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Catalytic Conversion of Caffeine into Molecules of Valuable Interest Via N-Demethylation Reaction

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This work aims to apply catalytic processes to promote the conversion of caffeine into valuable dimethylxanthines via N-demethylation reaction. Thus, we seek to evaluate the products formed and propose the reactions involved and their mechanisms. Reaction conditions were evaluated including different concentrations of Fenton reagent and ascorbic acid to evaluate caffeine oxidation. Theobromine, paraxanthine, and theophylline formation was proven by HPLC-DAD and mass spectrometry analysis evidencing the N-demethylation reactions, via radicals, with about 1% yield. These results open the way for new N-demethylation reaction routes to be studied with applications for caffeine and other biomolecules in diverse areas such as biochemistry and medicinal chemistry.

Introduction

Methylxanthines are a group of purine alkaloids present in plants in the form of caffeine, theobromine and theophylline, biosynthesized for protection against pathogens and insects as chemical defense or by inhibition of the growth of other plants.[1] Caffeine is found in coffee (*Coffea spp*) in amounts of about 0.8 to 1.4% in *Coffea arabica*, 1.7 to 4% in *Coffea canephora* (dry basis), while in minor quantities in tea leaves (*Camelia sinensis*), yerba mate *(Ilex paraguariensis*) and guarana (*Paullinia cupana*).[2–5] Theobromine is found in cocoa (*Theobroma cacao*) with about 2% in the beans or 0.6% in the husk, in young leaves of cocoa tea (*Camellia ptilophylla*) with around 5 to 6%, in tea leaves (*Camellia sinensis*) with a content of up to 0.8%, in yerba mate (*Ilex paraguariensis*) with around 0.08 to 0.16% and traces in kola nut (*Cola acuminata*) and in coffee (*Coffea spp*). Theophylline is found mainly in tea leaves, and also in coffee and cocoa beans.[6]

In plant metabolism, caffeine is biosynthesized from *xanthosine* with three steps of methylation, mediated by *synthase* enzymes, including theobromine as an intermediate.[7] In coffee leaves, caffeine turns into theobromine or theophylline, mediated by enzymes and can continue until generating CO_2 and NH_3.[3]

The pharmacologic activities of methylxanthines depend on the methylation pattern. They have been used since early the 19th century and are still being studied for many applications.[6] Their physiological effects include stimulating of central nervous system (CNS), increasing muscle strength and resistance to fatigue, improving functions for cardiovascular diseases, and increasing blood pressure and heart rate. They also present anti-inflammatory and anti-oxidative properties,[8] and neuroprotective effects.[9] Caffeine is more lipophilic with less influence on diuresis and has greater CNS stimulating action being the chemical substance with physiological activity most consumed by the population related to providing alertness, both in food and medicines. Theobromine has a small stimulating action, while it has a greater effect on diuresis and relaxation of the lung muscles. It has been used by the pharmaceutical industry in cold medicines and respiratory treatments.[10] Theophylline has a greater bronchodilator effect and also acts as a diuretic and it is used in the treatment of asthma and other lung diseases.[11]

Theobromine and theophylline can be obtained naturally by extractions but in low concentration, requiring additional steps as different sample preparations, and solvents, including assisted by microwaves or supercritical fluids,[4] presenting major disadvantages such as the use of toxic reagents, low specificity, high cost of installations and operations.[6,12] Paraxanthine, other methylxanthine, is not found in natural sources being a product of the caffeine catabolism.[13] Differently, caffeine is present in greater quantities in different parts of the coffee (bean, pulp, husk and leaves), which has a large source of biomass. It is already extracted by the coffee industry from the coffee bean and is even easily found purified by the industry and is available for supply, whether in the area of food, cosmetics, or medicines.[14]

Methylxanthines can be synthesized in the laboratory from uric acid or 4-amino-6-hydroxypyrimidine and 4,6-diaminopyrimidine, as earlier proposed by Fischer in 1895 and Traube in 1900 respectively.[15,16] Posteriorly, theobromine was synthesized by methylation of 3-methylxanthine with dimethyl sulfate.[17]

