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Oxidation of Caffeine and Related Methylxanthines in Ascorbate and Polyphenol-Driven Fenton-type Oxidations

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Caffeine and related methylxanthines were subjected to free radical mediated oxidation by incubation with Fe^{3+}-EDTA/ascorbate and Fe^{3+}-EDTA/polyphenolics. The reaction mixtures were analysed by reverse-phase HPLC, revealing the corresponding C-8 hydroxylated analogues as the major products of hydroxyl radical mediated attack. Further oxidation products of caffeine, analysed by liquid chromatography – mass spectrometry (LC-MS), were the N1-, N3- and N7-demethylated methylxanthine analogues theobromine, paraxanthine and theophylline, respectively. Isolable amounts of the imidazole ring opened 6-amino-5-(N-formylmethylamino)-1,3-dimethyl-uracil (1,3,7-DAU) derivative were also detected, which was characterised by ^1H NMR and mass spectroscopy. The identified products indicate that the pertinent chemical reactions, i.e. C-8 hydroxylation, demethylations, and C8-N9 bond scission, are comparable to the primary metabolic pathways of caffeine in humans. The influence of pH, transition metals, hydrogen peroxide, free radical scavengers and metal chelators on caffeine oxidation was studied. This report illustrates that natural food-borne reactants can aid in identifying specific chemical markers of free radical induced damage. Furthermore, potentially anti- and pro-oxidative reactions can be elucidated which may be important in assessing the impact of nutrient additives and supplements on the shelf life and stability of foods and beverages.

Key words: Caffeine, 1,3,7-trimethyluric acid, 6-amino-5-(N-formylmethylamino)-1,3-dimethyluracil, ascorbate, phenolics, catechins

INTRODUCTION

Caffeine is a naturally occurring dihydroxypurine alkaloid and a dietary constituent of many widely consumed beverages such as coffee, tea and cola-based refreshments. Because of the immense popularity enjoyed by consuming caffeine-rich beverages world-wide, research has focused strongly on the physiological and health effects of caffeine.[1] In this context, ample proof is at hand demonstrating that caffeine consumed at average daily intake equivalent to several cups of coffee or tea does not lead to mutagenic or carcinogenic effects in humans.[2,3] Moreover, caffeine has been reported to be an efficient scavenger of oxygen free radicals,[4] and a recent report has demonstrated this antioxidative efficacy *in situ* with instant and brewed coffee.[5]

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There is only limited information on the reaction of caffeine with oxygen free radicals in model systems.[4,6] One study, addressing the decomposition of caffeine under vigorous Fenton chemistry, showed that caffeine is degraded via demethylation and incorporation of an oxygen atom at the carbon-8 position, yielding primarily 1-methyluric acid and 1,3-dimethyluric acid.[6] However, 'physiologic' amounts of polyphenolic reductants at concentrations as present in coffee led to site-specific C-8 hydroxylation of caffeine and thus the formation of 1,3,7-trimethyluric acid (8-oxocaffeine) when the coffee brew was fortified with low levels of ferric iron salt.[5] This observation suggests that the major route of oxidation of caffeine exposed to hydrogen peroxide (H_2O_2) and oxygen free radicals is by site-specific hydroxylation at the nucleophilic C-8 position of the imidazole ring.

Similarly, exposure of cellular DNA to deleterious oxygen radicals results in mutagenic lesions, a major one being 8-oxoguanine.[7–10] This modified purine base is an important biochemical marker of *in vivo* oxidative damage, and may play a role in carcinogenic events.[11,12] The *in vitro* formation of 8-oxoguanine from guanine can be catalysed by the pro-oxidant mixture iron/H_2O_2/ascorbate,[8,13–15] or procured with polyphenolics acting as reducing agents of ferric iron chelates in the presence of oxygen.[13,16–18]

This work now addresses the influence of natural reductants, such as ascorbate and major tea and coffee polyphenolics, on the oxidation of caffeine and related methylxanthines in the presence of transition metals. Moreover, such cyclic Fenton-type reactions using low levels of reactants at physiological pH in model systems may help to identify novel chemical markers of oxidation which, in turn, could aid in the determination of shelf-life and stability of the food products. Furthermore, the employment of food-endogenous molecules as chemical markers could allow easier assessment of the effect of food additives and preservatives, such as for example citrate, EDTA, iron, ascorbate etc., on oxidative reactions *in situ*.

MATERIALS AND METHODS

Chemicals

All reagents were prepared fresh before use. Hydrogen peroxide (30% wt), $CuSO_4$, $ZnCl_2$, L-(+)-ascorbic acid and caffeine were purchased from Merck (Darmstadt, Germany). Citric acid, guanosine, 1,3,7-trimethyluric acid (8-oxo-caffeine), hypoxanthine, paraxanthine (1,7-dimethylxanthine), theophylline (1,3-dimethylxanthine), theobromine (3,7-dimethylxanthine), β-hydroxyethyltheophylline, thymol (5-methyl-2-(1-methylethyl)-phenol), orcinol (5-methyl-1,3-benzenediol), L-glutathione, 5-aminosalicylic acid (5-ASA), butylated hydroxyanisole (BHA), bovine liver catalase (EC 1.11.1.6), superoxide dismutase (SOD, EC 1.15.1.1), xanthine oxidase (XOD, EC 1.1.3.22), desferrioxamine mesylate (desferal), diethylenetriaminepentaacetic acid (DTPA), ethyleneglycol-bis-(2-aminoethyl)-tetraacetic acid (EGTA), nitrilotriacetic acid (NTA), Tris (2-amino-2-hydroxymethyl-propane-1,3-diol), and $FeCl_3.6H_2O$ were from the Sigma Chemical Co. (Buchs, Switzerland). $CuCl_2$ and $MnCl_2$ were purchased from Aldrich Chemie (Buchs, Switzerland). Caffeic and chlorogenic acids were obtained from Fluka (Buchs, Switzerland). (–)-Epigallocatechin, (–)-epigallocatechin-3-gallate, (–)-epicatechin, (–)-epicatechin-3-gallate, and (+)-catechin were purchased from Roth (Karlsruhe, Germany). 4-Ethylpyrocatechol was from Lancaster (Strasbourg, France). Chelex 100 resin (100–200 mesh) was from Biorad, CA, USA. The internal standard 2,6-diamino-8-purinol was purchased from Janssen Chimica (Geel, Belgium).

The reference compound 8-oxoguanosine was synthesised according to standard procedures[19] with a purity of at least 98% as determined by HPLC and GC-MS analysis.

