instant, Roasted and Ground (R&G), and Green Coffees by HPLC-ECD and LC–MS/MS Techniques

coffee brand	8-oxocaffeine (ppm) LC–MS/MS	HPLC-ECD
instant A, light roast	13.1	15.7
instant B, medium roast	11.2	13.3
instant C, medium roast	4.6	4.8
instant D, medium roast	19.7	14.7
instant E, medium roast	21.6	31.6
instant F, medium roast	11.5	9.5
instant G, dark roast	10.7	17.1
instant H, decaffeinated	nd[c]	15.5
R&G brand A	nd	2.9
R&G brand B	nd	6.5
green coffee beans, fresh	<0.5[d]	<0.5
green coffee beans, stored[e]	1.8	0.9

[a] Each coffee sample (2.5 g/150 mL of water) was spiked with three different concentrations of 8-oxocaffeine, linear regression calculation. [b] European shelf brands. [c] nd, not determined. [d] Level of detection 0.5 ppm. [e] Green beans stored for a period of 2 years.

3.4% of coffee powder) in the individual coffee brands revealed absolutely no correlation of 8-oxocaffeine content and the concentration of caffeine. This is also reflected by the relatively large amount of 8-oxocaffeine (15.5 ppm) present in the decaffeinated coffee powder (Table 1).

The extraction procedure applied for 8-oxocaffeine from R&G coffees was analogous to that described for the instant coffee brands (see Experimental Procedures). The levels of 8-oxocaffeine in the R&G brands were lower compared to the instant coffees and are calculated as per total coffee grounds and not as per soluble filtered solids. As exemplified by R&G brand A, a slight increase in the 8-oxocaffeine level was observed after the brew was kept on a hot plate at 75 °C for 1 h (from 2.9 to 3.9 ppm), having taken into account the loss of water during this time.

The presence of 8-oxocaffeine in roasted coffees prompted the investigation of green coffee beans to verify the origin of this uric acid analogue. Analysis of green coffee beans for the presence of 8-oxocaffeine was done by extracting the ground beans with boiling water for 10 min. The slurry was filtered and the filtrate cooled on ice, acidified, and subjected to C_{18} solid-phase extraction as described for the instant and R&G coffees (see Experimental Procedures). As depicted in Table 1, 8-oxocaffeine could not be detected in green beans, with only traces present in a batch of older beans that were stored for a period of 2 years.

Influence of pH, Oxygen, Hydrogen Peroxide, and Transition Metals. To demonstrate the free radical scavenging activity of caffeine *in situ*, the freshly prepared brew was fortified with transition metals and EDTA. Instant coffees were incubated in an 80 mM potassium phosphate buffer to give a final assay concentration of coffee corresponding to that in a standard cup (i.e. 2.5 g/150 mL), and the formation of 8-oxocaffeine was monitored by HPLC-ECD. The dependency of C-8 oxidation of caffeine on pH is portrayed in Table 2. The relative formation of 8-oxocaffeine in coffee treated with iron–EDTA was evident at all pH values tested and reached a maximum at pH 6.8. This result shows that Fenton chemistry takes place under acidic (pH 5.5) conditions and is even potentiated at neutral and slightly alkaline pH values, probably due to more facile autoxidation of polyphenolics and subsequent ease of reduction of Fe^{3+} to the active Fe^{2+} state. Also, there

Table 2. Influence of pH on 8-Oxocaffeine Formation[a] in Coffee

pH	8-oxocaffeine (ppm)	pH	8-oxocaffeine (ppm)
5.5	12.5 ± 1.6	7.4	16.8 ± 1.5
6.0	17.1 ± 2.0	8.2	15.6 ± 1.2
6.8	25 ± 2.2		

[a] Quantification by HPLC-ECD; values represent averages of three independent determinations ± SD. Coffees (16.7 mg/mL) were in a 80 mM potassium phosphate buffer. Final reaction concentrations: Fe^{3+}, 5.6 μg/mL; EDTA, 500 μM. Incubation conditions were as described under Experimental Procedures.