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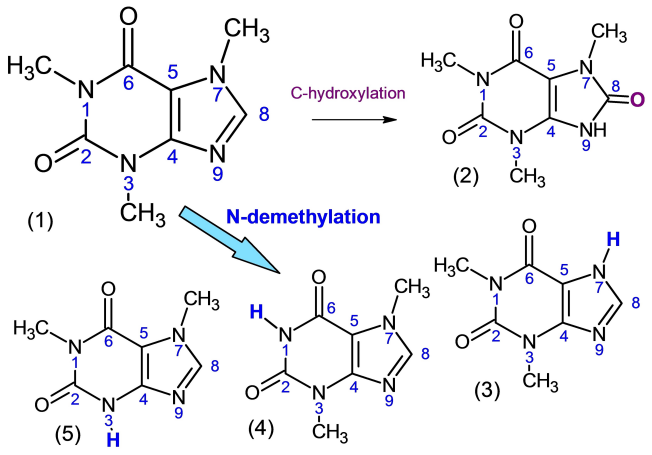
Other methods involve N-demethylation reaction, like the von Braun reaction, in which cyanogen bromide (BrCN) or chloroformate are the demethylating agents, to generate the secondary amines after hydrolysis.[18] Most works applying N-demethylation reactions are focused on alkaloid morphine derivatives. Polonovski reaction promotes the N-demethylation, modified in 2 steps: i) oxidation of the substrate to the corresponding N-oxide; ii) treatment with catalyst (activation) and decomposition of the demethylated product.[19–22] Disadvantages include the use of expensive and toxic reagents, the number of reaction steps under severe conditions, and hazardous by-products. Some recent reports use metal-catalyzed,[23] photocatalyst-free,[24] anodic oxidation[25] highlighting difficulties and revealing the search for new practical discoveries of N-demethylation methods.

In bacteria, caffeine degrades by enzymatic N-demethylation to generate the related methylxanthines.[1] Thus the use of biocatalysis is another way to promote the formation of methylxanthines from caffeine, by genetic engineering using microorganisms modified through enzymatic catalysis,[12,26–29] the active centers can be constituted by non-heme iron or cobalt.[30] Although these processes have high selectivity and are environmentally correct, there are difficulties due to caffeine inhibition in some microorganism strains, the great technological demand, the need for selection and maintenance of strains, and the limited quantity of products generated.

The Fenton reaction was evaluated as a synthetic tool for N-demethylation to xanthines to simulate hepatic metabolism.[31] The evaluation of caffeine degradation products by radical reactions was reported, indicating 1,3,7-trimethyluric acid as the major product,[32] in addition to theobromine and theophylline, in trace amounts.[33–37] In the human body, N-demethylation reactions are often related to the metabolism of nitrogen-containing drugs. The four main pathways of caffeine metabolism comprise N demethylation reactions at positions N1, N3 and N7, and oxidation reaction at C8 (Scheme 1), mediated by *cytochrome P450* (CYP) and *flavin monooxygenase*.[38,39] In vitro methods using non-enzymatic strategies have been developed as promoters of reactions simulating enzymatic processes, but



Scheme 1. Representation of caffeine (1) and transformation products: 1,3,7-trimethyluric acid (2), theophylline (3), theobromine (4) and paraxanthine (5).

few scientific works approach the Fenton process.[40] The use of isolated enzymes has the disadvantage of being unstable, and losing their activity, in addition to their high production cost.[1] All the processes to produce methylxanthines have inherent difficulties related to reagents and starting material, low yields and purification steps, operations and installation costs, justifying the current need for new alternatives to obtain them. On the other hand, the need for research in the chemical and biochemical areas that encompass N-demethylation reactions.

Considering the demand for environmentally friendly processes, we intend to apply catalytic reactions for the conversion of caffeine into dimethylxanthines, taking advantage of natural resources to obtain molecules with added value, using technological innovation processes with fewer steps, avoiding the use of high-cost or toxic reagents, in line with the precepts of "Green Chemistry". In this context, this study aims to identify the products generated from the oxidation of caffeine, elucidate the obtention of dimethylxanthines, and propose their transformation routes.

Results and Discussion

The caffeine transformation products were generated under different reaction conditions, monitored by HPLC-DAD, as shown in Table 1, for the methylxanthines, and Table S1. It was evidenced that 14 chromatography peaks were described numerically according to the elution order (Table S2), one of them being theobromine ("Peak 7") confirmed by standard injection. The other products were not compared to an analytical standard and could only be inferred. It is observed that theobromine is formed in Fenton reactions, including with the addition of ascorbic acid (AA), or in the presence of AA (with or without Fe^{2+} ions). Theobromine was not formed in a solution containing only iron ions, or by alkaline hydrolysis.

