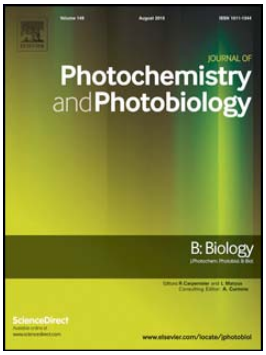

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PII: S1011-1344(16)30160-9
DOI: doi: 10.1016/j.jphotobiol.2016.08.042
Reference: JPB 10547

To appear in:

Received date: 11 March 2016
Revised date: 23 August 2016
Accepted date: 25 August 2016

Please cite this article as: R.S. Scurachio, F. Mattiucci, W.G. Santos, L.H. Skibsted, D.R. Cardoso, Caffeine metabolites not caffeine protect against riboflavin photosensitized oxidative damage related to skin and eye health, (2016), doi: 10.1016/j.jphotobiol.2016.08.042

Caffeine metabolites not caffeine protect against riboflavin photosensitized oxidative damage related to skin and eye health

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ABSTRACT

Caffeine metabolites were found to bind riboflavin with dissociation constant in the millimolar region by an exothermic process with positive entropy of reaction, which was found by ^{1}H NMR and fluorescence spectroscopy to occur predominantly by hydrogen bonding with water being released from riboflavin solvation shell upon caffeine metabolite binding to riboflavin. The caffeine metabolites 1-methyl uric acid and 1,7-dimethyl uric acid were shown by transient absorption laser flash photolysis to be efficient as quenchers of triplet riboflavin with second-order rate constant of 1.4 10^{8} L $mol^{-1}s^{-1}$ and 1.0 10^{8} L $mol^{-1}s^{-1}$, respectively, in aqueous solution of pH 6.4 at 25 ^{o}C and more efficient than the other caffeine metabolite 1,7-dimethyl xanthine with second-order rate constant of 4.2 10^{7} L $mol^{-1}s^{-1}$. Caffeine was in contrast found to be non-reactive towards triplet riboflavin. Caffeine metabolites rather than caffeine seem accordingly important for the observed protective effect against cutaneous melanoma identified for drinkers of regular but not of decaffeinated coffee. The caffeine metabolites, but not caffeine, were by time resolved single photon counting found to quench singlet excited riboflavin through exothermic formation of ground-state precursor complexes indicating importance of hydrogen bounding through keto-enol tautomer's for protection of oxidizable substrates and sensitive structures against riboflavin photosensitization.

Keywords: caffeine metabolites, riboflavin, photosensitization, skin and eye health

1. Introduction

Recently epidemiological evidence has been presented indicating that higher coffee intake is associated with a decrease in risk of cutaneous melanoma (E. Loftfield et al., 2015). Melanoma is the leading cause of skin cancer with exposure to UV components of sunlight as the important exogenous risk factor (B. K. Armstrong and A. Kricker, 2001). Accordingly, constituents in coffee seems to protect the skin against UV light, and since decaffeinated coffee was not found to be protective, caffeine seems to have been identified as the protective constituent in coffee (E. Loftfield et al., 2015).

Both UV-A (315-400 nm) and UV-B (290-350 nm) are part of the radiation that reaches the earth from the sun and are both associated with sunlight damage to skin and eye (E. Loftfield et al., 2015 and H. R. Taylor et al., 1992). UV-B is considered a main cause of sunburn, photo-ageing and skin cancer as UV-B is absorbed directly by proteins and DNA, but UV-A is of higher intensity and may penetrate deeper into tissue (A. C. Chen, D. L. Damian and G. M. Halliday, 2014). UV-A is absorbed by other compounds in skin and eye such as melanin, porphyrins and flavins, which may act as photosensitizers (A. C. Chen, D. L. Damian and G. M. Halliday, 2014; G. T. Wondrak, M. K. Jacobson; E. L. Jacobson, 2006). Riboflavin, vitamin B2, is an effective photosensitizer widely found in tissue and food leading to oxidative damage of proteins, other vitamins, sterols and lipids upon absorption of UV-A and visible light (D. R. Cardoso, S. H. Libardi and L. H. Skibsted, 2012).

Caffeine is only absorbing a minor fraction of the UV-A component of daylight and a simple filter effect of caffeine, as present in tissue does not explain the protective affect seen for coffee against the development of cutaneous melanoma. Caffeine has also been found, when topically applied as eyes drops, to protect against cataractogenesis that is known at least partially to be induced by light exposure (E. Loftfield et al., 2015 and M. Kronschläger et al., 2013).

Caffeine, not being protective simply by absorbing damaging light shielding sensitive tissue components, may accordingly be protective against cataract and cutaneous melanoma through other mechanisms. Caffeine or caffeine metabolites may, as it is known for uric acid, be quenchers of photosensitizers like the bioactive forms of riboflavin present in skin tissue and eye liquids. Riboflavin becomes

strongly oxidizing upon light absorption in a relatively long-lived triplet state, and efficient quenchers of this state may be strongly protective against photosensitized oxidative damage of tissues (D. R. Cardoso, S. H. Libardi and L. H. Skibsted, 2012).

Aiming to provide a better understand on the mechanism behind the photoprotection of sensitive structures in eyes and skin against UV-A photoinduced damage sensitized by flavins, herein, we report result of kinetic studies of the deactivation of triplet-excited riboflavin by caffeine and its biological relevant metabolites, Scheme 1.