Table 3. Influence of Oxygen and Transition Metals on 8-Oxocaffeine Formation in Coffee

conditions	8-oxocaffeine (ppm)
oxygenation[a]	
instant brand B, untreated	13.3
instant brand B, open jar[b]	14.2
instant brand C, untreated	4.8
instant brand C, oxygenated[c]	11.6
green coffee, untreated	<0.5
green coffee, oxygenated[c]	1.9
transition metals[d]	
control[e]	4.6 ± 0.2
only H_2O_2	5.3 ± 0.3
only iron(III)	30.3 ± 4.2
only manganese(II)	4.5 ± 0.3
only copper(II)	7.6 ± 0.5
iron(III) + H_2O_2	83.8 ± 8.2
manganese(II) + H_2O_2	5.2 ± 0.8
copper(II) + H_2O_2	14 ± 0.4

[a] Analysis by HPLC-ECD; values were obtained by spiking the coffee samples (2.5 g in 150 mL of water) with known concentrations of 8-oxocaffeine, linear regression calculation. [b] Jar left open for 16 days. [c] Purged with oxygen for 16 h at room temperature. [d] Coffees (16.7 mg/mL) were in a 80 mM potassium phosphate buffer, pH 6.8. Final reaction concentrations: metal ions, 5.6 μg/mL; EDTA, 500 μM; H_2O_2, 4.4 mM. Quantification by HPLC-ECD; values represent averages of three independent determinations ± SD; incubation conditions were as described under Experimental Procedures. [e] Untreated coffee.

was no difference in the pH optimum of 8-oxocaffeine formation in two other coffee brands incubated under identical conditions.

The importance of oxygen in the production of 8-oxocaffeine was demonstrated by purging an aqueous green bean slurry with oxygen for 16 h at room temperature. This showed a significant increase in 8-oxocaffeine, which was not detected in the same beans that were stirred for the same period of time (16 h) but without oxygenation, substantiating that this enhancement was not due to gradual extraction of possible endogenous 8-oxocaffeine. Analogously, instant coffee that was purged with oxygen at ambient temperatures also displayed a prominent increase (factor 2) in the level of 8-oxocaffeine (Table 3). This result again reflects the importance of oxygen and hydrogen peroxide in free radical mediated oxidation reactions. However, there was basically no increase in 8-oxocaffeine in dry instant coffee powders in which the coffee jar was left open to the atmosphere for a longer period of time (16 days), suggesting that the free radical reactions leading to 8-oxocaffeine are pertinent only under aqueous conditions.

The effect of addition of transition metals on 8-oxocaffeine formation in instant coffee is portrayed in Table 3. As anticipated, the most potent increase in the level of 8-oxocaffeine was observed when the brew was supplemented with ferric iron (Fe^{3+}). Even low levels of Fe^{3+} (5.5 ppm relative to the total reaction volume) increased the 8-oxocaffeine amount 6–7-fold after an

Table 4. Influence of Antioxidant Enzymes and Hydroxyl Radical Scavengers on 8-Oxocaffeine Formation[a] in Coffee

conditions	8-oxocaffeine (ppm)
antioxidant enzymes[b]	
only iron(III)	43.1 ± 1.4
iron(III) plus catalase (2 units)	37.9 ± 2
iron(III) plus catalase (10 units)	20.1 ± 2.6
iron(III) plus boiled catalase (10 units)	34 ± 5.1
iron(III) plus SOD (2.2 units)	11.3 ± 1.3
iron(III) plus SOD (11 units)	6.8 ± 0.5
iron(III) plus SOD (11 units) plus H_2O_2 (4.4 mM)	5.2 ± 1.4
iron(III) plus HRPase (11 units)	4.8 ± 0.5
hydroxyl radical scavengers[c]	
control	89 ± 6.6
mannitol (0.2 M)	11.7 ± 2.1
2-propanol (0.1 M)	27.7 ± 3.8
ethanol (1.7 M)	5.5 ± 1.1
DMSO (1.2 M)	4.1 ± 0.2

[a] Quantification by HPLC-ECD; values represent averages of three independent determinations ± SD. [b] Coffees (16.7 mg/mL) were in a 80 mM potassium phosphate buffer, pH 6.8. Final reaction concentrations: Fe^{3+}, 5.6 μg/mL; EDTA, 500 μM. Incubation conditions were as described under Experimental Procedures. [c] Coffees (16.7 mg/mL) were in a 80 mM potassium phosphate buffer, pH 5.5. Final reaction concentrations: Fe^{3+}, 5.6 μg/mL; EDTA, 500 μM; H_2O_2, 4.4 mM. Incubation conditions were as described under Experimental Procedures.