Using the retention factor (*K*) value, it is possible to relate peaks from different analyses. Thus, the chromatographic peak order was estimated based on evidence from other studies that used C18 reversed-phase column and similar polar mobile phase.[41–43] Based on the retention time (RT) of xanthines and uric acid derivatives and comparing the *K* values,[37,43,44] we proposed the identification of the peaks, with the probable elution order: xanthine (X), 7-methylxanthine (7X), 3-methylxanthine (3X), 1-methylxanthine (1X), theobromine (Tb), paraxanthine (Px), theophylline (Tf) and caffeine (Cf). A theobromine degradation experiment was conducted to better understand the products formed. The "peak 1" was formed in 3.1 minutes of retention, in both caffeine and theobromine experiments. Based on the notes above, and knowing that theobromine does not have methyl in position 1, it is inferred xanthine (X), or a monomethylxanthine (7X or 3X) or even derivatives of uric acid (3U, 7U), due to shorter retention time. This eliminates the possibility of 1-methylxanthine, 1-methyluric acid, and 1,3-dimethyluric acid, helping to identify the other peaks. Considering the assumed elution order, "peak 2" could be a 7-methylxanthine (7X) or 7-methyluric acid (7U). Peaks "3", "4", "5" and "6" could be: 3-methyluric acid (3U), 3-methylxanthine (3X),

Table 1.
Methylxanthines generated under experimental reactions of caffeine monitored by HPLC-DAD.

Experiment[a]	Peak	RT (min)	UV max. (nm)	Id. proposal	S (%)[b]	Y (%)
1	4	5.0	215	3-methylxanthine	1.2	1.2
2	4	4.9	216	3-methylxanthine	0.9	0.8
	7	5.8	203, 272	Theobromine	0.2	0.2
	9	7.7	202, 269	Paraxanthine[c]	0.9	0.8
	10	8.2	194, 270	Methylxanthine[c]	[] < 0.1	< 0.1
	14	13.6	203, 272	Caffeine		
3	2	3.6	199, 276	7-methylxanthine	0.5	0.5
	4	5.0	215	3-methylxanthine	3.0	3.0
	6	5.5	215	1-methylxanthine	0.5	0.5
	12	11.4	207, 276	Theophylline	0.8	0.8
4	7	5.7	204, 253	Theobromine	2.3	0.7
	9	7.7	202, 269	Paraxanthine[c]	2.7	0.8
	14	13.5	204, 272	Caffeine		
5	9	7.7	201, 269	Paraxanthine[c]	20.0	1.0
	14	13.5	204, 272	Caffeine		
6	2	3.8	200, 222	7-methylxanthine	< 0.1	< 0.1
	4	4.6	190, 269	3-methylxanthine	< 0.1	< 0.1
	7	5.7	200, 269	Theobromine	0.5	0.4
	9	7.7	201, 269	Paraxanthine[c]	0.9	0.7
	14	13.5	205, 272	Caffeine		
	15	21.7	190, 270	Methylxanthine[c]	0.2	0.1
7	2	3.8	200, 222	7-methylxanthine	0.1	< 0.1
	4	4.6	190, 269	3-methylxanthine	< 0.1	< 0.1
	7	5.7	200, 232, 269	Theobromine	0.3	0.3
	9	7.7	200, 269	Paraxanthine[c]	0.6	0.5
	14	13.5	205, 272	Caffeine		
	15	21.7	190, 270	Methylxanthine[c]	0.1	0.1
8	7	6.0	204, 272	Theobromine	0.6	0.6
	10	8.2	202, 272	Paraxanthine[c]	0.1	0.1
	14	14.7	204, 272	Caffeine		
9	4	4.7	– [d]	3-methylxanthine	< 0.1	< 0.1
	7	6.1	203, 271	Theobromine	0.6	0.5
	10	8.3	– [d]	Paraxanthine[c]	0.4	0.3
	14	14.9	204, 271	Caffeine		
10	7	6.0	201, 272	Theobromine	0.7	0.2
	10	8.2	199, 269	Paraxanthine[c]	0.7	0.2
	12	11.3	207, 275	Theophylline[c]	< 0.1	< 0.1
	13	12.3	207, 277	Theophylline[c]	< 0.1	< 0.1
	14	14.5	200, 270	Caffeine	0.1	< 0.1
	15	25.7	206, 274	Methylxanthine[c]	< 0.1	< 0.1

