Experimental and Theoretical Study of the Stability of the Complex Fisetin–Cu(II) and A Comparative Study of Free Ligand and Complex Interaction with Molecular Singlet Oxygen

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ABSTRACT

In this work, the flavonol fisetin was selected in order to study its reactivity against Cu(II), a metal ion of interest in biological media and industry. The stoichiometry and apparent formation constant of the complex in ethanolic medium at 25°C were evaluated using spectrophotometric techniques. The resulting stoichiometry was a 1:1 ligand:metal complex, and a log $K = 5.17 \pm 0.12$ was determined. Since two possible chelation sites can be proposed for the complex formation, quantum chemistry calculations were performed on these structures. Calculations suggest that the hydroxyl-keto site is more stable for the complex formation than the catechol site. Flavonoids could exert protection against oxidative damage caused by reactive oxygen species, and this biological activity could be affected by chelation with metal ions. This led us to perform a study on the interaction of both, free flavonoid and complex, with reactive oxygen species. Our results showed both compounds quench molecular singlet oxygen photogenerated with visible light, mainly in a physical fashion. In order to analyze a possible protective effect of flavonoid and its complex against oxidative damage in biological environments, the amino acid tryptophan was selected as a model oxidation system. Free flavonoid does not have a marked protective effect, whereas its complex showed a relevant protective effect.

Abbreviations: Fis, Fisetin; Fis-Cu(II), Fisetin–Cu(II) complex; Rf, riboflavin; RB, Rose Bengal; ROS, reactive oxygen species; FFAc, furfuryl acetate; SOD, superoxide dismutase; CAT, catalase; Trp, tryptophan.

INTRODUCTION

Several authors have reported flavonoids, secondary plant metabolites, as responsible of numerous biological properties: activity against viruses (1,2) and bacteria (3) as well as antiinflammatory (4), antithrombotic (5), anti-inflammatory (6) and antitumor (7) activities. Their antioxidant activity (8) has also been studied, activity exerted by quenching (9,10) or by inhibiting the formation of reactive oxygen species (11,12). The fact that these compounds are able to complex metal ions (13,14) affects their biological activity (15-18) and is the reason why characterizing the formed complexes is a mandatory stage. The synthesis and characterization by means of spectroscopic data and thermal analysis of several metal–flavonoid complexes in solid state have been reported (18,19). However, data on flavonoid complexation ability in solution are minimal, one of the reasons for the relevance of this work.

Fisetin (3,7,3',4'-tetrahydroxyflavone) is a bioactive flavonol present in fruits and vegetables such as strawberries, apples, grapes and onions (20,21). The structure of this flavonol is shown in Fig. 1. Fisetin (Fis) exhibits several properties attributed to flavonoid family, such as anti viral (22), anticarcinogenic (23,24) and anti-inflammatory (20) activities, but also has other biological effects; for example, it stimulates signaling pathways that improve long-term memory (17). The antioxidant capacity of Fis has been studied in recent years, but there is not many

Figure 1. Structure of Fis and numbering of atoms.
information about its activity in the presence of other compounds (25).

The flavonoids may form complexes with metal ions, and the number and position of their substituents affect the properties of the formed complexes as well as the type of coordination of metal ion (26-28). There are studies regarding the ability of Fis to complex metal ions; for example, Dimitrić Marković et al. (29), who studied the Fis-Al(III) complex, carried out theoretical and experimental studies that allowed determining the most important chelation sites in the ligand: the groups 3-hydroxy-4-carbonyl and 3',4'-dihydroxy. The study of the complexation of metal ions such as Cu(II), Fe(II) or Mn(II) by flavonoids continues to be of great scientific interest due to their role in living systems (30).

In this work, Cu(II) was selected as metal ion and its complex with Fis was studied. Copper is a substance present in nature and plays an essential role in all living organisms. Some researchers have studied the effect of Cu(II) complexes with proteins and have observed a possible antioxidant capacity, finding a new perspective about copper complexes as antioxidants and possibly as therapeutic agents for neurodegenerative diseases (31).