incubation time of 1 h. Addition of hydrogen peroxide (final assay concentration 4.4 mM) simultaneously with Fe^{3+} revealed up to 18 times more 8-oxocaffeine than detected in the control brew after the same incubation time. However, adding equivalent amounts of Cu^{2+} increased the level of 8-oxocaffeine only by a factor of close to 2, and Mn^{2+} fortification of the brew had practically no effect at all, in accord with the inability of Mn to participate in Fenton chemistry (Gutteridge and Bannister, 1986). Moreover, in model systems, Cu^{2+}–EDTA complexes have been reported to be less effective Fenton catalysts as compared to Fe^{3+}–EDTA complexes (McCord and Day, 1978), also observed here by the lower concentration of 8-oxocaffeine after treatment with Cu^{2+} vs Fe^{3+}. Omission of the metal chelator EDTA to the coffee solutions that were fortified with Fe^{3+} at pH 5.5 did not significantly alter 8-oxocaffeine formation over time (11.5 ± 1.6 vs 12.5 ± 2.3 ppm). However, at pH 6.8, the 8-oxocaffeine content was clearly lower in those samples without EDTA (7.6 ± 1.8 vs 25 ± 2.2 ppm), even though EDTA favorably influences the redox potential of iron to facilitate the reaction with hydrogen peroxide (Grootveld and Halliwell, 1986). Addition to the coffee solution of hydrogen peroxide (4.4 mM) alone without transition metals resulted in only a very slight increase in 8-oxocaffeine levels (5.3 ± 0.3 ppm), again emphasizing the importance of availability of transition metals in this Fenton-catalyzed reaction sequence.

Influence of Antioxidant Enzymes and Hydroxyl Radical Scavengers. The antioxidant enzymes catalase and superoxide dismutase (SOD) were incubated with an instant coffee solution (equivalent to 2.5 g/150 mL) in a potassium phosphate buffer, pH 6.8, in the presence of Fe^{3+} and EDTA (for details see Experimental Procedures). As shown in Table 4, the most potent inhibitory effects on 8-oxocaffeine production were portrayed by SOD, decreasing the amount of 8-oxocaffeine generated to near that of the control. Catalase (10 units) addition resulted in *ca.* 50% less 8-oxocaffeine formation relative to the positive control. The failure of catalase to completely prevent the formation of

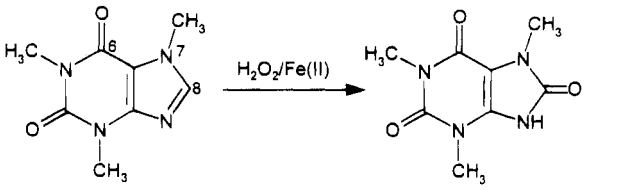

Figure 4. Formation of 8-oxocaffeine (1,3,7-trimethyluric acid) in the presence of hydrogen peroxide and ferrous iron.

8-oxocaffeine in transition metal ion fortified coffee may be due to the inhibition of the enzyme by Fenton's reagent (Orr, 1967) or extremely rapid interaction of the oxidizing species (ferrous iron or oxo–iron complex) with endogenously generated hydrogen peroxide. In contrast, addition of horseradish peroxidase (HRPase), which uses hydrogen peroxide as a cosubstrate in oxidation reactions, resulted in "background" levels of 8-oxocaffeine, completely nullifying the effect of Fe^{3+}–EDTA.

The involvement of hydroxyl radical species in the formation of 8-oxocaffeine was corroborated by the addition of mannitol, ethanol, 2-propanol, and DMSO, which are known to be efficient scavengers of the hydroxyl radical (Laughton *et al.*, 1989; Shi *et al.*, 1991). As shown in Table 4, incubation of coffee brew in the presence of high levels of ethanol and dimethyl sulfoxide (DMSO) resulted in complete inhibition of radical-mediated attack upon caffeine. "Lower" concentrations of mannitol also decreased the 8-oxocaffeine level *ca.* 7.5-fold vs the positive control.