[a] Experimental conditions: 1. 5 mM H_2O_2 (250 μL 3.5% sol.), 1.5 mM Fe^{2+} (41 mg Fe_2SO_4), pH 3; 2. 5 mM H_2O_2 (250 μL 3.5% sol.), 0.3 mM Fe^{2+} (9 mg Fe_2SO_4), pH 3; 3. 30 mM H_2O_2 (50 μL 35% sol.), 9 mM Fe^{2+} (250 mg Fe_2SO_4), pH3; 4. 2 mM AA (35 mg), pH 7; 5. 2 mM AA (35 mg), pH 3; 6. 2 mM H_2O_2 (100 μL 3.5% sol.), 0.6 mM Fe^{2+}(16.7 mg Fe_2SO_4), pH 7, 2 mM AA (35 mg), methanol 10 % (10 mL); 7. 2 mM H_2O_2 (100 μL 3.5% sol.), 0.6 mM Fe^{2+} (16 mg Fe_2SO_4), pH 3, 2 mM AA (35 mg), methanol 10 % (10 mL); 8. 2 mM H_2O_2 (100 μL 3.5% sol.), 0.6 mM Fe^{2+} (16 mg Fe_2SO_4), pH 7, 2 mM AA, ethanol 10 % (10 mL); 9. - 2 mM H_2O_2 (100 μL 3.5% sol.), 0.6 mM Fe^{2+} (16 mg Fe_2SO_4), pH 7, 2 mM AA, acetonitrile 10 % (10 mL); 10. 10 mM H_2O_2, 3 mM Fe^{2+}, pH 7. [b] S and Y refers selectivity and yield. [c] Spectra similarity. [d] Baseline.

or 3,7-dimethyluric acid (37U), 1-methyluric acid (1U), 1-methylxanthine (1X). Peaks "8", "9", "10", "11" and "12" show greater retention than theobromine, which may correspond to paraxanthine (1,7-dimethylxanthine – 17X), theophylline (1,3-dimethylxanthine – 13X) and some other component such as 1,3-dimethyluric acid (13U), 1,7-dimethyluric acid (17U) and 1,3,7-trimethyluric acid (137U).[37,44] Thus, the suggested possibilities for peaks "8", "9", "10", "11" and "12" would be: 13U, 17X, 17U, 13X, and 137X, although some peaks did not show a clear UV spectrum with the possibility of different analytes eluting at the same RT.

The UV spectra of the caffeine transformation products were evaluated from the data generated by the DAD. The reference spectra in the UV region of xanthines and uric acid derivatives (1,7-dimethyl uric acid, 3,7-dimethyl uric acid, 1,3,7-trimethyl uric acid) were consulted in a database platform NIST (National Institute of Standards and Technology) showing maximums absorption at approximately 230 and 270 nm to xanthines and 220 and 300 nm to uric acid derivatives.[45] By evaluating these UV spectra (Table 1) and considering the probable elution order of the analytes, the following attribution were inferred in these samples: theobromine (5.7 minutes) with a yield of up to 0.7%; "peak 8" at 6.9 minutes with UV bands common to xanthines and uric acids; "peak 9" assigned to paraxanthine (7.7 minutes) with a yield of up to 1.0%; elution region of acid derivatives between 8.7 to 9.6 min (UV band at 291 nm); "peak 12" assigned to theophylline (11.4 minutes) with a yield of up to 0.8%. It was evidenced that the Fenton condition favors the formation of "peak 8" at 6.9 minutes. The formation of the "peak 9" occurs under both Fenton and AA conditions. Fenton condition with the addition of AA and methanol favors the formation of uric acid derivative (8.7 and 9.0 minutes). Caffeine exposed only in AA favors the formation of uric acid derivatives (about 9 minutes, UV max 290 nm), while Fenton in high concentration promoted "peak 12" (11.4 minutes).