Fe^{3+}-EDTA/H_2O_2/Ascorbate Catalysed Oxidation of Dihydroxypurines

Unless otherwise stated, standard incubations contained the following compounds (final assay

concentrations): dihydroxypurines (0.5 or 2.5 mM), $FeCl_3$ hexahydrate (100 μM), EDTA (500 μM), potassium phosphate buffer (70 mM) at pH as specified, H_2O_2 (4.4 mM) and ascorbic acid (1 mM). Ascorbate, $FeCl_3$ and H_2O_2 were always prepared fresh before use. The H_2O_2 concentration was verified before use by calculation with $\varepsilon = 67\ M^{-1}cm^{-1}$ at $\lambda = 230$ nm.[17] Iron and EDTA were premixed, and the reaction was started by the addition of H_2O_2. Incubation was done in the dark at 37°C for 1 hour. The reaction was terminated by addition of catalase (500 U) and then ethanol to a final concentration of 2.86 M. Immediately thereafter, β-hydroxyethyltheophylline (6.67 μg/mL) or 2,6-diamino-8-purinol (3.3 μg/mL) were added as internal standards for UV and electrochemical detection (ECD), respectively. The samples were stored at −75°C until direct analysis by HPLC. Where applicable, Chelex treatment of phosphate buffer was performed as described by Buettner[20] using 5 g of column packed resin per 20 mL of buffer.

Oxidation of Caffeine by Fe^{3+}-EDTA/H_2O_2/Phenolics

Polyphenols and other reductants were prepared as 10 mM stock solutions in water, and heated to dissolve if necessary. The polyphenols 4-ethylpyrocatechol and pyrocatechol were dissolved in 10 mM HCl. Caffeic and chlorogenic acids which were prepared in hot 0.1 M potassium phosphate buffer at pH 7.4. BHA was dissolved in hot water and introduced into the assays immediately as a clear aqueous solution. Sample preparation, concentration of the oxidant (Fe^{3+}-EDTA/H_2O_2), incubation time and termination of the reactions were as described for the ascorbate catalysed reactions.

Analytical HPLC and Quantification of Dihydroxypurines/Purines and Their C-8 Oxygenated Analogues

Analytical HPLC was performed with a Hewlett Packard 1050 instrument equipped with a HP1050 autoinjector, a HP1050 diode array detector, and an Antec Decade electrochemical detector with an integrated pulse dampener. Instrument control, data acquisition and data evaluation of UV and electrochemical signals were performed with the HPLC^{2D} ChemStation software. HPLC separation was achieved with two Supelcosil LC-ABZ columns in series (each column 25 × 4.6 cm, 5 μm), with a LC-ABZ precolumn, and injecting 20 μL of the reaction mixtures without prior clean-up or dilution. Unless otherwise stated, all values given represent means of two single measurements of duplicate incubation mixtures. Repetitive analysis of the same incubation mixture revealed s.d.<5% of the measured values for all analyses reported.

HPLC Conditions A

These conditions were employed for analysis with UV-diode array detection only. The mobile phase consisted of solvents A = 20 mM potassium phosphate, pH 2.3 adjusted with concentrated phosphoric acid, solvent B = acetonitrile (Lichrosolv grade), at a flow rate of 0.8 mL/min. The gradient commenced with 4% solvent B for 25 min, going to 10% solvent B oyer 10 min and resting at 10% B for 15 min before increasing to 35% B within 2 min and resting at 35% B for 8 min, and finally returning to initial conditions over 5 min. Absorbance was monitored at 288 and 260 nm. Under the above mentioned conditions, typical retention times (t_R) in minutes of the pertinent analytes were: β = hydroxyethyl theophylline: 46.5; 8-oxocaffeine: 47.4; caffeine: 53.0; 1,3-dimethyl uric acid: 32.5; theophylline: 43.2; 3,7-dimethyluric acid: 25.2; theobromine: 34.5; guanosine: 16.6; 8-oxoguanosine: 24.3.

HPLC Conditions B

These conditions were used for dual ECD and UV-diode array detection of reaction products, and comprised the identical HPLC columns as stated for conditions A, with the same solvents except that solvent A was adjusted to a pH of 2.6. Solvent flow was initially isocratic commencing

with 10% B for the first 25 min, increasing to 35% B over 5 min and resting at 35% B for 15 min. The gradient then returned to initial conditions over 15 min to ensure baseline stability of the electrochemical response. These conditions eluted the internal standard 2,6-diamino-8-purinol after t_R = 6.3 min, 8-oxocaffeine after t_R = 23.8 min and caffeine after t_R = 32.1 min. ECD was performed with a glassy carbon electrode at 30°C, the potential set at 0.9 eV vs the AgCl/KCl reference electrode. UV absorbance was measured as stated under HPLC 'Conditions A'.

The methylxanthines and purines as well as their C-8 hydroxylated analogues were quantified by recording standard curves (50 to 400 µg, r^2>0.999 for UV detection; 0.1 to 2 ng for ECD of 8-oxocaffeine, r^2>0.999) and extrapolation of the peak areas of the pertinent products obtained in the reaction mixture. Corrections for recovery were calculated with the aid of the internal standards β-hydroxyethyl theophylline (for UV detection) and 2,6-diamino-8-purinol (for ECD).

Purification of the Major Products by Semi-Preparative HPLC

Twenty standard reaction mixtures of caffeine with the oxidant mix Fe^{3+}-EDTA/H_2O_2/ascorbate were prepared without adding stop reagents or the internal standards. After incubation (19 h, 37°C), the reaction mixes were pooled and concentrated under speed vacuum to a small volume (ca. 1 mL) and the major products subsequently purified by semi-preparative HPLC. Chromatography employed a semi-preparative Macherey & Nagel column (200/10 Nucleosil C-18, 5 µm), attached to a HP 1090 HPLC chromatograph equipped with a diode array detector. Samples were injected manually (100 µL), and chromatographic conditions used solvents A = 10 mM ammonium acetate (pH 6.8), B = methanol, at a flow rate of 1.5 mL/min. The gradient commenced with 3% solvent B for 10 min, then increased to 20% B over 15 min and remained at 20% B for 10 min, then increased to 50% B over 5 min and rested at 50% B for another 5 min. The column was washed with 80% B over 5 min and the flow remained at 80% B for further 5 min before returning stepwise over 10 min to initial conditions. UV detection was at 288 and 260 nm. Typical t_R (min) of the major reaction products were as follows: unknown peak I (34.8), 8-oxocaffeine (37), 1,3,7-DAU (two broad peaks, 38–40), 3,7-dimethylxanthine (44.9), 1,7-dimethylxanthine (48.8), 1,3-dimethylxanthine (49.4).

Liquid Chromatography-Mass Spectroscopy (LC-MS)

A typical reaction mixture of Fe^{3+}-EDTA/H_2O_2/ascorbate and caffeine (1 h incubation, 37°C) was subjected to a HPLC clean-up procedure before actual LC-MS analysis. This entailed injection (4 × 100 µL) onto an analytical Macherey & Nagel column (Nucleosil 100/5, C-18) with solvents A = 10 mM ammonium acetate, pH 6.8, solvent B = methanol. The gradient started at 5% solvent B for 10 min, then increased to 20% B over 15 min and remained at 20% B for 5 min, increasing to 50% B over 5 min. The column effluent was collected from t_R 4–32 min. This step achieved removal of iron-EDTA and other polar contaminants eluting within the first 4 min and also all residual non-reacted caffeine which eluted after 32 min. Methanol was removed from the collected effluent under a stream of nitrogen at 40°C. The remaining aqueous solvent was removed by lyophilisation and the sample redissolved in 100 µL water, equivalent to a 4-fold concentration relative to the standard incubation assay.