DISCUSSION

Only a few uric acids have been detected and quantified in green coffee beans, namely 1,3,7,9-tetramethyluric acid (theacrine), 1,9-dimethyl-2-methoxyuric acid (liberine), and 1,7,9-trimethyl-2-methoxyuric acid (methylliberine), the levels of which range between 7 and 110 ppm (dry wt) for liberine and at *ca.* 11 ppm for theacrine (Kappeler and Baumann, 1985; Suzuki *et al.*, 1992). The uric acid analogue 8-oxocaffeine is not a product of purine metabolism in the coffee plant, since it was absent in fresh green coffee beans. The detection of 8-oxocaffeine in roasted coffees suggests that it is generated during the roasting process, and one can envisage its formation as an addition of the hydroxyl radical at the carbon-8 position of the methylxanthine nucleus (Figure 4). This chemical reaction is analogous to the formation of 8-oxoguanine from guanine in DNA subjected to oxygen free radical attack (Kasai and Nishimura, 1984). It has been reported that the reaction of caffeine and related methylxanthines such as theobromine and theophylline with Fenton's reagent results in the formation of uric acid derivatives that are largely N-demethylated (Zbaida *et al.*, 1987). However, in our model test systems that employed the same ratios of oxidants as mentioned in the coffee experiments but with only pure caffeine as a substrate, we observed that 8-oxocaffeine makes up to 60% of the total of the oxidized reaction products (R. H. Stadler and J. Richoz, unpublished observation).

The generation of the highly electrophilic oxygen free radical species such as the hydroxyl radical involves reductive cleavage of O–O bonds (hydrogen peroxide or other alkyl peroxides) by catalysts such as ferrous iron (Fe^{2+}) or ferrous iron complexes. Therefore, if ferric iron (Fe^{3+}) is added exogenously to a freshly prepared

cup of coffee, the "Fenton inactive" ferric state can be reduced to the active ferrous state by coffee reductants present in the brew. Subsequent interaction with endogenously generated hydrogen peroxide can then result in the formation of the powerful oxidant(s), either the hydroxyl radical or, as recently proposed for Fenton-type reactions, the more selective metal-based oxidizing species defined as peroxo $Fe^{II}(OOH)$ and iron(IV)–oxo complexes (Wink *et al.*, 1994). This reductive capacity is comparative to that observed for numerous antioxidants, which reverse their beneficial antioxidative effects in the presence of transition metals. For example, both vitamin C and vitamin E can exert pro-oxidative effects by reducing transition metals such as iron and copper to their "Fenton active" state, thus catalyzing free radical generation in the presence of hydroperoxides (Kasai and Nishimura, 1984; Fischer-Nielsen *et al.*, 1992; Maiorino *et al.*, 1993). Moreover, the reducing equivalents needed for the production of hydrogen peroxide via oxygen reduction, and for the reduction of ferric iron, can originate either directly or indirectly (e.g. via the superoxide anion radical) from reductants which include compounds such as caffeic and chlorogenic acids and even vitamin C. This effect is very well demonstrated by the enhanced levels of 8-oxocaffeine after addition of chelated or nonchelated Fe^{3+} to the coffee brew. Notably, freshly prepared coffee not fortified with iron did not lead to an increase in 8-oxocaffeine over a period of 1 h, implying that hydroxyl radical production without addition of exogenous transition metals is negligible.

The endogenous formation of reactive oxygen species in coffee such as the superoxide anion radical has been suggested, a finding supported by the addition of SOD to the coffee brew, which resulted in an increase in hydrogen peroxide levels (Itagaki *et al.*, 1992). However, coffee can also efficiently scavenge superoxide radicals if these are added exogenously (Morrice *et al.*, 1993). The potent inhibition of hydroxyl radical mediated 8-oxocaffeine formation by SOD in coffees subjected to Fe^{3+}–EDTA treatment also implies that superoxide radicals play a key role in the reduction of chelated Fe^{3+} to active Fe^{2+}. It is well established that polyphenolics and semiquinones can give rise to superoxide radicals via one-electron reduction of oxygen (Marklund and Marklund, 1974; Ochiai *et al.*, 1984). An alternative or additional source of superoxide radicals could also be the reduction of the Fe^{3+}–EDTA complex by hydrogen peroxide (Gutteridge and Bannister, 1986). Therefore, 8-oxocaffeine formation in Fe^{3+}–EDTA challenged coffees seems exclusively superoxide driven.