In order to improve the spectra and elucidate the peaks of caffeine transformation products, experiment 10 was carried out using a high concentration of caffeine (20.0 $g\,L^{-1}$) submitted to the Fenton process (summarized in Table S3). Figure 1 depicts the chromatogram which presents the peaks of caffeine transformation products in elution order. By evaluating the UV spectra obtained, the formation of monomethylxanthines, dimethylxanthines, and uric acid derivatives are suggested and confirm the observations (Figure S1). The spectra similarity of some analytes eluted at 8.2; 11.3; 12.3 and 25.7 minutes for xanthines and about 9.5 minutes for 1,3,7-trimethyluric acid.

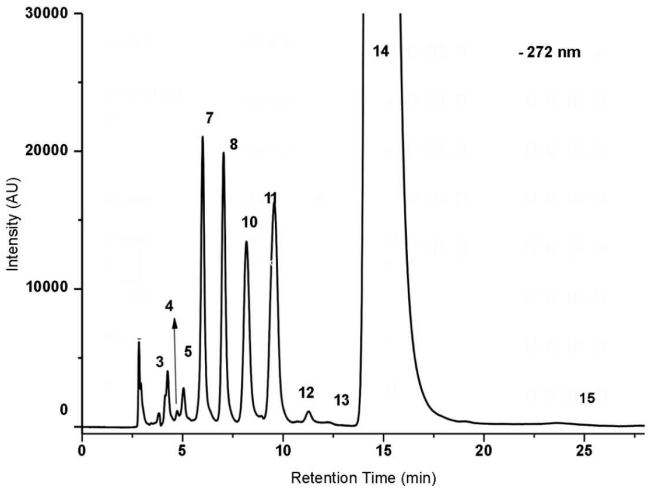
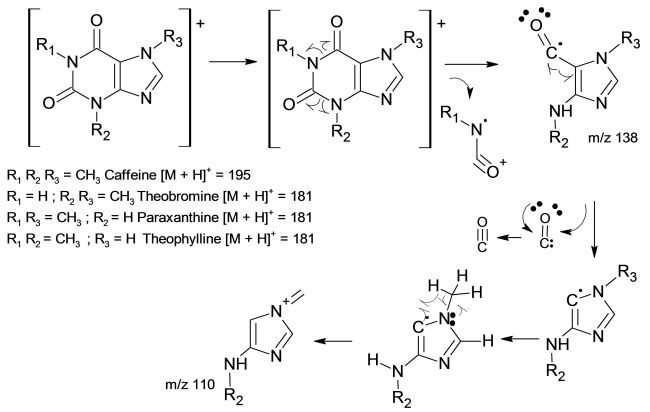


Figure 1. The HPLC chromatogram shows the presence of different caffeine transformation products at 272 nm in experimental 10.

Evaluation of caffeine transformation products by MS

To overcome difficulties in elution and comparison of analytes based on RT, it is used the association of liquid chromatography with spectrometry techniques. The MS in sequence allows to evaluate the fragmentation in the molecules by identifying the product ions.[44,46,47] The ions generated from the breakdown of caffeine and metabolites indicated the retro Diels-Alder fragmentation as dominant with ring contraction reaction by loss of methyl isocyanate (57 Da) or isocyanic acid (43 Da), with transitions from m/z 195 to m/z 138 in caffeine, m/z 181 to m/z 138 in theobromine and m/z 181 to m/z 124 in theophylline (Scheme 2).

In addition to the experiments described, other 2 tests were carried out under conditions that favored the formation of "Peak 10" at close to 8.2 minutes, and of "Peak 11" at about 9.2 minutes, in a region of the chromatogram that presented UV spectra similar to xanthines and uric acid derivatives, to evaluate the m/z signals (Figure S2). "Peak 10" could be 1,7-dimethyluric acid, molecular formula $C_7H_8N_4O_3$ with exact mass 196.0596 $g\,mol^{-1}$, or paraxanthine, molecular formula $C_7H_8N_4O_2$ with exact mass 180.0647 $g\,mol^{-1}$, which would generate molecular ion $[M+H]^+$ 197.0675 $g\,mol^{-1}$ or 181.0725 $g\,mol^{-1}$, respectively. The "Peak 11" could be theophylline or 1,3,7-trimethyluric acid, molecular formula $C_8H_{10}N_4O_3$ with exact mass 210.0753 $g\,mol^{-1}$, which would generate molecular ion $[M+H]^+$ 211,0831 $g\,mol^{-1}$.[48]