LC-MS was performed using a Waters 600-MS pump, a Waters 486-MS UV detector and a Waters 717 autosampler. Separation was achieved on a Macherey & Nagel Nucleosil C-18 column (5 µm, 250 × 4 mm) at a flow rate of 0.8 mL/min and injecting 20 µL of the sample. The mobile phase was comprised of solvent A = ammonium acetate, 10 mM, pH 6.8, B = methanol, and commenced with 5% B over the first 10 min and increased to 20% B over 15 min, remained at 20% B for 5 min

and then increased to 50% B over 15 min. The solvent was split before entering the mass spectrometer, admitting 80 μL/min into the source. Mass spectroscopy was performed with a Finnigan TSQ-700 mass spectrometer (Bremen, Germany), equipped with a Finnigan Electrospray interface working at a high voltage of 4.5 kV. The manifold temperature was 70°C and the heated capillary was set at 200°C. Nitrogen was used as sheath gas at a pressure of 4.8 bar. The acquired mass range was 50 to 300 Da.

Electron Impact-Mass Spectrometry (EI-MS)

Collected fractions from semi-preparative HPLC were analysed by EI-MS using a Finnigan MAT 8430 (Bremen, Germany) double focusing mass spectrometer. The samples were directly introduced into the ion source and heated from 20 to 300°C at 2°/s. The spectra were acquired from 20 to 400 Da with an electron energy of 70eV and a source temperature set at 180°C.

NMR Spectroscopy

Proton spectra were recorded at 360.13 MHz on a Bruker AM-360 Fourier transform spectrometer equipped with a selective 5 mm proton probe head at room temperature (21°C). DMSO-d_6 (99.95% isotopically substituted, Dr. Glaser AG, Basel, Switzerland) was used as the solvent, and tetramethylsilane (TMS) as an internal shift standard. The samples were prepared in a nitrogen gas flooded glove box.

RESULTS

Incubation of Guanosine/Dihydroxypurines with Fe^{3+}-EDTA/H_2O_2/Ascorbate

The methylxanthines caffeine, theobromine and theophylline were incubated at pH 6.8 for a period of 1 hour in the presence of Fe^{3+}-EDTA/H_2O_2 as described in 'Materials and Methods'. In each case, analytical HPLC analysis with UV-diode array detection showed the presence of only one major oxidation product of each of the three substrates (Figure 1), identified by on-line UV comparisons and t_R with reference compounds as the corresponding uric acid analogues.

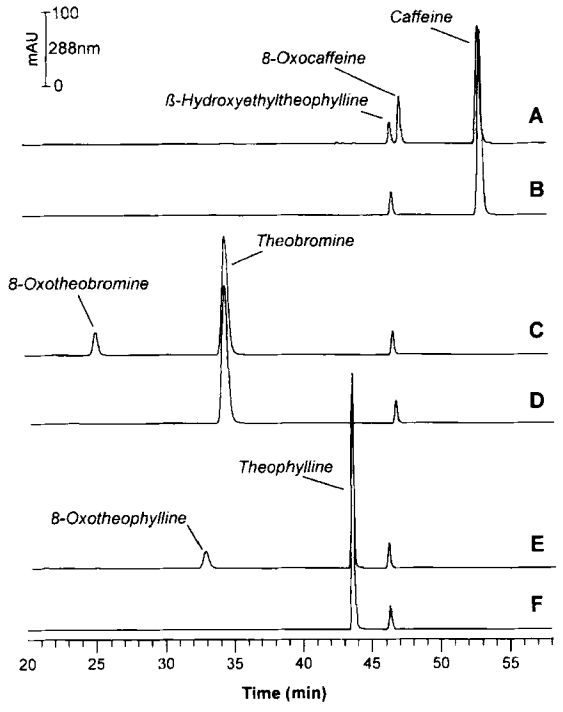

FIGURE 1 Reverse-phase HPLC profiles ('Conditions A') of dihydroxy-purine reaction mixtures after 1 h incubation in Fe^{3+}-EDTA/H_2O_2/ascorbate at pH 6.8. HPLC traces A and B of caffeine, B representing the control without ascorbate; traces C and D of theobromine, D the control as for B above; traces E and F of theophylline, F of the control as for B above. All uric acid products were matched with reference standards with respect to retention time and on-line UV. Experimental details are described in 'Materials and Methods'.

Controls (same substrates as above) without the addition of ascorbate but in the presence of Fe^{3+}-EDTA/H_2O_2 at an H_2O_2 assay concentration of 4.4 mM did not yield hydroxylated products. Furthermore, good recovery of the starting material in the range of 97–98% was found in all the reductant-deficient controls. However, omission of Fe^{3+} and incubation with ascorbate alone resulted in a certain degree of site-specific oxidation (0.2–1.2% of 8-oxo analogues of the total

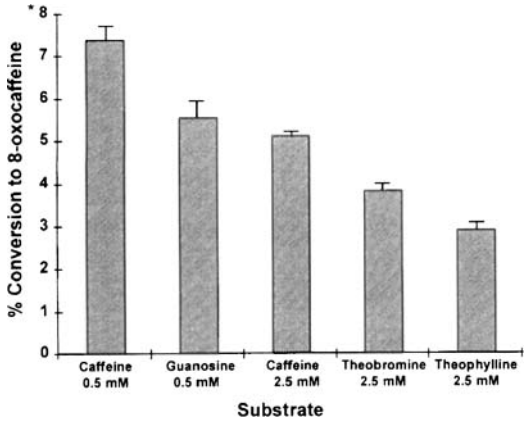

FIGURE 2 Oxidation of guanosine/dihydroxypurines by Fe^{3+}-EDTA/H_2O_2/ascorbate. Products were quantified by HPLC using 'Conditions A' with β-hydroxyethyltheophylline as internal standard as described in 'Materials and Methods'. All entries are averages of three independent incubations each with single determinations (n = 3) ± s.d. (*8-oxocaffeine or 8-oxo analogue)

methylxanthines employed). Such reactions were probably due to trace amounts of contaminant transition metals such as iron, which are difficult t remove completely from the reactants.

As portrayed in Figure 2, reaction of Fe^{3+}-EDTA/H_2O_2/ascorbate mix with the target molecules afforded the corresponding C-8 oxygenated analogues, with conversion rates after 1 hour of incubation ranging from 2.9 to 5.1%. Notably, the total % recovery of the substrates – defined as the molar % dihydroxypurine converted to the major quantified product (8-oxo analogues) plus the molar % substrate found after the reaction time (t_{60}) – was in the range of 86.2 ± 3% and 85.6 ± 0.65% for theophylline and theobromine, respectively (n = 3), but significantly lower for caffeine (67 ± 3.4%, n = 3). Thus, a considerable portion of caffeine was transformed into other oxidation products which were not easily detectable under the given HPLC conditions. For comparative purposes, the purine nucleoside guanosine was also tested under identical assay conditions, albeit at a 5-fold lower assay concentration (0.5 mM) due to limited solubility. As depicted (Figure 2), turnover to the C-8 hydroxylated analogue 8-oxoguanosine reached 5.53 ± 0.5% (n = 3) compared to 7.4 ± 0.78% (n = 3) for equimolar amounts of caffeine.