Since complexation may change biological properties of Fis, a comparative study of free flavonoid and complex was carried out. The antioxidant activity of both compounds was studied, particularly the scavenging activity against molecular singlet oxygen (O₂(1g)), O₂(Δg) is a species which has attracted the attention of numerous researchers in last decades due to its physical, chemical and biological properties. Photosensitized production of O₂(Δg) is important in a wide range of areas from photodynamic cancer therapy (32) to polymer uses and its applications (33). Photosensitization uses the capacity of a given substance to absorb light radiation and generate electronically excited singlet and triplet states. The latter can react with molecular oxygen O₂(Σg⁻) and produce reactive oxygen species (ROS). O₂(Δg), superoxide radical anion (O₂⁻), hydroxyl radical (HO•) and hydrogen peroxide (H₂O₂) constitute the so-called ROS which could act on biological targets present in the environment causing their oxidation (34). The presence of other substrates could exert a photoprotective effect deactivating these ROS.

In this work, the formation and stability of fisetin–Cu(II) complex were studied. The investigation involves the characterization of the complex using UV-Vis and FTIR techniques and a molecular modeling analysis to gain insight into the spectroscopic properties of the system. In addition, ability of free fisetin and the complex to deactivate ROS, mainly molecular singlet oxygen, through photosensitized processes was measured in a system that simulates a natural environment in the presence of visible light.

**MATERIALS AND METHODS**

**Materials.** Fisetin (CAS 528-48-3) was provided by Aldrich, while solutions of Cu(II) were prepared using CuSO₄·5H₂O p.a. Merck (CAS 7758-99-8). The sensitizer riboflavin (CAS 83-88-5) was acquired from Aldrich, while Rose Bengal (CAS 11121-48-5) was purchased from Anendra. Furfuryl acetate (CAS 623-17-6) was acquired from Aldrich as well as the specific quenchers superoxide dismutase (CAS 9054-89-1) and catalase (CAS 9001-05-2), while L-tryptophan (CAS 73-22-3) was purchased from Sigma Chem. All these compounds were used as received. As solvent, ethanol HPLC quality (Sintorgan, CAS 64-17-5) was used and triply distilled water. In order to enlarge O₂(Δg) phosphorescence lifetime in time-resolved phosphorescence detection (TRPD), deuterated water (D₂O, Aldrich, CAS 7789-20-0) and deuterated ethanol (EtOD, CAS 925-93-9) were employed (35). All experiments were made at room temperature and with freshly prepared solutions.

Spectrophotometric technique, FTIR spectra were recorded using an Agilent 8453 diode array spectrophotometer provided with an Agilent 89090A temperature controller and using 1 cm path quartz cell with hermetical Teflon cover.

FTIR spectra of the free ligand and its complex were recorded in the 4000–400 cm⁻¹ range with a 2 cm⁻¹ spectral resolution using a Shimadzu Affinity-1. Spectrum of the complex was recorded after deposition of the sample solution on the surface of KBr pellets, a procedure successfully applied for flavonoid metallic complexes (36,37).

Stoichiometry and apparent formation constant. The determination of stoichiometry of fisetin–Cu(II) was taken using methods based on the spectrophotometric techniques. The continuous variation method, also known as Job’s method, was used (38,39). This method requires the preparation of a set of solutions where the sum of total analytical concentration of Fis and Cu(II) is held constant while their ratio is varied. The absorbances of these solutions are measured at a wavelength where only the complex absorbs. A plot of mole fraction of ligand in the mixture vs. absorbance gives a triangular shaped curve. The legs of the triangle are extrapolated until they cross and the mole fraction at the point of intersection gives the stoichiometry of the complex.

This method determines the apparent formation constant, K, of the complex using the plot previously described and the following equation:

\[
K = \frac{(A/\langle\theta\rangle)}{[M] - (A/\langle\theta\rangle)C}/[L - (A/\langle\theta\rangle)C]
\]

where \((A/\langle\theta\rangle)\) is the ratio between the measured and the extrapolated absorbances and C is the concentration of the metal or ligand, whichever is the limiting concentration at the intersection point.