2. Materials and Methods

2.1 Chemicals

Caffeine (1,3,7-trimethyl xanthine), 1,7-dimethyl uric acid, 1,7-dimethyl xanthine, 1-methyl uric acid, and riboflavin, and tetrabutylammonium hexafluorphosphate salt were purchased from Sigma-Aldrich (Steinheim, Germany). Water was purified (18 MΩ·cm^{-1}) by means of a Milli-Q purification system from Millipore (Billerica, MA, USA).

2.2 Transient Absorption Laser Flash Photolysis

Laser flash photolysis (LFP) experiments were carried out with LFP-112ns laser flash photolysis spectrometer from Luzchem (Ottawa, Canada) using the third harmonic (355 nm) of a pulsed Q-switched Nd:YAG laser (Brilliant-B, LesUlis, France) attenuated to 10 mJ·cm^{-2} as the excitation source with 8 ns of pulse duration. The signal from the photomultiplier detection system was captured by a Tektronix TDS 2012 digitizer (Beaverton, OR, USA). The FFP-112ns and the digitizer were connected to a personal computer via General Purpose Instrumentation Bus (GPIB) and serial interfaces controlling all the experimental parameters and providing suitable processing and data storage capabilities using a proprietary software package developed in LabView environment and compiled as a stand-alone application (Luzchem, Ottawa, Canada). Each kinetic trace was averaged 16 times, and observed rate constants were determined by parameter fitting to exponential decay functions. All measurements were made with aqueous phosphate buffer solutions pH 6.4

(ionic strength = 0.2 mol L^{-1}), thermostatted at 25.0 ± 0.5 °C, and purged with high-purity N_2 for at least 30 min before the experiment.

2.3 Time Resolved Single Photon Counting

Time-resolved fluorescence were measured by time-correlated single-photon counting using an picosecond spectrometer equipped with Glan-Laser polarizers (Newport, Irvine, CA), a Peltier-cooled PMTMCP from Hamamatsu model R3809U-50 (Hamamatsu, Japan) as the photon detector, and Tennelec-Oxford (Oxford, Abingdon, UK) counting electronics. The light pulse was provided by frequency doubling the 200 fs laser pulse of a Mira 900 Ti-Sapphire laser pumped by a Verdi 5 W coherent laser (Santa Clara, CA), and the pulse frequency was reduced to 800 kHz using a Conoptics pulse picker. The fluorescence decays were taken at the magic angle (λ_{exc} = 400 nm) and analyzed by a re-convolution procedure with instrument response function with exponential decay models, and the goodness of the fit was evaluated by the statistical parameters $\chi 2$. The fluorescence was measured at 25.0 ± 0.1 ^{o}C in phosphate buffer solutions with pH = 6.4 and 0.2 mol L^{-1} of ionic strength.

2.4 Steady-state Fluorescence Spectroscopy

Fluorescence measurements were carried out using a Hitachi F-7000 Fluorescence Spectrometer (Hitachi High-Tech, Tokyo, Japan). The samples were excited in 1.0 cm x 1.0 cm fluorescence cuvettes from Hellma (Mulheim, Germany) at 440 nm. Emission spectra from 480 nm to 600 nm were recorded using a 2.0 nm band-pass for the excitation monochromator and a 4.0 nm band-pass for the emission monochromator. The recorded spectra were corrected for instrument response. The fluorescence was measured at 25.0 ± 0.2 ^{o}C in phosphate buffer solutions with pH = 6.4 and 0.2 mol L^{-1} of ionic strength.

2.5 Cyclic Voltammetry

Cyclic voltammetry of caffeine and its metabolites (1 ×10^{-3} mol L^{-1}) dissolved in nitrogen-purged phosphate buffer pH 6.4 containing 0.05 mol L^{-1} of tetrabutylammonium hexafluorphosphate were carried out using a PAR 264A Potentiostat (Princeton Applied Research, Princeton, NJ, USA) equipped with a glass carbon as the working electrode, a platinum wire as the auxiliary electrode, and employing an Ag/AgCl reference electrode. The scan rate was 100 mV s^{-1}, and the electrochemical cell was thermostated at 25.0 ± 0.2 ^{o}C.

2.6 ^{1}H NMR spectroscopy

^{1}H NMR spectra were acquired on an 11.7 T Agilent DD2 500 MHz NMR Spectrometer. A standard solvent suppression pulse sequence presat was used to saturate the residual water signal, using a 90° pulse width of 7.7 µs, a SW of 16.03 ppm, 4K scans, 32K total acquired complex points, and a relaxation delay of 2 s. The data were phase corrected, baseline corrected and the spectra was reference to TMSP-d4 (0 ppm) using VnmrJ 4.0 software (Agilent Technologies, USA).

3. Results and Discussion

Riboflavin, vitamin B2, and its biological active derivatives flavin adenine dinucleotide (FAD) and flavin mononucleotide (FMN) undergo intersystem crossing with a high quantum yield, ?? = 0.67, yielding a long-lived reactive triplet-excited state ($E^{o'}$ ~ +1.77 V vs. NHE, ?? = 15 ??s in aqueous solution) upon exposure to blue light or UV-A (D. R. Cardoso, S. H. Libardi and L. H. Skibsted, 2012; C. B. Martin, M. Tsao, C. M. Hadad and M. S. Platz, 2002; P. F. Heelis, 1982). Figure 1 shows the UV-vis absorption spectra recorded for riboflavin, caffeine and caffeine metabolites in aqueous phosphate buffer solution with pH 6.4. As may be seen from Figure 1, the absorption spectrum of riboflavin in aqueous solution consists of four peaks centered at 446, 375, 265, and 220 nm, and exposure to UV-A region was appropriate for excitation without any interference from caffeine and caffeine metabolites.