Identification of the Major Products of Caffeine Oxidation

In order to identify the isolable products of Fe^{3+}-EDTA/H_2O_2/ascorbate mediated oxidation of caffeine, a typical incubation mixture (1 h, 37°C) was subjected to an HPLC clean-up procedure prior to LC-MS analysis. This step ensured removal of all unreacted caffeine and non-alkaloidal polar contaminants. Subsequent LC-MS analysis of the concentrated effluent was as described in 'Materials and Methods'.

As portrayed in the LC-MS trace (Figure 3) the major reaction product was 8-oxocaffeine, with m/z = 211 $[M+H]^+$. This uric acid analogue was isolated and purified by semi-preparative HPLC, and then subjected to EI-MS analysis. The EI mass spectrum corroborated a trimethylated uric acid structure (see Figure 4) with a base peak at m/z = 210 corresponding to the molecular ion $[M^{+\cdot}]$, and fragments at m/z = 180 ($[M\text{-}2 \times CH_3]^+$), 153 ($[M\text{-}H_3CNCO]^+$), and 125 ($[M\text{-}H_3CN(CO)_2]^+$), in agreement with the reference compound.

Additional apolar products of caffeine oxidation were identified by on-line UV and LC-MS.

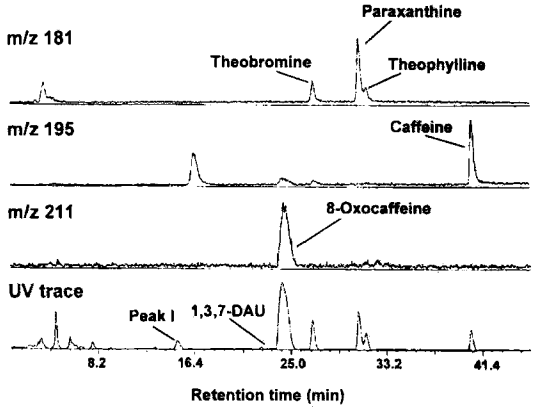

FIGURE 3 LC-MS profile of a 1 h reaction mixture of caffeine subjected to Fe^{3+}-EDTA/H_2O_2/ascorbate oxidation. The bottom trace shows the UV profile (detection at 250 nm from 0–18 min, then 270 nm from 19–42 min); upper ion traces at m/z 211, 195 and 181. Details are as described in 'Materials and Methods'.

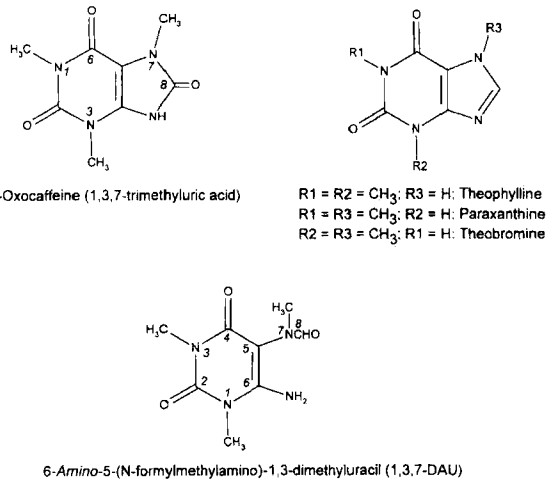

FIGURE 4 Chemical structures of the compounds identified in Fe^{3+}-EDTA/H_2O_2/ascorbate mediated oxidation of caffeine.

Comparison of UV spectra, t_R, and protonated molecular ions (m/z = 181 $[M+H]^+$) of three of the reaction products matched perfectly with the authentic standards theobromine, paraxanthine, and theophylline.

Two peaks were detected identical in t_R, on-line UV (λ_{max} = 268 nm) and m/z = 213 $[M+H]^+$ to the ring opened 1,3,7-DAU derivative (Figure 4). The reference compound showed the same chromatographic behaviour as the caffeine degradation product, in that it chromatographed as two fairly broad peaks, probably representing two isomeric forms of 1,3,7-DAU, and partly co-eluting with 8-oxocaffeine under the given HPLC conditions. This important caffeine derivative was isolated and subjected to a second semi-preparative HPLC step using the same HPLC conditions as before with the exception that the pH of the aqueous solvent was acidified to 5.0 with acetic acid. This resulted in removal of 8-oxocaffeine which showed a significant shift in retention time from 37.9 to 49.3 min. Mass spectroscopic (EI) analysis confirmed a methylated N-formylmethylamino-substituted pyrimidine with a molecular ion at m/z = 212 $[M^{+\cdot}]$, and fragments at m/z 184 ($[M-CO]^+$, base peak), 169 ($[M-COCH_3]^+$), 155 ($[M-CONCH_3]^+$), and 142 ($[M-CONCH_3CH]^+$). ^1H NMR analysis showed a spectrum characteristic for the occurrence of two isomeric forms in a ratio of approximately 80:20. It is likely that the N7–C8 bond presents some double bond character and that the 'formyl' proton can occur in *cisoid* or *transoid* conformation with respect to the N7–CH_3 group. Based on homonuclear decoupling experiments and on earlier in-house work (80 MHz proton spectra), comprising shift comparisons between a number of related compounds and homonuclear NOE measurements to identify the *cisoid* and *transoid* forms of 1,3,7-DAU,[21] the reference compound could be tentatively assigned as follows: major isomer (*transoid*): 2.813 ppm, doublet, J *ca.* 0.5 Hz, 3H, N7–CH_3, 3.118 ppm, singlet, 3H, N3–CH_3, 3.303 ppm, singlet, 3H, N1–CH_3, 7.107 ppm, broad singlet, 2H, D_2O exchangeable, 6-NH_2, 7.771 ppm, incompletely resolved quartet, 1H, 'formyl'-H; minor isomer (*cisoid*): 2.942 ppm, unresolved doublet, J ≤ 0.3 Hz, 3H, N7–CH_3, 3.106 ppm, singlet, 3H, N3–CH_3, 3.298 ppm, singlet, 3H, N1–CH_3, *ca.* 6.84 ppm, broad singlet, 2H, D_2O exchangeable, 6-NH_2, 8.055 ppm, incompletely resolved quartet, 1H, 'formyl'-H. The spectrum of the isolated microgram quantities of caffeine oxidation product was identical to within 0.002 ppm with that of the 1,3,7-DAU reference compound, although the exchangeable amine protons could not be clearly observed because of insufficient signal/noise ratio.