Computational details. The molecular geometry of Fis and the corresponding 1:1 Fis–Cu(II) complexes were fully optimized with the DFT functional UB3LYP (40,41) under an unrestricted scheme. The 6-31+Gd basis set was employed for the H, C and O atoms, and for the Cu(II) ion, the Los Alamos double-ζ LANL2DZ effective core potential was implemented. A vibrational analysis was performed on the free flavonoid and the metallic complex in order to obtain thermodynamic parameters and to make sure that the calculated structures were true minima. The solvent effect on the gas-phase optimized structures was analyzed using the polarizable continuum model with the integral equation formalism (IEF-PCM) (42), and the UAHF radii set were employed to build the solvent cavity. A thermodynamic cycle was employed to estimate the Gibbs energy change of the complex formation (37). Nonequilibrium time-dependent density functional theory (TD-DFT) calculations were performed to estimate vertical excitation energies and absorption wavelengths of the complexes (43). Four functionals were tested in these calculations B3LYP (40), PBE0 (44,45), M06 (46) and CAM-B3LYP (47). Finally, the electronic transitions were analyzed using natural transition orbitals (NTOs) (48) which provides a good representation of the electronic transitions in terms of single particles. All the calculations were performed with the Gaussian 09 software package (49).

Stationary aerobic photolysis. Experiments employing sensitizers were carried out in a homemade photolayer with filtered light from a 150-W quartz-halogen lamp. The sample was placed into a 1 cm path length cell, under magnetic stirring, and using cutoff filters at 480 nm. Under these conditions, neither Fis nor its complex absorbs any incident light.

The rate constant for the reaction O₂(1g) + Fis or Fis–Cu(II), k₉, (process (15), Scheme 12) was determined employing a previously described actinometric method (50). In this method, the slope of the first-order plot of oxygen consumption by Fis or Fis–Cu(II) by the reference compound R (slopeR) are spectrophotometrically determined in the same experimental conditions. k₉ of Fis or Fis–Cu(II) are calculated using the expression:

\[
slope/slope_R = k_R/\tau_R
\]

The reference used was FFAc, with a reported k₉ value of 7.8 × 10⁻³ L mol⁻¹ s⁻¹ (51).

Rates of oxygen uptake (ROU) were determined employing a specific oxygen electrode Orion 810 A¹, immersed in a 50 mL, hermetically sealed glass test tube, which contained the sample solution. The system was irradiated with the above-described photolysis device.

Time-resolved O₂(1g) Phosphorescence detection (TRPD). The rate constant kᵣ for overall quenching of O₂(1g) by the flavonoid and its complex was determined employing time-resolved phosphorescence detection (TRPD), deuterated water (D₂O, Aldrich, CAS 7789-20-0) and deuterated ethanol (EtOD, CAS 925-93-9) were employed (35). All experiments were made at room temperature and with freshly prepared solutions.
The O$_2$(1$D_g$) emission at 1270 nm was generated by excitation of RB solutions at 532 nm with a Nd:YAG laser (Spectron), after filtering with a 1270-nm interference and a Wratten filter. At right angle, the phosphorescence signal was detected using an amplified Judson J16/8Sp Germanium detector. O$_2$(1$D_g$) phosphorescence lifetimes were evaluated in the absence ($\tau_0$) and in the presence ($\tau$) of the quencher (Fis or Fis-Cu(II)). The data were plotted according to a simple Stern-Volmer treatment: $\tau_0/\tau = 1 + k_q \tau_0$ [Fis or Fis-Cu(II)]. Aerated solutions were employed in all cases.