Blue light excitation, 446 nm, of a nitrogen saturated aqueous phosphate buffer pH 6.4 solution of riboflavin resulted a fluorescence emission with a maximum at 517 nm, Figure 2. This fluorescence was not quenched by caffeine, however, caffeine metabolites reduced the emission intensity linearly with increasing concentration of the metabolites and accordingly to Stern-Volmer equation (eq.1), Figure 2.

$$\frac{I_o}{I} = 1 + k_q \times \tau_o \times [\text{metabolite}]$$

eq.(1)

The lifetime for the singlet-excited riboflavin as monitored by time-resolved single photon counting was, however, not altered by the presence of caffeine or its metabolites (data not shown). The lifetime for singlet-excited riboflavin in the present conditions at 25 ^{o}C was found to be $??_{o}$ = 4.51 ± 0.02 ns and it is in agreement with previous reports (R. S. Scurachio et al., 2015). From the fitting of Stern-Volmer equation (eq.1) to the plot of Figure 2, the Stern-Volmer constant for riboflavin fluorescence emission quenching was found and is reported in Table 1. The steady-state fluorescence quenching as observed in Figure 2 and the lack of effect of increasing concentration of caffeine metabolites in the time constant for fluorescence decay is in agreement with a static quenching of the singlet-excited state of riboflavin by caffeine metabolites, despite having $\Delta G^{o}_{ET,singlet}<0$ according to Rhem-Weller equation (Table 2). Formation of a ground-state precursor complex was further investigated by analyzing the steady-state fluorescence quenching of singlet-excite riboflavin with increasing concentration of caffeine metabolites at varying temperatures from 25 ^{o}C to 35 ^{o}C. The formation of the precursor ground-state complex may be described by the following reaction:

$$\text{n metabolite} + \text{riboflavin} \rightleftharpoons [(\text{metabolite})\text{n}...\text{riboflavin}] \qquad \text{eq.(2)}$$

The equilibrium constant, K_a, and the number of quenchers binding (n) to FMN was calculated according to (M. Weert, 2010):

$$\log\frac{I_o - I}{I} = logK_a + \text{n}\log[metabolite] \qquad \text{eq.(3)}$$

The experimental values, collected in Table 1, determined for the dissociation equilibrium constant ($K_d = 1/K_a$) and n for three different temperatures were calculated by fitting eq.3 to the experimental data and the thermodynamic parameters for the ground-state precursor complex formation were analyzed accordingly to the Van't Hoff equation for a 1:1 stoichiometry. The thermodynamic parameters for the ground-state precursor complex was were found to have values of ΔH° = -39 ± 5 kJ mol^{-1} and ΔS° = 9 ± 2 J mol^{-1} K^{-1} for 1-methyl uric acid, ΔH° = -44 ± 8 kJ mol^{-1} and ΔS° = 12 ± 1 J mol^{-1} K^{-1} for 1,7-dimethyl uric acid, and ΔH° = -16 ± 3 kJ mol^{-1} and ΔS° = 6 ± 1 J mol^{-1} K^{-1} for 1,7-dimethyl xanthine. The binding of caffeine metabolites to riboflavin is accordingly an exothermic process suggested occurring by hydrogen bonding and the positive entropy of

reaction an indication of water released from riboflavin solvation shell upon caffeine metabolite binding. Binding of caffeine metabolites to riboflavin does not resulted in observable changes in the flavin UV-vis absorption spectra. Aiming to provide a better evidence of the ground-state complex formation, ^{1}H NMR experiments were conduct for the mixture of riboflavin and 1-methjyl-uric acid and for the individual compounds. A noticeable upfield shift of $\Delta\delta_{H6}$ = -0.023 ± 0.002 and $\Delta\delta_{H9}$ = -0.010 ± 0.001 for the riboflavin aromatic hydrogens H6 and H9 is seen, Figure 3A, in the presence of 6 equivalents of 1-methyl-uric acid. Also a less perceptible upfield shift of $\Delta\delta_{H11}$ = -0.009 ± 0.001 and $\Delta\delta_{H12}$ = -0.008 ± 0.001 for the hydrogen's H11 and H12 of the ribityl side chain is observed, Figure 3B and 3C. This change in the chemical shift values of the ^{1}H NMR signals for nonexchangeable riboflavin hydrogen's in the presence of 6 equivalents of 1-methyl-uric acid indicates the formation of a weak π-stacking ground-state complex. On the other hand, as may be seen from Figure 3D, a more pronounced downfield shift is observed for the nonexchangeable methyl protons of 1-methyl-uric acid ($\Delta\delta_{CH3}$ = +0.054 ± 0.003) when in the presence of riboflavin (riboflavin:1-methyl-uric acid 1:6 equivalents). The later downfield shift observed for the signal of the nonexchangeable hydrogens of 1-methyl-uric acid is evidence for the formation of ground-state complex by hydrogen bonding which seems to be the predominant interaction for the ground-state precursor complex formation.