Another oxidation product of caffeine, designated as Peak 1 in Figure 3, was detected showing an on-line λ_{max} at 257–8 nm. The protonated molecular ion had a m/z = 213 $[M+H]^+$, the same molecular mass as 1,3,7-DAU, and therefore probably represented a closely related structure. However, characterisation by NMR was not possible due to the lack of sufficient material. A fifth apolar compound was visible in the HPLC trace (t_R = 29.2 min) and portraying an on-line λ_{max} = 275 nm, indicative of a methylated xanthine structure. Surprisingly, this compound was not visible in the LC-MS trace which was recorded after the HPLC clean-up step. Instead, a peak corresponding to

caffeine in t_R and molecular ion (m/z = 195 $[M+H]^+$) was visible in the LC-MS profile (Figure 3). This unknown product had apparently reverted back to caffeine during the HPLC clean-up procedure prior to LC-MS, probably during concentration and/or lyophilisation of the pooled effluent.

Influence of H_2O_2 on Caffeine Oxidation

Caffeine was incubated at different assay concentrations (0.5 or 2.5 mM) in the presence of Fe^{3+}-EDTA/ascorbate as described in 'Materials and Methods', and subjected to varying doses of hydrogen peroxide (0–4.4 mM). As depicted in Figure 5, C-8 hydroxylation of caffeine (0.5 mM) in the presence of ascorbate commenced in the absence of H_2O_2, and reached a maximum at 1.8 mM H_2O_2. At higher levels of H_2O_2, 8-oxocaffeine formation dropped steadily, either due to enhanced decomposition of 8-oxocaffeine or alternative degradation pathways of caffeine. Moreover, the total % recovery of caffeine, which takes into account the amount of 8-oxocaffeine generated, decreased at H_2O_2 concentrations above 0.44 mM (e.g. 92 ± 3.1% at the aforementioned concentration to 76 ± 3.8% at 4.4 mM).

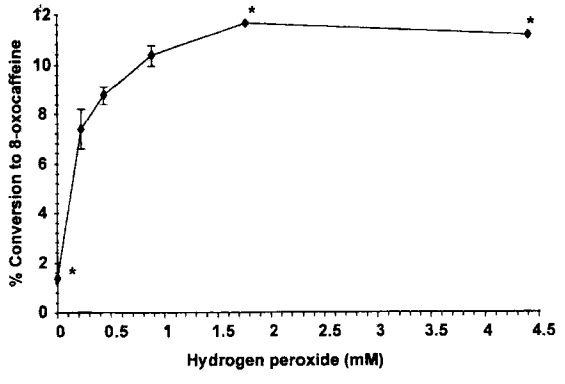

FIGURE 5 Influence of H_2O_2 on caffeine (0.5 mM) degradation and the formation of 8-oxocaffeine. 8-Oxocaffeine was quantified by HPLC using 'Conditions A' with β-hydroxyethyltheophylline as internal standard as described in 'Materials and Methods'. All entries are averages of three independent incubations each with single determinations (n = 3) ± s.d. Data points with an asterisk (*) represent a s.d. less than or equal to the size of the data point marker.

Caffeine employed at 5-fold higher levels resulted in a decrease of 8-oxocaffeine due to a lower oxidant:substrate ratio. However, the dose-response curves paralleled, with the relative maximum yield of 8-oxocaffeine registered at the same H_2O_2 level (1.8 mM).

Caffeine incubated without reductant, i.e. in the presence of only Fe^{3+}-EDTA/H_2O_2 and at different concentrations of H_2O_2, revealed stability of the molecule up to 17 mM of H_2O_2 at pH 6.8 (data not shown). At higher levels of H_2O_2, reduction of the ferric chelate was achieved by H_2O_2 alone, reflected in this experiment by augmented damage to the substrate with a total recovery of caffeine of only ca. 62% after a 1 hour incubation period. Surprisingly, conversion to 8-oxocaffeine was only minimal (0.2%), suggesting alternative pathways or intermediates of oxidation under such extreme conditions.

Influence of pH on Caffeine Oxidation Over Time

Classical Fenton oxidation's are usually favoured under strong acidic conditions to ensure that 'free' ferrous iron remains in solution. However, in the case of the oxidation mixture described here, solubility of iron is guaranteed by a surplus of EDTA and thus such reactions can be carried out efficiently at neutral pH. As portrayed in Figure 6, the hydroxylation of caffeine in the presence of Fe^{3+}-EDTA/H_2O_2/ascorbate was optimal over a broad pH range under slightly acidic to neutral conditions (pH 6 to 7.4). It was also evident that basic conditions led to an attenuation in both hydroxylating activity and in the overall degradation of the substrate. A more acidic pH of the reaction mix will not only increase the oxidising ability of hydrogen peroxide[22] (pK_a = 11.56),[23] but also ensure that reductants such as the superoxide anion radical reduce Fe^{3+}-EDTA to active Fe^{2+}-EDTA faster.[24]

The suppression of 8-oxocaffeine formation under alkaline conditions could be due to additional oxidative reactions, such as imidazole and

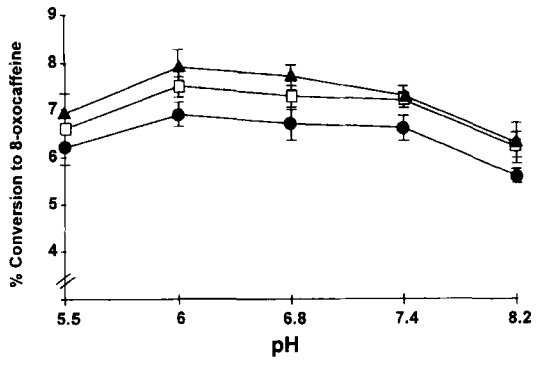

FIGURE 6 Effect of pH on the formation of 8-oxocaffeine over time (● = 1 h, □ = 3 h, ▲ = 6 h). Quantification was performed by HPLC using 'Conditions A' with β-hydroxyethyltheophylline as internal standard as described in 'Materials and Methods'. All entries are averages of three independent incubations each with single determinations (n = 3) ± s.d.

purine ring-opening. Furthermore, comparison of the total % recovery of caffeine showed a tendency towards higher recoveries at higher pH after the first hour of the reaction (75% recovery at pH 5.5 compared to 89% at pH 8.2). Thus, the reactive oxidants/intermediates leading to products other than 8-oxocaffeine and its demethylated analogues may be more labile under slightly alkaline conditions.

Influence of Enzymes and Free Radical Scavengers on Caffeine Oxidation

As shown in Table 1, introduction of SOD did not inhibit 8-oxocaffeine formation. Addition of catalase, however, resulted in a significant decrease in hydroxylation, which could be completely inhibited by 1000 U of catalase. This inhibition is, in fact, due to H_2O_2 and not to unspecific radical scavenging by the protein, because boiled catalase had no suppressive effect on caffeine oxidation. Reactive oxygen species can also be generated enzymically by using XOD, an enzyme which does not recognise caffeine as a substrate.[25] This oxygen dependent enzymic reaction affords superoxide anions and H_2O_2, and is an alternative to the cyclic Fe^{3+}-EDTA/H_2O_2/ascorbate approach. Incubation of caffeine with XOD/hypoxanthine but without

TABLE 1 Effect of anti- and pro-oxidative enzymes on 8-oxocaffeine formation.