Quenching of Rf electronically excited states. The rate constant referred to the quenching of first electronically excited singlet state of Rf (1$^1Rf^*$) by Fis or its complex (1$k_q$, process (4)) was determined employing a classical Stern-Volmer treatment according to the expression $I_0/I = 1 + K_{sv} [\text{Fis or Fis-Cu(II)}]$, where $I_0$ and $I$ are the respective stationary fluorescence intensities in the presence and in the absence of quencher. $K_{sv}$ in the equation above is the Stern-Volmer constant ($K_{sv} = 1/k_q \tau_0$) being 1$\tau_0$ = 5 ns, the Rf fluorescence lifetime (53). Fluorescence spectra were recorded using a FluoroMax 4 Horiba Jobin

\[ \text{Rf} + \text{hv} \rightarrow ^1\text{Rf}^* \]  
\[ ^1\text{Rf}^* + \text{Q} \rightarrow \text{Rf} + \text{Q} \oplus \text{P}(4) \]  
\[ ^1\text{Rf}^* \rightarrow ^3\text{Rf}^* \]  
\[ ^3\text{Rf}^* + \text{O}_2(\Sigma_g^+) \rightarrow \text{Rf}^{+} + \text{O}_2^{-} \]  
\[ ^3\text{Rf}^* + \text{Q} \rightarrow \text{Rf}^{+} + \text{Q}^{-} \]  
\[ \text{Rf}^{+} + \text{H}^+ \rightarrow \text{RfH}^+ \]  
\[ 2 \text{RfH}^+ \rightarrow \text{Rf} + \text{RfH}_2 \]  
\[ \text{RfH}_2^+ + \text{O}_2(\Sigma_g^+) \rightarrow \text{RfH}_2^{+} + \text{O}_2^{-} \]  
\[ \text{RfH}_2^{+} + \text{O}_2^{-} \rightarrow \text{Rf} + \text{H}_2\text{O}_2 \]  
\[ \text{O}_2^{-} + \text{Q} \rightarrow \text{P}(12) \]  
\[ \text{H}_2\text{O}_2 + \text{Q} \rightarrow \text{P}(13) \]  
\[ ^1\text{Rf}^* + \text{O}_2(\Sigma_g^+) \rightarrow \text{Rf} + \text{O}_2(\Delta_g) \]  
\[ \text{O}_2(\Delta_g) \rightarrow \text{O}_2(\Sigma_g^+) \]  
\[ \text{O}_2(\Delta_g) + \text{Q} \rightarrow \text{O}_2(\Sigma_g^+) + \text{Q} \]  
\[ \text{O}_2(\Delta_g) + \text{Q} \rightarrow \text{P}(17) \]

\[ \text{Rate constant } k_e = k_q + k_i \]

Scheme 1. Possible pathways in a Rf-photosensitized process in the presence of an electron donor transparent to the incident light (Q).
Yvon spectrophotometer. The excitation and emission wavelengths employed were 445 and 517 nm, respectively.

The rate constant accounting for the quenching of electronically excited RF (RF•) by Fis or Fis-Cu(II) (kq, process (5)) could not be determined employing the conventional method of Laser Flash Photolysis (LFP) due to the overlapped ground state absorption bands of RF and the quenchers at 337 nm, the available excitation wavelength in our LFP apparatus. Nevertheless, an alternative method was used to determine the rate constant considering that the anaerobic visible light-mediated degradation of RF in solution occurs mainly from RF• state, for which has a reported lifetime (τ0) of 15 μs (54). The rate of the degradation process can be estimated through the time evolution of the absorbance decrease in the RF absorption band at 445 nm. The decomposition rate of RF was evaluated in the absence (V0) and in the presence (V) of several Fis or Fis-Cu(II) concentrations, in N2 saturated atmosphere. Employing the Stern-Volmer treatment (V0/V = 1 + kqappτ0 [Fis or Fis-Cu(II)]), the apparent rate constant for the quenching of RF• by Fis and Fis-Cu(II) was roughly evaluated. This indirect method allows to determine kqapp values with a ±20% error.