As a result of the high quantum yield for intersystem crossing (Φ_{ISC} = 0.67) to the lowest energy triplet state of riboflavin (^{3}Rib), a highly reactive bi-radical is formed E° = 1.77 V; τ = 15 µs in water) upon UV-A or blue light-excitation (D. R. Cardoso, S. H. Libardi and L. H. Skibsted, 2012 and P. F. Heelis, 1982). Figure 4 displays the transient absorption spectra for the ^{3}Rib following a 355 nm laser pulse of 8 ns of duration and 10 mJ cm^{-2}. Photoexcitation of a mixture of caffeine metabolite and riboflavin lead instantaneously to formation of riboflavin neutral radical ($??_{max}$ = 540 nm), Figure 3B (H. Li, T. B. Melo and K. R. Naqvi, 2012). The triplet-triplet absorption band at 680 nm was used to monitor the decay of the reactive ^{3}Rib in the presence of increasing concentrations of caffeine metabolites (see Figure 5). Triplet riboflavin was found to shown a first-order exponential decay in aqueous phosphate buffer solution pH 6.4 according to eq.4 and the observed rate constant (k_{obs}) was determine for increasing concentration of metabolite.

$$\Delta A_t = \Delta A_{t=0} e^{-k_{obs}t}$$

eq.(4)

The first-order exponential decay for ^{3}Rib T-T absorption band was found to depend linearly on the metabolite concentration according to

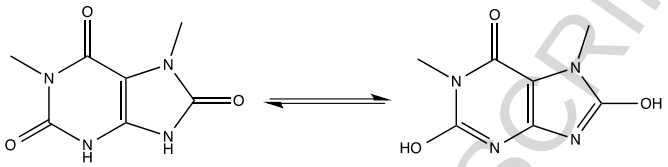
$$k_{obs} = k_o + k_2[metabolite] \quad \text{eq.(5)}$$

where k_o is the rate constant for the triplet-state self-decay and k_2 is the second-order rate constant for the ^{3}Rib reaction with the caffeine metabolites. The values for the bimolecular rate constant for ^{3}Rib deactivation by caffeine metabolites are collected in Table 2 together with the previously reported values for other purine bases (D. R. Cardoso et al.,2005). Caffeine was shown to be unreactive towards triplet riboflavin or to react with a rate constant < 10^4 L mol^{-1}s^{-1}. The specific rate constants obtained for triplet riboflavin deactivation by 1-methyl uric acid and 1,7-dimethyl uric acid are near diffusion control but slower for 1,7-dimethyl xanthine.

The second-order rate constant for quenching of triplet riboflavin is in Table 2 further compared to the same deactivation parameter for other purine derivatives. For xanthine, hypoxanthine and uric acid, the quenching of triplet riboflavin has been shown to be result of electron transfer from the purine derivative to triplet riboflavin rather than energy transfer as a physical process. Provided the structural similarities, see Scheme 1, and rate constants of similar magnitude, quenching of triplet riboflavin by the caffeine metabolites is also suggested to occur by electron transfer generating purine radicals as the first oxidation product. The caffeine metabolites are subsequently oxidized to stable oxidation products. The tendency to oxidation were measured by cyclic voltammetry (CV) and expressed as the half-wave potentials, E_{ox}, also found in Table 2, rather than standard reduction potentials for the radicals, since the oxidation process were found to be irreversible by CV.

From a comparison between the second-order rate constant and the oxidation potentials presented for purine, caffeine, caffeine metabolites, xanthine, hypoxanthine, and uric acid, it may be concluded that there is no simple relationship between driving force, $\Delta G^o_{ET,triplet}$, and rate constant for quenching of triplet riboflavin. Caffeine is not active as quencher in contrast to hypoxanthine despite

the comparable driving force, $\Delta G^{o}_{ET,triplet}$, as calculated from E_{ox}. Caffeine is like purine, also not active as a quencher, without the structural element capable of forming enols though tautomerization as for 1,7-dimethyl uric acid:



eq.(6)

The number of possible enol groups formed though tautomerization, is also listed in Table 2, seems to correlate with the rate constant of quenching. Accordingly, it is suggested that the electron transfer depends on the presence of such enol groups and their pK_{a1}-values and occurs as a proton-coupled electron transfer (PCET):

$$^{3}Rib^{*} + HO\text{-}R \longrightarrow [^{3}Rib^{*}\ldots H\ldots O\text{-}R]^{\ddagger} \longrightarrow {}^{2}Rib\text{-}H\bullet + \bullet O\text{-}R \qquad \text{eq.(7)}$$

Only hypoxanthine is reacting faster than expected accordingly to the classification using the number of potential enol groups. However, the 6-membered ring of hypoxanthine is fully conjugated making the tautomeric hydroxyl group more reactive. The rate constant of quenching of triplet riboflavin is accordingly not primary depending on thermodynamic factors like oxidation potentials but rather on kinetic factors.

After consumption of coffee, tea, or caffeine rich food, caffeine is extensively metabolized in the liver via N-3 demethylation by cytochrome P450 (CYP1A2) to 1,7-dimethyl xanthine and further by secondary metabolism of 1,7-dimethyl xanthine to yield 1-methyl xanthine, 1-methyl uric acid and 1,7-dimethyl uric acid (Thorn et al., 2012). The caffeine metabolites are then transported from the liver by the general blood system and are efficiently distributed across human tissues (tissue-to-blood partition coefficient of 1 for skin and highly perfused tissues) before being excreted in the urine (M. Kronschläger et al., 2014; T. Delahunty and D. Schoendorfer, 1998).