Conditions[a]	% 8-Oxocaffeine[b]	% Recovery of caffeine
Control A	2.9	91
Control B	1.0	96
Catalase (40 U)	2.8	89
Catalase (200 U)	2.1	92
Catalase (1000 U)	0	90
Catalase boiled[c]	2.6	82
SOD (44 U)	3.0	90
SOD (110 U)	3.1	90
XOD (0.18 U)[d]	0.5	88
XOD (0.18 U)[e] without Fe^{3+}	0	90

[a]All reaction mixtures incubated at pH 7.2 in the presence of Fe^{3+}-EDTA/ascorbate unless otherwise stated. Control A = positive control with Fe^{3+}-EDTA/ascorbate. Control B = without Fe^{3+}.
[b]Quantified by HPLC with UV detection using 'Conditions A' as described in 'Materials and Methods' with β-hydroxyethyltheophylline as internal standard.
[c] = Catalase (1000 U) in boiling water for 10 min.
[d] = without H_2O_2/ascorbate, with hypoxanthine (0.5 mM) and Fe^{3+}-EDTA.
[e] = without Fe^{3+}-EDTA/H_2O_2/ascorbate, with hypoxanthine (0.5 mM).

added H_2O_2 resulted in the formation of 8-oxocaffeine, although at low levels (Table 1). 8-Oxocaffeine was not formed when Fe^{3+}-EDTA was omitted, substantiating the importance of ferric chelate reduction in the generation of the hydroxylating species.

Addition of typical hydroxyl radical (OH) scavengers mannitol, ethanol, 2-propanol and DMSO at different concentrations corroborated the involvement of the OH in the hydroxylation reaction (data not shown). The most potent inhibition of 8-oxocaffeine formation was observed in the presence of dimethylsulfoxide (DMSO), with levels of 50 mM DMSO (20-fold molar excess relative to caffeine) showing more than 96% inhibition relative to that of the positive control without radical scavenger. Recoveries of total caffeine after a 1 h incubation period were slightly higher in the reaction mixes treated with different mannitol concentrations (95 ± 3.6%) as compared to the three ethanol, 2-propanol and DMSO incubated

samples (83 ± 1.5; 88 ± 2.9 and 89 ± 4.7%, respectively).

It should be noted that the reactive oxidising species may involve not only ·OH,[26] but other oxidants of ferryl (FeO^{2+}) or iron-peroxo [FeII-(OOH)] nature,[27] which may show selectivity in resistance to certain alcohols and other free radical scavengers.[28] Furthermore, ethanol has been claimed not to inhibit C-8 hydroxylation of the DNA nucleoside 2'-deoxyguanosine in Udenfriend oxidation systems, which consist of the oxidant mixture Fe^{3+}-EDTA/ascorbate in the presence of oxygen.[13]

Influence of Metal Chelators and Transition Metals on Caffeine Oxidation

Ascorbate is a potent oxidant if exposed to transition metal chelates and oxygen.[13,29,30] However, certain chelators such as desferal (desferrioxamine) prevent the reduction of Fe^{3+} due to blockage of all co-ordination sites.[31,32] Under such conditions, ascorbate or the superoxide anion radical do not have access to iron and therefore homolytic cleavage of H_2O_2 to generate reactive radical species will not occur. As shown in Table 2, iron chelated with desferal instead of EDTA had a significant effect on the catalytic activity of iron, decreasing the rate of reaction by 74% at 0.1 mM and 100% at 0.2 mM compared to the standard Fe^{3+}-EDTA chelate.

In the presence of a desferal-copper chelate, reactivity was lowered, but the rate of reaction, reflected by C-8 hydroxylation, was still measurable at desferal concentrations of up to 0.5 mM (74% reduction of reactivity *vs* control without chelator). At equimolar concentrations, DTPA and EGTA showed a significant decrease of caffeine oxidation as compared to Fe^{3+}-EDTA. In contrast, the synthetic chelator NTA, which has been reported to promote ·OH production and C-8 hydroxylation of the purine base guanine,[33] enhanced the reactivity towards caffeine, although to a lesser extent than Fe^{3+}-EDTA. Recoveries (%) of total caffeine in NTA chelates were comparable to incubation mixtures containing Fe^{3+}-EDTA (75–77%), clearly lower than in DTPA, EGTA and desferal chelates (87–97%).

TABLE 2 Influence of metal chelators on 8-oxocaffeine formation.

Conditions[a]	% 8-Oxocaffeine[b]	% Recovery of caffeine
With Fe^{3+}:		
Positive control[c]	6.5 (0)	71
Desferal (0.1 mM)	1.7 (74)	90
Desferal (0.2 mM)	0 (100)	96
Desferal (0.5 mM)	0 (100)	97
EGTA (0.1 mM)	2.0 (69)	87
EGTA (0.5 mM)	2.2 (66)	89
DTPA (0.1 mM)	2.2 (66)	87
DTPA (0.5 mM)	1.3 (80)	93
NTA (0.1 mM)	3.9 (40)	77
NTA (0.5 mM)	4.4 (32)	75
With Cu^{2+}:		
Positive control[d]	1.6 (0)	85
Desferal (0.1 mM)	1.1 (31)	85
Desferal (0.2 mM)	1.0 (38)	87
Desferal (0.5 mM)	0.42 (74)	87

[a]All reaction mixes incubated at pH 6.8 in the presence of Fe^{3+}-chelator/H_2O_2/ascorbate; Fe^{3+}(as $FeCl_3$) and Cu^{2+} (as $CuSO_4$) at 5.6 μg/mL. Values in parenthesis are % age reduction of C-8 hydroxylation activity *vs* the positive control.
[b]Quantification of 8-oxocaffeine by HPLC with UV detection using 'Conditions A' as described in 'Materials and Methods' with β-hydroxyethyltheophylline as internal standard. Values in parenthesis represent inhibition (%) calculated relative to the positive control which was taken as 100% conversion.
[c]Incubation with Fe^{3+}-EDTA/H_2O_2/ascorbate.
[d]Incubation with Cu^{2+}-EDTA/H_2O_2/ascorbate.

Damage to caffeine in the presence of only H_2O_2/ascorbate was reduced significantly (45%) after partial removal of contaminant metal ions from the phosphate buffer by treatment with Chelex resin (Table 3). Total recovery of caffeine was also much higher in the samples treated with Chelex (98 vs 91%). Thus, hydroxylation of caffeine in samples not subjected to Chelex treatment is probably due to trace contamination of the substrate and/or reductant with metal ions. EDTA ligated to Zn^{2+} and Mn^{2+} did not promote caffeine degradation, revealing 'background' levels of 8-oxocaffeine comparable to the metal-free control (0.65%). However, a slight increase in the 8-

TABLE 3 Influence of transition metals on 8-oxocaffeine formation.