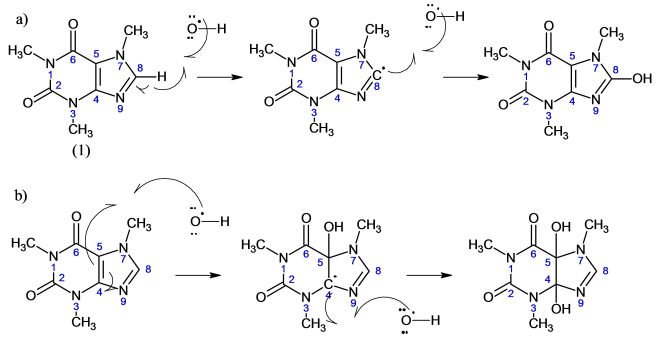
Scheme 2. Representation of molecular ions and fragmentation proposals for methylxanthines.

In sample 1 (Fenton + AA + Methanol) fraction 1, a peak at 1.6 minutes, signal m/z 181.0466 and its UV spectrum with maximum absorption at 267 nm were verified, being attributed to the dimethylxanthines. In sample 2 (Fenton + AA + Acetonitrile) fraction 1, a peak was observed at 1.7 minutes with a base ion of m/z 239.1475 which was not attributed to any of the expected products, and the ion m/z 181.0371 which may correspond to theophylline, at a lower intensity verified in the total ion chromatogram (TIC). In fraction 2 of samples 1 and 2, was evidenced small signal in approximately 1.5 minutes, showing a UV spectrum band at 205 nm and mass spectrum with base ion m/z 181 compatible with dimethylxanthines. Possibly it is paraxanthine because it has a longer retention time than theobromine and lower than theophylline (Table S4). The fractionation of analytes performed in the HPLC-DAD system can promote an excessive dilution of the sample, hindering the following study by mass spectrometry.

These results suggest the formation of paraxanthine and theophylline in the chromatographic peaks close to 1.5 and 1.7 minutes, respectively. Thus, it is inferred that Fenton reactions plus ascorbic acid preferentially promote N-demethylation reactions in relation to C-hydroxylation. Can be established N-demethylation reaction occurs in order of 0.8% yield for theobromine, paraxanthine, and theophylline formation, corresponding to about 8×10^{-4} mmol formed from 1 mmol caffeine, depending on the reaction conditions.

Reaction mechanisms proposals

According to literature reports, the main oxidation product of caffeine is 137U, indicating that the C8 hydroxylation reaction occurs to a greater extent, and the formation of methylxanthines is cited just in a few studies.[33,36,37,49] It is also proven that C8 oxidation predominates over N-demethylation reactions in caffeine degradation by the action of the *flavin monooxygenase* enzyme.[38] Considering the probable degradation products of caffeine, we present in Scheme 3 the proposals of the reaction mechanism for the formation of 1,3,7-trimethyluric acid (a) mostly, and also a minor product that can also be formed by radical attack on the double bond C4=C5 with m/z 228 (b).

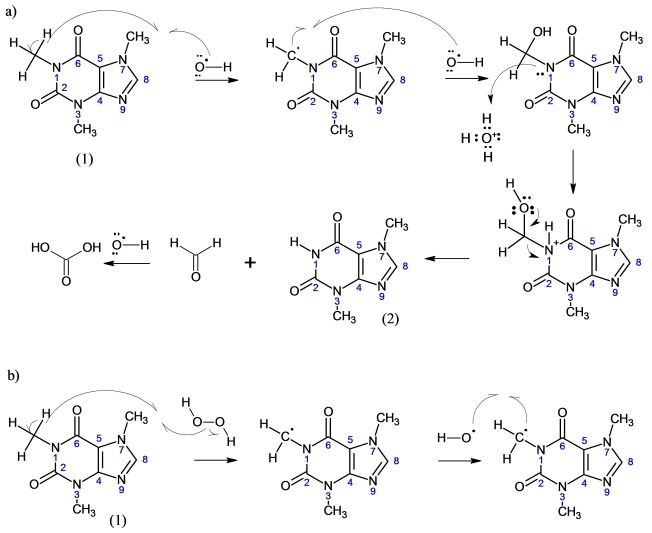


Scheme 3. Proposal of a reaction mechanism for the oxidation of caffeine, mediated by HO• radicals, with the formation of 1,3,7-trimethyluric acid (a) mostly, and minor product in the C4 = C5 double bond (b).