Partition of caffeine metabolites into skin and eye may accordingly leads to presence of efficient quenchers of triplet riboflavin in light exposed tissue (M. Kronschläger et al., 2014; T. Delahunty and D. Schoendorfer, 1998). The caffeine metabolites are quenching triplet excited riboflavin at rates which are competitive with quenching by peptides and proteins and especially with nucleotides and will protect these molecules against oxidative damage by excited state riboflavin, flavin mononucleotide (FMN), and flavin adenine dinucleotide (FAD) as biological active forms of the B2 vitamin. Flavin sensitized photodamage to skin and eye may otherwise led to pathological conditions such as skin photoaging and cancer and to age-related macular degeneration (AMD) and cataract in eyes (E. Loftfield et al., 2015; H. R. Taylor et al. 1992; A. C. Chen, D. L. Damian and G. M. Halliday, 2014; G. T. Wondrak, M. K. Jacobson and E. L. Jacobson, 2006; M. Kronschläger et al., 2014).

4. Conclusions

Flavins including riboflavin (vitamin B2) are widely distributed in skin and in the eye, and exposure of eyes and skin to UV-A and visible light leads to formation of triplet flavin to a varying degree. Endogenous chromophores in human skin and eyes seem to be serving as sensitizers involved in tissue photodamage. In this view, photochemically reactive excited states in skin and eyes should be targets for development of antioxidant therapies for inhibition of light-induced pathological conditions. Topical application of caffeine and consumption of caffeinated coffee has been shown by clinical intervention and epidemiologic studies to delay development of cataract and to reduce the risk of cutaneous melanoma induced by UV-B and UV-A light, respectively. Following ingestion of a single dose (3.5 g) of a coffee product providing 71 mg of caffeine, health subjects were shown to reach a plasma maximum concentration of 3.4 μM (T_{max} = 6.4 h) of 1,7-dimethyl-xanthine and 1.3 μM (T_{max} = 0.54 h) (S. Martínez-Lopez et al., 2014). Both caffeine and its metabolites were shown to display half-life of around 3 - 5 hours, being distributed in the body water, and present in all body tissues (A. Lelo, et al., 1986; Cappelletti et al., 2015; M. Kot and W. A. Daniel, 2008). Thus, caffeine is revealed to be completely absorbed following ingestion and rapidly metabolized mainly to 1,7-dimethyl-xanthine, which may be found in eyes fluid at relevant concentration. However, the mechanistic aspects for photoprotection following coffee consumption were unknown and assigned to the presence and absorption of the damaging light by caffeine. Our results clearly suggest that the photoprotective effect is related to the caffeine

metabolites which may act as chemical quenchers of triplet excited flavins protecting vital structures from light induced oxidation otherwise sensitized by flavins. The current dietary recommendations of an caffeine intake of up to 400 mg per day as recent published by the European Food Safety Authority (EFSA) to decrease the risk of pathological conditions such as age-related cognitive decline and Alzheimer's disease may also have a potential benefit against skin photoaging and cutaneous melanoma and also protect against development of age-related macular degeneration (AMD) and cataract.

5. Acknowledgements

This research is part of the bilateral Brazilian/Danish Food Science Research Program "BEAM - Bread and Meat for the Future" supported by FAPESP (Grant 2011/51555-7) and by the Danish Research Council for Strategic Research (Grant 11-116064). D.R.C. thanks the Brazilian National Research Council - CNPq for the research Grant (305385/2009-7).

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Table 1. Stern-Volmer constant for riboflavin fluorescence emission quenching (K_{SV}) by caffeine metabolites at different temperatures and dissociation constant (K_d) and number of binding quenchers (*n*) for formation of ground-state precursor complex at different temperatures in aqueous phosphate buffer pH 6.4

purine derivative	Temp. (°C)	K_{SV} (mol L^{-1})	K_d (mol L^{-1})	*n*
1,7-dimethyl xanthine	25	139 ± 5	$3.60 \pm 0.01\ 10^{-3}$	0.94
	30	131 ± 5	$3.30 \pm 0.01\ 10^{-3}$	0.97
	35	122 ± 5	$2.95 \pm 0.02\ 10^{-3}$	1.06
1,7-dimethyl uric acid	25	631 ± 10	$3.38 \pm 0.01\ 10^{-3}$	0.92
	30	446 ± 5	$2.73 \pm 0.01\ 10^{-3}$	0.97
	35	433 ± 9	$2.06 \pm 0.01\ 10^{-3}$	1.12
1-methyl uric acid	25	189 ± 9	$3.46 \pm 0.01\ 10^{-3}$	0.89
	30	185 ± 5	$3.03 \pm 0.01\ 10^{-3}$	0.99
	35	176 ± 4	$2.34 \pm 0.01\ 10^{-3}$	1.11

Table 2. Second-order rate constant, ^3k_q, for bimolecular quenching of triplet riboflavin by purine derivatives in aqueous phosphate buffer pH 6.4 at 25.0 ± 0.2 °C, half-wave oxidation potential (E_{ox}) for purine derivatives, free energy change for oxidation of purine derivative by triplet and singlet riboflavin calculated according to the Rhem-Weller equation ($\Delta G^o_{ET,singlet}$ and $\Delta G^o_{ET,triplet}$), acid dissociation constant (pK_{a1}) for purine derivative, and number of possible tautomeric enol groups in the purine derivative (N)

purine derivative	^3k_q ($L\ mol^{-1}\ s^{-1}$)	E_{ox} (V *vs* NHE)	$\Delta G^o_{ET,singlet}$ ($kJ\ mol^{-1}$)[b]	$\Delta G^o_{ET,triplet}$ ($kJ\ mol^{-1}$)[b]	pK_{a1}[a,c]	N
purine	not reactive	> 2	-18	> 0	-	0
caffeine	not reactive	1.7	-47	-16	14	0
1,7-dimethylxanthine	$4.2 \pm 0.3\ 10^7$	1.2	-95	-64	8.8	1
1,7-dimethyl uric acid	$1.0 \pm 0.5\ 10^8$	0.9	-124	-90	5.7	2
1-methyl uric acid	$1.4 \pm 0.4\ 10^8$	0.6	-153	-124	5.4	2
hypoxanthine[a]	$1.7 \pm 0.2\ 10^8$	1.65	-52	-21	8.8	1
xanthine[a]	$1.2 \pm 0.4\ 10^9$	1.11	-104	-63.5	7.7	2
uric acid[a]	$2.9 \pm 0.1\ 10^9$	0.69	-145	-114	5.4	3

[a] from D. R. Cardoso et al., 2005.