Conditions[a]	% 8-Oxocaffeine[b]	% Recovery of caffeine
Fe^{3+}-EDTA/H_2O_2	0	98
H_2O_2/ascorbate	1.17	91
H_2O_2/ascorbate (Chelex treated)[c]	0.65	98
Fe^{3+}-EDTA/H_2O_2/ascorbate	5.9	69
Fe^{3+}-EDTA/H_2O_2/ascorbate (Chelex treated)	5.6	82
Cu^{2+}-EDTA/H_2O_2	0	96
Cu^{2+}-EDTA/H_2O_2/ascorbate	1.14	91
Cu^{2+}-EDTA/H_2O_2/ascorbate (Chelex treated)	0.82	95
Zn^{2+}-EDTA/H_2O_2/ascorbate (Chelex treated)	0.63	96
Mn^{2+}-EDTA/H_2O_2/ascorbate (Chelex treated)	0.63	96

[a]All reaction mixtures incubated at pH 6.8, all metal ions employed as their chloride salts at 5.6 μg/mL assay concentration.
[b]Quantified by HPLC with UV detection using 'Conditions A' as described in 'Materials and Methods' with β-hydroxyethyltheophylline as internal standard.
[c]Potassium phosphate buffer treated with Chelex 100 resin as described in 'Materials and Methods'.

TABLE 4 Catalysis of 8-oxocaffeine formation by polyphenols/reductants in the presence of Fe^{3+}-EDTA/hydrogen peroxide.

Conditions[a]	% 8-Oxocaffeine[b]	% Recovery of caffeine
Control[c]	0	98
Chlorogenic acid	5	67
Caffeic acid	6.1	59
4-Ethylpyrocatechol	7.1	54
(−)-Epicatechin	2.2	83
(−)-Epicatechin gallate	2.2	83
(+)-Catechin	2.1	88
(−)-Epigallocatechin	6.1	70
(−)-Epigallocatechin gallate	5.6	77
Thymol	0	91
Orcinol	0.1	92
5-ASA	0.4	89
BHA	0	92
L-Glutathione	0.4	93
Ascorbate	5.9	78

[a]Reaction mixes incubated at pH 7.2 with Fe^{3+}-EDTA/H_2O_2; all reductants/phenols at 1 mM final concentration.
[b]Quantified by HPLC with UV detection using 'Conditions A' as described in 'Materials and Methods' with β-hydroxyethyltheophylline as internal standard.
[c]Only Fe^{3+}-EDTA/H_2O_2.

oxocaffeine level was recorded with Cu^{2+}-EDTA, although several fold lower than in the reaction mixes with Fe^{3+}-EDTA.

Oxidation carried out in Tris and phosphate buffers with the standard reaction mixture (Fe^{3+}-EDTA/H_2O_2/ascorbate) was suppressed in the former buffer, with only 0.71% conversion to 8-oxocaffeine. In contrast to the phosphate buffered reaction mixtures, increasing concentrations of Fe^{3+} (0.1 to 1 mM) did not elevate but instead decreased the 8-oxocaffeine levels by nearly 20% in the presence of Tris. Total recoveries of caffeine were in the range of 96–97% when incubated in Tris, compared to 70–79% in the presence of potassium phosphate buffer. These results again corroborate ·OH mediated oxidation of the substrate, and in addition the potent scavenging action of the organic Tris buffer.[34]

Polyphenol Mediated Oxidation of Caffeine

The oxidation of caffeine induced by natural polyphenols and other reductants/antioxidants was performed both in the presence (Table 4) and absence (Table 5) of exogenous H_2O_2. All compounds with catecholic moieties (1,2-benzenediols) as well as ascorbate proved effective in reducing the Fe^{3+} ligand and thereby generate reactive oxygen species. Notably, 8-oxocaffeine levels recorded in incubation mixtures with the flavanoids (−)-epigallocatechin and (−)-epigallocatechin-3-gallate were nearly 3 times higher as compared to the other catechins tested. On the other hand, caffeine degradation was completely inhibited upon incubation with phenolic compounds with only one hydroxyl function, such as thymol and the synthetic food preservative BHA. The 1,3-benzenediol orcinol was comparably ineffective in mediating caffeine

TABLE 5 Catalysis of 8-oxocaffeine formation by polyphenols/reductants in the presence of Fe^{3+}-EDTA.

Conditions[a]	8-Oxocaffeine (nmoles)[b]
Control[c]	0
Chlorogenic acid	1.2
Caffeic acid	2.6
4-Ethylpyrocatechol	4.5
Pyrocatechol	1.5
(–)-Epicatechin	4.4
(–)-Epicatechin gallate	7.7
(+)-Catechin	1.6
(–)-Epigallocatechin	0.5
(–)-Epigallocatechin gallate	27.8
Thymol	0
Orcinol	0.1
5-ASA	0.4
Sesamol	0.1
BHA	0
Ascorbate	42.4

[a]Standard incubation conditions with Fe^{3+}-EDTA, potassium phosphate buffer at pH 7.2, without exogenous H_2O_2. Caffeine at an assay concentration of 2.5 mM and polyphenols/reductants at 1 mM.
[b]Quantified by HPLC with ECD using 'Conditions B' as described in 'Materials and Methods' with 2,6-diamino-8-purinol as internal standard.
[c]Only Fe^{3+}-EDTA.

hydroxylation, with slight catalysis observed in reaction mixtures containing 5-ASA and L-glutathione.

Caffeine was incubated with the same polyphenols/reductants and at the same assay concentrations as listed in Table 4 but without exogenous H_2O_2 (Table 5). In these experiments, oxidation of the substrate reflects the redox capacity of the reductants, and thus also the formation of hydrogen peroxide via 2-electron reduction of available molecular oxygen. Due to the comparably low level of degradation of caffeine without H_2O_2 added to the mixture, 8-oxocaffeine was detected and quantified by HPLC with ECD.[5] This analytical technique allowed detection of ≥ 1 ng 8-oxocaffeine (4.8 pmol) in the total reaction mixture, i.e. 0.0002% conversion of the substrate, compared to 0.08% achieved by UV detection under the given HPLC conditions. Accurate quantitation of 8-oxocaffeine by ECD was made possible with the use of the electrochemically active internal standard 2,6-diamino-8-purinol, already successfully employed in the quantitation of 8-oxoguanine by the highly sensitive HPLC-ECD technique.[35]

As portrayed in Table 5, catechins harbouring a substituted trihydroxy-benzene moiety such as epigallocatechin-3-gallate and epicatechin-3-gallate catalysed the formation of significantly higher levels of 8-oxocaffeine than the other catechins tested, reaching 1.1 and 0.31% turnover of total caffeine to the uric acid analogue, respectively. Notably, epigallocatechin catalysed 55-fold less 8-oxocaffeine than its 3-gallate substituted congener, and still significantly less than the other catechins as well as the phenylpropanoids tested.

As already evident in the previous H_2O_2 treated mixtures, hydroxylation activity was lower or totally absent with simple monomeric catechols as potential reductants of the Fe^{3+}-EDTA chelate. Recoveries of caffeine in all of the above mentioned phenol/reductant assays were in the range of 98–100%, attributable to the lack of exogenous H_2O_2 which, as was evident in the previous experiment (Table 4), enhanced degradation of the substrate.