ROS deactivation. The possible photogeneration of ROS in solutions containing RF and/or their possible deactivation caused by Fis or Fis-Cu(II) can be evaluated qualitatively through oxygen consumption in the presence of specific quenchers of ROS. In this work, three specific quenchers were used: sodium azide, a physical quencher of O2 (ΔG), superoxide dismutase, which dismutates O2•− (process (1)), and catalase, an enzyme responsible for the decomposition of H2O2 (process (2)) (55-57).

\[
\begin{align*}
20_2^- + 2H^+ &\rightarrow SODO_2^-(\Sigma_g^+) + H_2O_2 \\
2H_2O_2 &\rightarrow CAT2H_2O + O_2(\Sigma_g^+) 
\end{align*}
\]

(1) (2)

Photoprotection of tryptophan by Fis and Fis-Cu(II). Relative rates for RF and RB sensitized photooxidation of the systems, Trp, Fis and Fis-Cu(II) and their mixtures were evaluated through the initial slope of oxygen consumption as a function of photoinduced time, employing the specific oxygen electrode already described. Normalized rates for each sensitizer family were obtained as the quotient between the respective ROF for a given sample and that for the faster oxygen-consumer sample of the family. The experiments were carried out until 10–15% of oxygen consumption as a measure of the photooxidability of each studied system.

RESULTS
Complex stoichiometry and apparent constant formation determination

The absorption spectrum of Fis in ethanol medium is showed in Fig. 2. A shift to higher wavelength is observed when the metallic complex is formed. In order to study Fis-Cu(II) complex, solutions of different ligand:metal ratio were prepared. The spectra of these solutions were recorded and are shown in Fig. 2.

The stoichiometry of the complex was determined by Job’s method, recording the spectra of a set of solutions where the ligand and metallic concentrations varied in the $1 \times 10^{-4}$–$1 \times 10^{-5}$ mol L$^{-1}$ range and total analytical concentration of Fis and Cu(II) was held constant at $1 \times 10^{-4}$ mol L$^{-1}$. Absorbances were measured at 475 nm, and a wavelength was only the complex absorbs. Data were used to build the graphic shown in Fig. 2 (inset), from which a 1:1 L:M stoichiometry was determined. A similar stoichiometry was reported for Fis-Al(III) complex at pH ≤ 5 in aqueous media (29), while mono- and dinuclear species were reported for Fis-Cu(II) complexes in a 40:60 v/v methanol/water solvent (58).

The apparent formation constant of Fis-Cu(II) was determined in triplicate according to Job’s method, previously described. The K values were obtained using Eq. (1) and data in Fig. 2. The log K value obtained at 25°C was 5.17 ± 0.12. A value of 36.51 was reported for the overall stability constant of FisH+ and Cu(II) complex in a 40:60 v/v methanol/water solvent (42). Considering the ionization constant of FisH+ species reported in the same work, whose value is 29.87, the apparent formation constant of the complex is 6.64 ± 0.03, slightly higher than the value obtained in this work in ethanol medium.

The formation constant of Fis-Cu(II) was also estimated by means of quantum chemistry calculations. Since Fis exhibits two possible chelating sites for Cu(II) ions, different structures were employed in the calculations. The first structure corresponds to the formation of a cationic complex where the chelation takes place in the 3-OH and 4-oxo site (i), and the second one is for a neutral complex where the chelation takes place in the catechol group (ii). The ΔG (Gibbs energy change in ethanol) were calculated for both complexes using a thermodynamic cycle (see Figure S1). This method has been successfully applied in the calculation of Gibbs energies for other flavonoid metallic complexes (37). For the site (i), a complex ΔG = −66.62 kJ mol$^{-1}$ (log K = 11.67) was obtained, while for the site (ii), complex ΔG = 16.04 kJ mol$^{-1}$ (log K = −5.58). These results indicate that the complexation Fis by Cu(II) is thermodynamically more stable when the metal interacts with the 3-OH-4-oxo site compared with the chelation in the catechol group. It is important to note that the interaction of Cu(II) with the 3-hydroxyl-4-oxo site involves the dissociation of one OH group of Fis, while the other chelation site (catechol) involves the dissociation of two OH groups. In addition, one of the complex has cationic nature, and the other is neutral (see Figure S1). The energy difference can be rationalized in terms of these features. Similar results were obtained by E. Jabeen et al. for the complexations of Cu(II) and Fe(III) with quercetin and morin (59). These authors reported negative ΔG values for the chelation site i and positive ΔG values for the chelation site ii using DFT calculations.