[b] $\Delta G^o_{ET} = 96.48 \times \left(E_{ox} - E_{red} - \frac{e^2}{\varepsilon_a}\right) - \Delta E_{0,0}$ in which $\frac{e^2}{\varepsilon_a}$ the Coulombic term can be neglected due to high dielectric constant of the solvent. $\Delta E_{0,0}$ is the energy level of singlet-excited and triplet-excited state of riboflavin (239.3 and 208.2 kJ mol^{-1}, respectively). $E_{ox}\ and\ E_{red}$ are the half-wave potentials of the electron donor (purine derivative) and acceptor (riboflavin; $E_{red} = -0.29\ V\ vs\ NHE$) (K. Huvaere et al., 2010).

[c] from F. Bergmann and S. Dikstein, 1954; E. A. Johnson,1952.

Figure captions

Scheme 1. Chemical structure of riboflavin and purine derivatives.

Figure 1. Absorption spectra of riboflavin, caffeine and caffeine metabolites in aqueous phosphate buffer pH 6.4 solution at room temperature.

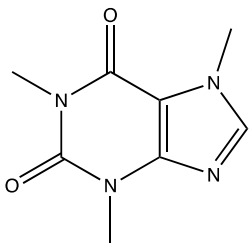
Figure 2. A) Emission spectra of riboflavin excited at 446 nm at a concentration of 1.0 10^{-5} mol L^{-1} in absence and presence of increasing concentration of 1-methyl uric acid ranging from 1.6 10^{-4} mol L^{-1} to 5.0 10^{-3} mol L^{-1} at 25.0 ^{o}C in aqueous phosphate buffer pH 6.4. B) Stern-Volmer plot for quenching of singlet-excited riboflavin emission at 517 nm by increasing concentration of 1-methyl uric acid.

Figure 3. A) Upfield shift of the riboflavin (1.02 10^{-4} mol L^{-1}) aromatic protons (H6) and (H9) in D_2O (pD 7.2, 0.2 M phosphate buffer) in the absence (top-red spectra) and presence (bottom-blue spectra) of 1-methyl-uric acid (5.80 10^{-4} mol L^{-1}). B) Upfield shift of the riboflavin (1.02 10^{-4} mol L^{-1}) ribityl protons (H11) in D_2O (pD 7.2, 0.2 M phosphate buffer) in the absence (top-red spectra) and presence (bottom-blue spectra) of 1-methyl-uric acid (5.80 10^{-4} mol L^{-1}). C) Upfield shift of the riboflavin (1.02 10^{-4} mol L^{-1}) ribityl protons (H12) in D_2O (pD 7.2, 0.2 M phosphate buffer) in the absence (top-red spectra) and presence (bottom-blue spectra) of 1-methyl-uric acid (5.80 10^{-4} mol L^{-1}). D) Downfield shift of the methyl protons of 1-methyl-uric acid (5.80 10^{-4} mol L^{-1}) in D_2O (pD 7.2, 0.2 M phosphate buffer) in the absence (top-green spectra) and presence (bottom-blue spectra) of riboflavin (1.02 10^{-4} mol L^{-1}).

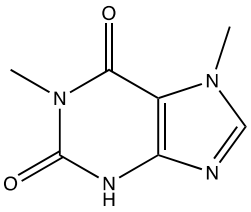
Figure 4. A) Transient absorption spectra of riboflavin (1.0 10^{-4} mol L^{-1}) in presence of 1-methyl uric acid (1.0 10^{-4} mol L^{-1}) in aqueous phosphate buffer pH 6.4 at 25 ^{o}C. Arrow indicates the wavelength (680 nm) used to probe the T-T transition, see Figure 4. B) Decay and growth/decay curve for triplet riboflavin (680 nm) and for riboflavin neutral radical (540 nm) as probed by transient absorption laser flash photolysis, same conditions as in Figure 3A.

Figure 5. A) Time decay profiles of transient absorption at 680 nm for triplet riboflavin in aqueous solution at 25 ^{o}C in absence or presence of increasing concentration of 1-methyl uric acid from 1 10^{-4} mol L^{-1} to 5 10^{-3} mol L^{-1}. B) Observed first-order rate constant for exponential decay of ^{3}Rib in presence of 1-methyl uric acid in aqueous phosphate buffer at 25 ^{o}C.