8-Oxocaffeine is a normal byproduct of caffeine metabolism in the human liver and is excreted in the urine of man and rodents after the ingestion of caffeine (Yesair *et al.*, 1984). Interestingly, 8-oxocaffeine has also been classified as a good radical scavenger and potent antioxidant in model systems (Nishida, 1991). In addition, 8-oxocaffeine may be useful as a chemical marker of oxidation in quality assessment and shelf-life determination of coffee and caffeine-rich foods and beverages. The mechanisms of radical formation during storage have been proposed to involve homolytic cleavage of organic peroxides (Santanilla *et al.*, 1981), which could be created during lipid breakdown. As EPR techniques have already shown, free radical production can to a certain extent reflect the degree of decomposition of coffee beans (Ikeya *et al.*, 1989). Further studies are now underway to ascertain the significance of related methylxanthines as radical scavengers and uric acid derivatives as markers of free radical generation in foods and beverages and their potential application also *in vivo*.

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LITERATURE CITED

Abraham, S. K. Inhibition of in vivo genotoxicity by coffee. *Food Chem. Toxicol.* **1989**, *27*, 787–792.

Abraham, S. K. Antigenotoxicity of coffee in the Drosophila assay for somatic mutation and recombination. *Mutagenesis* **1994**, *9*, 383–386.

Aeschbacher, H. U. Mutagenic and antimutagenic compounds in beverages. In *Mutagens in Food: Detection and Prevention*; Hayatsu, H., Ed.; CRC Press: Boca Raton, FL, 1991; pp 181–191.

Alejandre-Duran, E.; Alonso-Moraga, A.; Pueyo, C. Implication of active oxygen species in the direct acting mutagenicity of tea. *Mutat. Res.* **1987**, *188*, 251–257.

Ariza, R. R.; Dorado, G.; Barbancho, M.; Pueyo, C. Study on the causes of direct-acting mutagenicity in coffee and tea using the Ara test in S. typhimurium. *Mutat. Res.* **1988**, *201*, 89–96.

Barone, J. J.; Grice, H. C. Seventh international caffeine workshop, Santorini, Greece 13–17 June 1993. *Food Chem. Toxicol.* **1994**, *32*, 65–77.

Brajter-Toth, A.; Goyal, R. N.; Wrona, M. Z.; Lacava, T.; Nguyen, N. T.; Dryhurst, G. 421—electrochemical and enzymic oxidation of biological purines. *Bioelectrochem. Bioenerg.* **1981**, *8*, 413–435.

Conney, A. H.; Wang, Z-Y.; Huang, M-T.; Ho, C-T.; Yang, C. S. Inhibitory effect of green tea on tumorigenesis by chemicals and ultraviolet light. *Prev. Med.* **1992**, *21*, 361–369.

D'Ambrosio, S. M. Evaluation of the genotoxicity data on caffeine. *Regul. Toxicol. Pharmacol.* **1994**, *19*, 243–281.

Farooqi, Z.; Kesavan, P. C. Radioprotection by caffeine pre- and post-treatment in the bone marrow chromosomes of mice given whole body gamma-irradiation. *Mutat. Res.* **1992**, *269*, 225–230.

Fischer-Nielsen, A. F.; Poulsen, H. E.; Loft, S. 8-Hydroxydeoxyguanosine in vitro: effects of glutathione, ascorbate, and 5-aminosalicylic acid. *Free Radical Biol. Med.* **1992**, *13*, 121–126.

Gandhi, R. K.; Khanduja, K. L. Action of caffeine in altering the carcinogen-activating and -detoxifying enzymes in mice. *J. Clin. Biochem. Nutr.* **1992**, *12*, 19–26.

Grootveld, M.; Halliwell, B. An aromatic hydroxylation assay for hydroxyl radicals utilizing HPLC. Use to investigate the effect of EDTA on the Fenton Reaction. *Free Radical Res. Commun.* **1986**, *1* (4), 243–250.