TD-DFT results
The optimized molecular structures of the Fis-Cu(II) are shown in Fig. 3. TD-DFT calculations were performed on complexes to
simulate the UV-Vis absorption spectra in ethanolic solution. For this purpose, the B3LYP, PBE0, M06 and CAM-B3LYP functionals were employed in combination with the IEF-PCM formalism. Table 1 shows the predicted absorption wavelengths ($\lambda_{TD-DFT}$) and oscillator strength ($f$) calculated for the two complexes. Taking into account the open-shell nature of the Fis-Cu(II) complex, spin contamination is observed in the TD-DFT results particularly in site (ii) complex. A measure of spin contamination is provided by the extent to which the calculated spin operator ($S^2$) differs from the value expected from the same operator for a doublet state, which is 0.750 (60). Following an empirical rule, the absorption wavelengths reported in Table 1 have spin contaminations lower than 5% of the expected value.

Table 1. Calculated absorption wavelengths ($\lambda_{TD-DFT}$) in nm, oscillator strength ($f$) for the Fis-Cu(II) complexes from TD-DFT/PCM simulations. $\Delta \lambda$ is the difference between the calculated and experimental wavelengths in absolute value.

|                  | B3LYP         | PBE0         |                        | M06          | CAM-B3LYP  |
|------------------|---------------|--------------|------------------------|--------------|------------|
|                  | $\lambda_{(exp)}$ | $\lambda_{TD-DFT}$ | $f$ | $\Delta \lambda$ | $\lambda_{TD-DFT}$ | $f$ | $\Delta \lambda$ |
| Fis-Cu(II)(i)    | 429.6         | 491.2        | 0.3861                 | 61.6         | 480.0      | 0.4400          | 50.44 |
|                  | 495.70        | 522.3        | 0.0682                 | 93.6         | 522.3      | 0.1483          | 92.65 |
|                  | 429.6         | 523.2        | 0.1280                 | 91.7         | 495.7      | 0.3527          | 66.1  |
|                  | 491.2         | 523.2        | 0.3861                 | 61.6         | 522.3      | 0.1483          | 92.65 |

To elucidate the nature of the electronic transitions of the Fis-Cu(II) complex UV-Vis spectrum, a natural transition orbital (NTO) analysis was performed. This approximation provides a compact representation of the transition density between the ground and excited states in terms of an expansion into single-particle transitions (47). These orbitals were constructed with the CAM-B3LYP functional since the most accurate $\lambda_{TD-DFT}$ value was obtained with this method. The NTOs of the Fis-Cu(II) complex are depicted in Fig. 4. Since these calculations are performed under an unrestricted scheme, the $\pi$ and $\beta$ spin orbitals are computed separately. For the $i$-Fis-Cu(II) complex, both spin orbitals have almost identical shape and energy for the analyzed transition. The adsorption band located at 429.6 nm ($\lambda_{TD-DFT} = 459.07$ nm) corresponds to $\pi-\pi^*$ transition, with a small contribution of a metal-to-ligand charge transfer (MLCT). The spin orbitals of the $ii$-Fis-Cu(II) complex have different shapes and energy, with a higher contribution of the $\beta$ orbitals. The NTOs for the same absorption band calculated for $ii$-Fis-Cu(II) ($\lambda_{TD-DFT} = 495.70$ nm) show that this is mainly a $\pi-\pi^*$ transition-mixed metal-to-ligand charge transfer (MLCT).