This work proved the formation of dimethylxanthines from caffeine under Fenton conditions and with AA, via the HO• radical with a caffeine/HO• molar ratio of 1:2, according to the reaction mechanism proposed in Scheme 4.a, and pointing by Chen[39] that N-dealkylation reactions proceed with the hydroxylation of $C_\alpha H$ and the subsequent breaking of the C–N bond to release formaldehyde. First, methyl oxidation occurs by a radical mechanism. In a second step, with the oxidized methyl, the lone pairs of electrons on the oxygen assist in the detachment of the methyl by heterolytic cleavage, in which the breaking of the bond is favored by acidic means, with the release of formaldehyde. Thus, 2 mols of HO• are consumed to promote the N-demethylation of caffeine. Formaldehyde continues to be oxidized by HO• radicals generating formic acid and later carbonic acid, which can decompose into H_2O and CO_2.

Theobromine was also formed in a solution containing AA and with your association plus ferrous ions. AA promotes the formation of reactive species such as H_2O_2, which can act in the conversion of caffeine to theobromine. In this case, the reaction of N-demethylation mediated by H_2O_2 presents a molar ratio of caffeine/H_2O_2 1:1, whose initial steps proposed are depicted in Scheme 4.b.

It is noteworthy that all methyl groups can be oxidized similarly, giving the N-demethylation reaction at positions 1, 3, and 7 to generate theobromine, paraxanthine and theophylline, respectively. Likewise, C-hydroxylation reactions can occur to form mono- and dimethylated uric acid derivatives, in addition to smaller molecules such as dimethylparabanic acid, justifying a great number of caffeine transformation products. The cleavage of the C–N bond of amines leads to a major advance in organic chemistry that affects other major areas such as biochemistry, medicinal chemistry, and medicine.



Scheme 4. Representation of the N-demethylation reaction mechanism of caffeine, mediated by HO• radical, for theobromine (2) formation (a); and mediated by H_2O_2 (b).

Conclusions

The 14 caffeine transformation products formed under oxidative reactions were determined by HPLC. The formation of the dimethylxanthines theobromine, paraxanthine, and theophylline was confirmed by the verification of ion m/z 181, corroborating the RT and UV spectra inferences. Based on the results, the Fenton process plus ascorbic acid favors N-demethylation reactions by a radical mechanism with formation of theobromine, paraxanthine, and theophylline with about 8×10^{-4} mmol formed from 1 mmol caffeine. The reaction mechanisms of N-demethylation and C-hydroxylation were proposed, via the $HO^{\bullet}$ radical, with a caffeine/$HO^{\bullet}$ molar ratio of 1:2 and also H_2O_2-mediated with a molar ratio of 1:1. Due to the importance of the N-demethylation reaction, which covers several areas, added to the benefits provided by catalysis, the work demonstrates a potential opportunity of a new route to further investigations into N-demethylation reactions.

Experimental Section

Catalytic experiments

The experiments were carried out in a 250 mL glass reactor, under magnetic stirring at 25 °C. To the reactor were added 100 mL of 1 mM aqueous caffeine solution (20 mg), the adequate dose of H_2O_2 (50–250 μL), and catalyst load ($FeSO_4.7\ H_2O$) or 35 mg *L*-ascorbic acid (AA). Alternatively, ethanol, methanol, and acetonitrile were used with the Fenton system and with AA. After adding the reactants, aliquots were collected for 24 h to monitor the reaction.

Determination of caffeine transformation products by HPLC

A chromatographic system constituted by an HPLC coupled to a DAD detector (Shimadzu), a C18 column (250×4.6 mm) with 5 micrometers of particle size (Supelco), a mixture of water and acetonitrile (9:1, v/v) as mobile phase and a flow rate of 1.0 ml min^{-1} during 30 min of running. To perform the HPLC analysis, aliquots were collected in eppendorf flasks containing excess Na_2SO_3 to stop the reaction, then filtered through a 0.45 or 0.20 μm filter membrane into a vial and injected. The concentration of caffeine was determined using the calibration curve of caffeine 2.0×10^{-3} to 1.0×10^{-2} g L^{-1}, coefficient of determination (R^2) of 0.9996. The concentration of caffeine transformation products was determined using the calibration curve of theobromine 1.1×10^{-3} to 5.2×10^{-3} g L^{-1}, R^2 of 0.9998.