Gutteridge, J. M. C.; Bannister, J. V. Copper and zinc + manganese SOD's inhibit deoxyribose degradation by the SOD-driven Fenton reaction at two different stages. Implications for the redox states of copper and manganese. *Biochem. J.* **1986**, *234*, 225–228.

Ikeya, M.; Baffa, F. O.; Mascarenhas, S. Quality Assessment of coffee beans with ESR and gamma-ray irradiation. *Appl. Radiat. Isot.* **1989**, *40*, 1219–1222.

Itagaki, S. K.; Kobayashi, Y.; Kitagawa, S.; Iwata, H.; Nukaya, H.; Tsuji, K. Cytotoxicity of coffee in human intestinal cells in vitro and its inhibition by peroxidase. *Toxicol. in Vitro* **1992**, *6*, 417–421.

Kappeler, A. W.; Baumann, T. W. Purine alkaloid pattern in coffee beans. *Proc. 11th Coll. ASIC* **1985**, 273–279.

Kasai, H.; Nishimura, S. Hydroxylation of deoxyguanosine at the C-8 position by ascorbic acid and other reducing agents. *Nucleic Acids Res.* **1984**, *12*, 2137–2145.

Kroyer, G. Th.; Kretschmer, L.; Washüttl, J. Antioxidant properties of tea and coffee extracts. *Agric. Food Chem. Consum., 5th Proc. Eur. Conf. Food Chem.* **1989**, 433–437.

Laughton, M. J.; Halliwell, B.; Evans, P. J.; Hoult, J. B. S. Antioxidant and pro-oxidant actions of the plant phenolics Quercetin, Gossypol and Myricetin. *Biochem. Pharmacol.* **1989**, *38*, 2859–2865.

Macku, C.; Shibamoto, T. Volatile antioxidants isolated from brewed coffee. *Proc. 14th Coll. ASIC* **1991**, 146–154.

Maiorino, M. A.; Zamburlini, A.; Roveri, A.; Ursini, F. Prooxidant role of vitamin E in copper induced lipid peroxidation. *FEBS Lett.* **1993**, *330*, 174–176.

Marklund, S.; Marklund, G. Involvement of the superoxide anion radical in the autoxidation of pyrogallol and a convenient assay for superoxide dismutase. *Eur. J. Biochem.* **1974**, *47*, 469–474.

McCord, J. M.; Day, E. D. Superoxide dependent production of hydroxyl radical catalyzed by iron–edta complex. *FEBS Lett.* **1978**, *86* (1), 139–142.

Morrice, A. E.; Deighton, N.; Glidewell, S. M.; Goodman, B. A. Free radical scavenging reactions in coffee. *Proc. 15th Coll. ASIC* **1993**, 644–649.

Nagao, M.; Fujita, Y.; Wakabayashi, H.; Nukaya, T.; Kosuge, T.; Sugimura, T. Mutagens in coffee and other beverages. *Environ. Health Perspect.* **1986**, *67*, 89–91.

Nakasato, F.; Nakayasu, M.; Fujita, Y.; Nagao, M.; Terada, M.; Sugimura, T. Mutagenicity of instant coffee on cultured hamster lung cells. *Mutat. Res.* **1984**, *141*, 109–112.

Nehlig, A.; Debry, G. Potential genotoxic, mutagenic and antimutagenic effects of coffee: a review. *Mutat. Res.* **1994**, *317*, 145–162.

Nishida, Y. Inhibition of lipid peroxidation by methylated analogues of uric acid. *J. Pharm. Pharmacol.* **1991**, *43*, 885–887.

Obana, H.; Nakamura, S.; Tanaka, R. Suppressive effects of coffee on the SOS response induced by UV and chemical mutagens. *Mutat. Res.* **1986**, *175*, 47–50.

Ochiai, M.; Nagao, M.; Wakabayashi, K.; Sugimura, T. Superoxide dismutase acts as an enhancing factor for quercitin mutagenesis in rat liver cytosol by preventing its decomposition. *Mutat. Res.* **1984**, *129*, 19–24.

Orr, C. W. M. Studies on ascorbic acid. I. Factors influencing the ascorbate-mediated inhibition of catalase. *Biochemistry* **1967**, *6*, 2995–2999.