DISCUSSION

Udenfriend-type oxidations using the dihydroxypurine theophylline as a substrate were first described in 1954.[29] In these early experiments, hydroxylation was induced by Fe^{3+}-EDTA/ascorbate under an atmosphere of oxygen, and in the case of theophylline afforded the corresponding C-8 oxygenated analogue 1,3-dimethyluric acid in approximately 6% isolable yield.

The results of the experiments described here show that the major products of dihydroxypurine degradation in the presence of the oxidant Fe^{3+}-EDTA/ascorbate are the corresponding uric

acids. Closer inspection of the caffeine reaction products (Figure 4) revealed the presence of, amongst others, isolable amounts of the imidazole ring-opened adduct 1,3,7-DAU. Due to the poor HPLC behaviour of the 1,3,7-DAU isomers, attempts were made to quantify this compound by GC/MS techniques, and thereby establish a product ratio of 1,3,7-DAU to 8-oxocaffeine. However, rapid dehydration and ring-closure to caffeine was observed during derivatization (silylation), making direct quantification by GC-MS impossible.

The results presented here also illustrate a close analogy to the DNA base guanine which, when exposed to hydroxyl radicals, forms substantial amounts of the C-8 hydroxylated analogue 8-oxoguanine[33,36] as well as the imidazole ring-opened 2,6-diamino-4-hydroxy-5-formamidopyrimidine (FAPy) adduct.[37,38] The product profile suggests that the mechanism of oxidation of caffeine follows a similar pattern. A redox ambivalent intermediary carbon centred radical with a C-8 hydroxyl function could be the precursor of both 8-oxocaffeine and the corresponding ring-opened adduct, by either electron abstraction or reduction, respectively. Besides, caffeine reacts with the highly electrophilic $^{\cdot}OH$ at a reaction rate constant[4,39] of ca. $5.9–6.9 \times 10^9\ M^{-1}\ s^{-1}$, comparable to that of DNA bases which scavenge $^{\cdot}OH$ a rate constant [38,40] ranging from $4.5–9 \times 10^9\ M^{-1}\ s^{-1}$.

As illustrated in this report, exposure to reducing agents such as polyphenols or ascorbate alone will not result in significant damage to the substrate. On the other hand, addition of low levels of chelated iron results in rapid degradation of caffeine. Exchange of Fe^{3+}-EDTA with Cu^{2+}-EDTA in ascorbate initiated reactions yielded far less 8-oxocaffeine and also a higher recovery of total caffeine, corroborating the suppressive action of EDTA in Cu^{2+} catalysed oxidation of purines.[14] However, $^{\cdot}OH$ mediated damage can easily be suppressed by certain chelators, for example desferal, which blocks all iron co-ordination sites and thus inactivates the metal catalyst.[32] In contrast, the metal chelator and free radical promoter NTA[33,41] potentiates damage to caffeine, as reflected by increased levels of 8-oxocaffeine with increasing NTA levels.

In accordance with previously published results, hydroxyl radicals in 'free solution' generated by this iron-EDTA/ascorbate model can be efficiently scavenged by the typical $^{\cdot}OH$ scavengers[42,43] and organic buffers such as Tris.[32] Furthermore, the enzymic antioxidant SOD did not inhibit 8-oxocaffeine formation, confirming earlier observations that such reactions are not exclusively superoxide driven due to the ability of ascorbate to directly reduce ferric iron chelates.[44] In comparison, polyphenol initiated oxidation of caffeine *in situ* was shown to be effectively suppressed by SOD.[5]

It is well established that natural dietary polyphenolic compounds can act as pro-oxidants and generate deleterious oxygen free radicals when exposed to oxygen and transition metals such as iron. This seemingly contradictory behaviour to act as both free radical scavengers and free radical generators in certain *in vitro* systems is well documented.[43,45–47] Incubation of caffeine with the major individual coffee and tea polyphenols in the presence of iron-EDTA under physiologic pH clearly supports the pro-oxidative role of certain catechols, which easily autoxidize to generate superoxide anion radicals and H_2O_2.[48–50] Many polyphenolics, especially *o*- and *p*-dihydrols are good reductants of ferric ion chelates, thus catalysing $^{\cdot}OH$ formation[51] and subsequent purine hydroxylation.[13] Similarly, ascorbic acid in an oxidative environment with 2'-deoxyguanosine or DNA as substrate will induce significant damage to yield 2'-deoxy-7,8-dihydro-8-oxoguanosine.[15,18] In the experiments presented here, employment of analytical HPLC with ECD made possible the detection of very low levels (pmol) of 8-oxocaffeine, enabling accurate assessment of caffeine damage by the individual antioxidants and catechols/phenolics tested. Autoxidation of the polyphenols chlorogenic and caffeic acids can enhance $^{\cdot}OH$ formation if they are concomitantly exposed to metal salts.[16,51]

It is interesting to note that both caffeic and chlorogenic acids induced far lower levels of 8-oxocaffeine compared to the flavanols epigallocatechin-3-gallate and epicatechin-3-gallate in experiments where exogenous H_2O_2 was omitted. This result is supportive of a greater proclivity of the gallate-substituted catechins to undergo autoxidation, and thereby generate more reactive oxygen species that can attack caffeine via $^{\cdot}OH$ formation. However, it needs to be mentioned that pro-oxidative effects of certain polyphenolic antioxidants in some model systems reflect their redox capacity, and not necessarily their biological activity *in vivo*.[17,43,52]

Numerous foods and beverages are rich in ascorbate and/or polyphenolics, and many also harbour Ca^{2+}-EDTA and metal ions as supplements.[47,53] Uncontrolled fortification of foods with iron or copper could have the effect that pro-oxidative reactions predominate.[53,54] Simple model systems such as the one described here, using natural food constituents as reactants, can simulate potentially anti- and pro-oxidative processes in the food or beverage. As already demonstrated *in situ* for coffee[5], natural dietary chemicals as substrates/indices of $^{\cdot}OH$ induced attack such as caffeine/8-oxocaffeine will enable quick and facile identification of pro-oxidative additives and ingredients, similar to the deoxyribose assay which has been adopted to measure deleterious free radical reactions in foods.[47,55]

The non-enzymic catalysis of 8-oxocaffeine from caffeine due to oxidative stress may also have important implications in biological systems. The primary pathways of caffeine metabolism in humans[56,57] involve 8-hydroxylation (CYP3A), demethylations (CYP1A2) to dimethylxanthines, and C8-N9 bond scission to afford 1,3,7-DAU, comparative to the product spectrum of the chemical reactions observed in this model system.

However, in light of the data presented here, it cannot be excluded that unspecific Fenton-type reactions participate to a certain degree in the C-8 hydroxylation and further transformation of caffeine *in vivo*. Therefore, the proposed usage of 8-oxocaffeine as an indicator of CYP3A activity[56] should also take into account dietary contributions[5] and potential free radical mediated reactions in the body *en route* to the liver.

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