In order to compare analytes eluted under different chromatographic conditions, the k value was determined using Equation (1), establishing the interaction of the analyte with stationary and mobile phases, and indicating how long a compound can be retained by the stationary phase, which depends on RT of the analyte and the time taken for the mobile phase to pass through the column (t_0), obtained by Equations (2–3).

$$k = {}^{(RT - To)}/_{To} \quad (1)$$

$$V_0 = \pi \cdot r^2 \cdot length \cdot porous \quad (2)$$

$$t_0 = {}^{V_0}/_{flow} \quad (3)$$

where *flow* is the flow rate of the chromatographic method, *r*, *length* and *porous* are the chromatography column features.

The conversion of caffeine (X_{caf}) was calculated according to Equation (4).

$$X_{caf} = {}^{(mol_{caf,i} - mol_{caf,f})}/_{mol_{caf,i}} \quad (4)$$

where mol_i and mol_f refer to mole of caffeine at the beginning and at the end of the reaction, respectively.

The selectivity to each product p from caffeine (S_p) was calculated from the ratio between the mole of the product (mol_p) and the variation in the number of moles of caffeine, as detailed in Equation (5). The yield of each product (Y_p) was calculated from the ratio between the mole of product and the number of moles of caffeine at the beginning of the reaction, as given in Equation (6).

$$S_p = {}^{mol_p}/_{(mol_{caf,i} - mol_{caf,f})} \quad (5)$$

$$Y_p = {}^{mol\ _p}/_{mol_{caf,i}} \quad (6)$$

Methylxanthines elucidation by MS

The HPLC-QTOF consists of a Shimadzu chromatographic series 20 equipment, DAD coupled to a triple quadrupole/TOF hybrid mass detector with ESI Bruker ionizer in positive mode: end plate offset 500 V, capillary source 5500 V, N_2 nebulizer gas pressure at 4 bar, drying gas flow rate of 10 L.min^{-1} and temperature of 200 °C, with a collision energy of 25 eV, using Compass software. The chromatographic method used a C18 column (150×2.1 mm) and a mobile phase consisting of 0.1% (v/v) formic acid and acetonitrile (9:1, v/v), with a flow rate of 0.5 mL min^{-1}, at 50 °C, monitored at 272 nm for 10 minutes.

A mixed standard solution of caffeine 0.004 g L^{-1} (2.06×10^{-2} mM) and theobromine 0.002 g L^{-1} (1.11×10^{-2} mM) was used for quantification. Samples were prepared using 0.2 g L^{-1} caffeine (1.03 mM) solution over 24 h using 1) 10% (v/v) methanol and 2) 10% (v/v) acetonitrile as solvents (keeping rest of operating conditions equal: 2 mM H_2O_2, 0.6 mM Fe^{2+}, 1 mM AA, neutral pH and room temperature). After 24 h of reaction time under agitation, aliquots were removed from the reactor and treated to eliminate interferences: pH check (approximately 3.4), alkalinization step (pH close to 10) with the addition of 3 drops of 1 M NaOH, centrifugation at 3000 rpm for 10 minutes, collection of the supernatant and dilution 25 times, in order to obtain the theoretical concentration of 0.008 g L^{-1} of caffeine. Then was conducted an isolation for the formed analytes (see supporting information), with post-DAD fractional collection. Subsequently, each fraction was injected into the HPLC-QTOF.

Supporting Information Summary

The Supporting Information includes Figure S1, Chromatogram of caffeine degradation by HPLC- DAD; Figure S2, Chromato-

gram obtained by HPLC-MS/TOF; Table S1, Reactions conditions and caffeine transformation products; Table S2, Chromatography peak verified in experiments; Table S3, Peaks verified on Fenton condition using 20.0 $g\,L^{-1}$ of caffeine; Evaluation of caffeine transformation products by mass spectrometry; Table S4, UV spectra and mass/charge ratio for peaks detected by HPLC-QTOF.

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Conflict of Interests

The authors declare no conflict of interest.

Data Availability Statement

Data sharing is not applicable to this article as no new data were created or analyzed in this study.

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