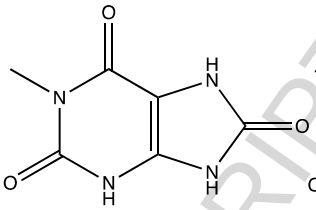
Scheme 1



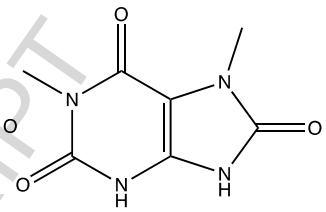
caffeine



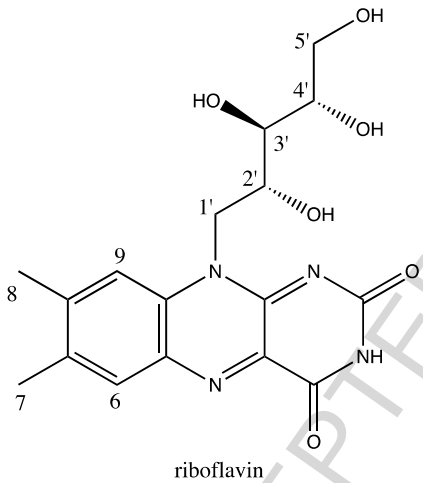
1,7-dimethyl-xanthine



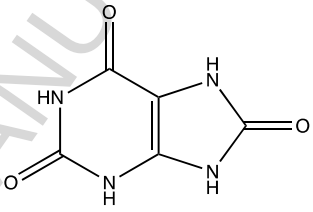
1-methyl-uric acid



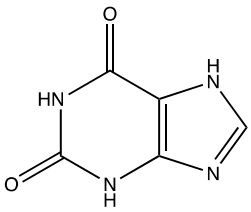
1,7-dimethyl-uric acid



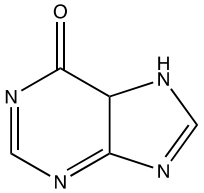
riboflavin



uric acid



xanthine



hypoxanthine

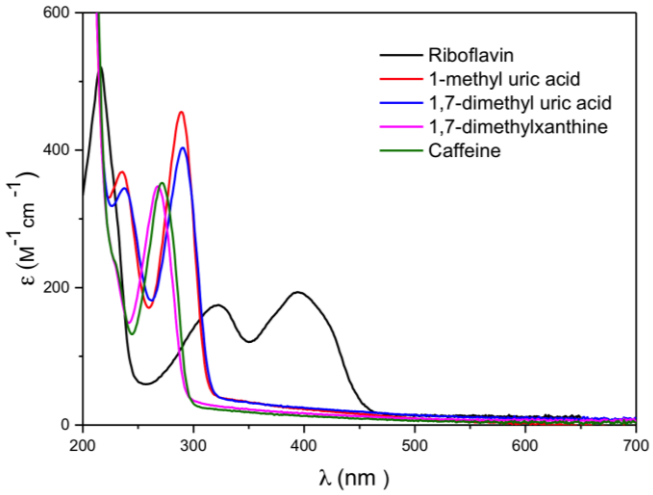
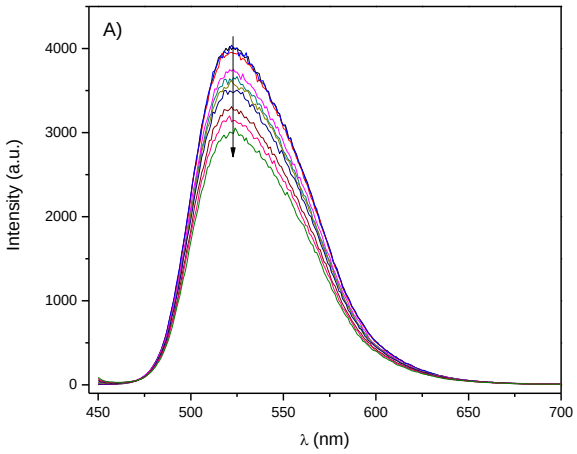


Figure 1.



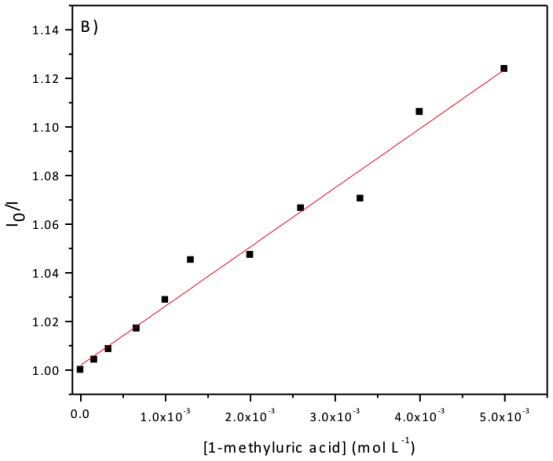


Figure 2.