Sadzuka, Y.; Mochizuki, E.; Takino, Y. Caffeine modulates the antitumor activity and toxic side effects of adriamycin. *Jpn. J. Cancer Res.* **1993**, *84*, 348–353.

Santanilla, J. D.; Fritsch, G.; Müller-Warmuth, W. Electron spin resonance Experiments on raw and roast coffee samples. *Z. Lebensm. Unters. Forsch.* **1981**, *172*, 81–86.

Shi, X.; Dalal, N. S.; Jain, A. C. Antioxidant behaviour of caffeine:efficient scavenging of hydroxyl radicals. *Food Chem. Toxicol.* **1991**, *29*, 1–6.

Shiraki, M.; Hara, Y.; Osawa, T.; Kumon, H.; Nakayama, T.; Kawakishi, S. Antioxidative and antimutagenic effects of theaflavins from black tea. *Mutat. Res.* **1994**, *323*, 29–34.

Stadler, R. H.; Turesky, R. J.; Müller, O.; Markovic, J.; Leong-Morgenthaler, P.-M. The inhibitory effects of coffee on radical-mediated oxidation and mutagenicity. *Mutat. Res.* **1994**, *308*, 177–190.

Stalder, R.; Bexter, A.; Würzner, H. P.; Luginbühl, H. A carcinogenicity study of instant coffee in Swiss mice. *Food Chem. Toxicol.* **1990**, *28*, 829–837.

Stich, H. F. The beneficial and hazardous effects of simple phenolic compounds. *Mutat. Res.* **1991**, *259*, 307–324.

Suzuki, T.; Ashihara, H.; Waller, G. R. Purine and purine alkaloid metabolism in Camellia and Coffea plants. *Phytochemistry* **1992**, *31*, 2575–2584.

Takayama, S.; Kuwabara, N. Long-term study of the effect of caffeine in wistar rats. *Gann* **1982**, *73*, 365–371.

Troup, G. J.; Hutton, D. R.; Dobbie, J.; Pilbrow, J. R.; Hunter, C. R.; Russel Smith, B.; Bryant, B. J. Free radicals in coffee, but not in tea. *Med. J. Aust.* **1988**, *148*, 537–538.

Troup, G. J.; Pilbrow, J. R.; Hutton, D. R.; Hunter, C. R.; Wilson, G. L. EPR detection of free radicals in (I) coffee and (II) gamma-ray irradiated foodstuffs. *Appl. Radiat. Isot.* **1989**, *40*, 1223–1226.

Tsuji, S.; Shibata, T.; Ohara, K.; Ito, Y. Studies on the factors affecting the formation of hydrogen peroxide in coffee. *J. Food Hyg. Soc. Jpn.* **1991**, *32*, 504–512.

Viani, R. Physiologically active substances in coffee. In *Coffee Volume 3: Physiology*; Clarke, R. J., Macrae, R., Eds.; Elsevier Applied Science: London, 1988, pp 1–25.

Welsch, C. W.; DeHoog, J. V.; O'Connor, D. H. Influence of caffeine and/or coffee consumption on the initiation and promotion phases of 7,12-dimethylbenz(a)anthracene-induced rat mammary gland tumorigenesis. *Cancer Res.* **1988**, *48*, 2068–2073.

Wink, D. A.; Nims, R. W.; Saavedra, J. E.; Utermahlen, W. E.; Ford, P. C. The Fenton oxidation mechanism: reactivities of biological relevant substrates with two oxidising intermediates differ from those predicted for the hydroxyl radical. *Proc. Natl. Acad. Sci. U.S.A.* **1994**, *91*, 6604–6608.

Yesair, D. W.; Branfam, A. R.; Callahan, M. M. Human disposition and some biochemical aspects of methylxanthines. In *The Methylxanthine beverages and foods: chemistry, consumption, and health effects*; Spiller, G. A., Ed.; Alan R. Liss: New York, 1984; pp 215–235.

Zbaida, S.; Kariv, R.; Fischer, P.; Gilhar, D. Reactions of theophylline, theobromine and caffeine with Fenton's reagent–simulation of hepatic metabolism. *Xenobiotica* **1987**, *17*, 617–621.

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