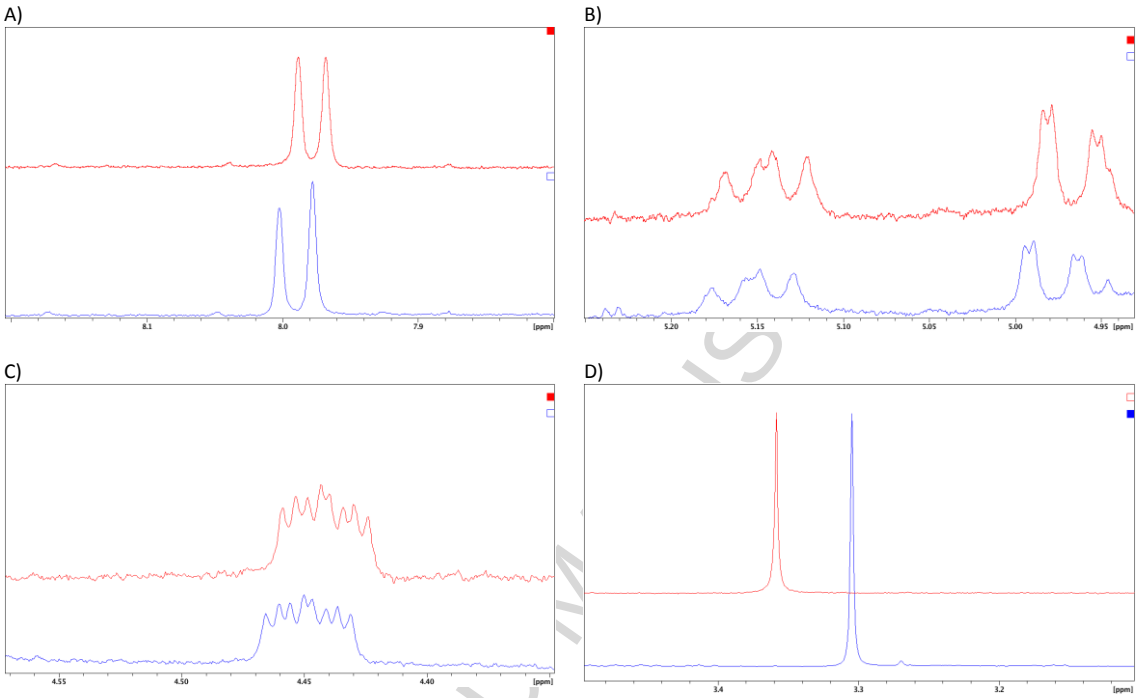


Figure 3.

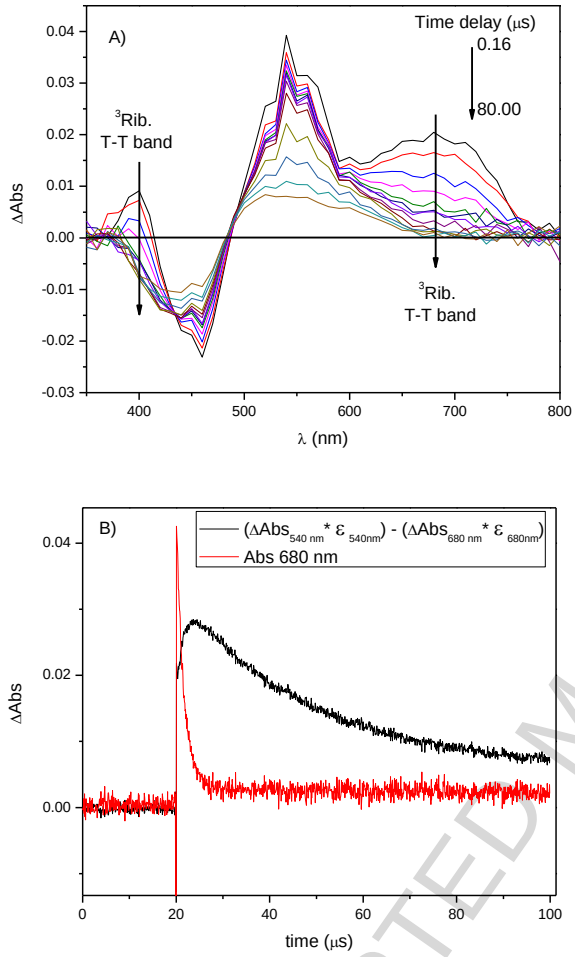


Figure 4.

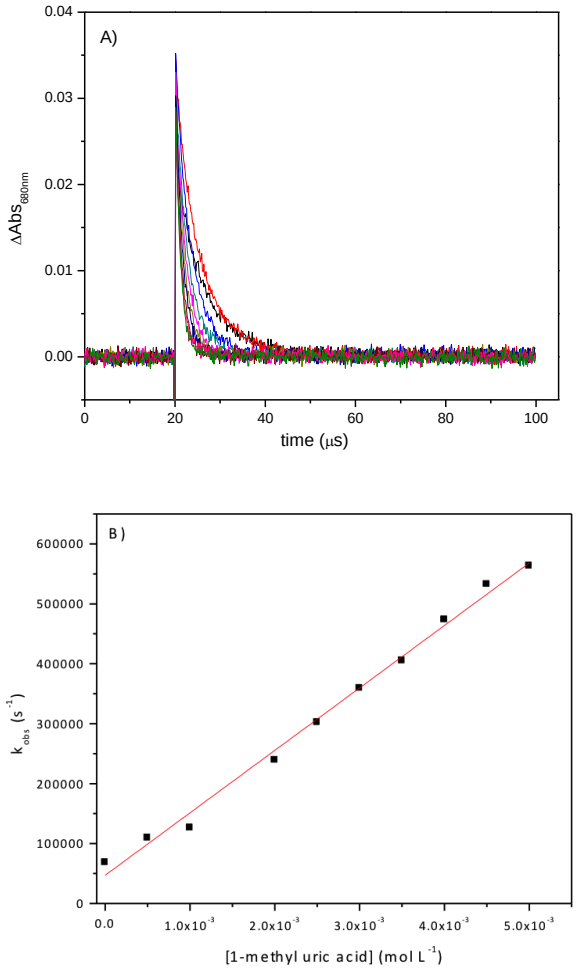


Figure 5.

Highlights

- Caffeine metabolites was found to quench singlet-excited riboflavin
- Singlet-excited riboflavin was quenched by ground-state precursor complex formation
- 1-methyl uric acid and 1,7-dimethyl uric acid are efficient quenchers of triplet riboflavin
- Caffeine was accordingly found to be non-reactive towards triplet riboflavin
- Caffeine metabolites seems responsible for the photo-protection identified for coffee drinkers