FTIR results

The FTIR spectra of Fis and the metallic complex were recorded to evaluate the structural features of these compounds. The complexation with Cu(II) ions induces important changes in the fisetin spectra. The spectra of the free ligand and the complex are shown in Fig. 5. The sharp bands located at 1630 and 1606 cm$^{-1}$ in Fis spectrum are assigned to the overlapping of the C=O and C2=C3 stretching modes (29). These are the most intense bands of Fis spectrum. In the 1500–1300 cm$^{-1}$ range, the in-plane C-H bending and wagging vibrations can be found. The presence of vibration bands in the 3500–3200 cm$^{-1}$ range is attributed to the stretching vibrations of the many OH groups of the ligand. The bending vibrations of the different OH groups contribute to several modes located at 1440, 1340, 1183 and 1167 cm$^{-1}$ (61). The formation of the metallic Fis-Cu(II) complex induces some important changes in the FTIR spectrum of the flavone. The C=O stretching vibration is shifted 10 cm$^{-1}$ to...
a higher wavenumber (1616 cm\(^{-1}\)), and a new band (weak) is observed at 617 cm\(^{-1}\). This new band can be assigned to the Cu-O stretching vibration mode, which is consistent with previously reported wavenumbers for other Cu(II) complexes with flavonoids. In addition, there is an important reduction in the intensity (in relation to the most intense C=O vibration) of the bands located at 1340, 1183 and 1167 cm\(^{-1}\) upon complexation. These results are not conclusive enough to elucidate the preferential chelation site of Fis-Cu(II) complex. However, they are indicative of a clear interaction between Cu(II) and fisetin and the formation of a metal complex.

Interaction of electronically excited states of RF and Fis or Fis-Cu(II)

Flavonoids are involved in the generation and deactivation of oxidative specie (7,62). Study of photoprocesses in the presence of natural sensitizers can be used as an approach to real situations in living environments. RF is a pigment considered as a possible sensitizer for photodegradation of natural substrates present in food and living organism (53). Since flavonoids and RF are found in the same natural locations, information of the influence between this compounds will allow to gain insight into the behavior of this species in nature.

RF in solution is able to photogenerate \(\text{O}_2^-\) (63) and \(\text{O}_2(1\Delta_g)\) (64), a behavior which may suffer variations in the presence of potential electron donors, as Fis or Fis-Cu(II). It is interesting then to study the interaction between this compounds and photexcited RF in order to determine whether Fis or Fis-Cu(II) is able to inhibit or favor the production of ROS.

Photoirradiation of RF, RF + Fis or RF + Fis-Cu(II) in water: ethanol 70:30 v/v at wavelength where only the vitamin absorbs produces the spectral changes shown in Fig. 6. Time evolution of the spectra indicates the occurrence of chemical changes involving the studied substrates and/or the photosensitizer. Even in the 450–500 nm range, there are spectral changes, a zone which clearly corresponds to RF. These data definitively indicate electronically excited states of RF, or the species produced from them with or without molecular oxygen participation are responsible for the observed changes.

A systematic kinetic study was carried out to evaluate and characterize the nature and mechanism of the possible processes involved in the sensitized degradation of RF in the presence of Fis or Fis-Cu(II). Scheme 12 is used in order to discuss and evaluate the photophysical–photochemical results obtained from this study.

Scheme 12 shows a reaction sequence which includes photoprocesses in the presence and in the absence of a quencher (Q),
an electron donor transparent to the incident light, which in this case corresponds to Fis or Fis-Cu(II). P(n) are products for each corresponding (n) reaction, and O2(3Δg) represents ground state molecular oxygen. Experimental conditions and nature of the involved compounds will determine the prevalence of a given photoprocess.

**Interaction of Fis and Fis-Cu(II) with O2(1Δg)**

Both, flavonol and complex, were able to quench O2(1Δg). $k_i$ values were obtained using TRPD, measuring the lifetime of O2(1Δg) generated from RB in a deuterated water:deuterated ethanol 70:30 v/v mixture according to technics described before. In the presence of Fis or Fis-Cu(II), a decay in the phosphorescence of O2(1Δg) was observed, evidencing the interaction of these compounds with O2(1Δg). The phosphorescence lifetimes of O2(1Δg) measured were in agreement with those reported in the literature (35). Figure 7 (inset) shows obtained results, and Table 2 shows determined $k_i$ values.

Rate constant $k_i$ involves both physical deactivation and chemical deactivation of O2(1Δg). In order to discriminate the fraction of this deactivation involved in each of these processes, $k_i$ values (process 15, Scheme 12) must be determined. This can be achieved measuring substrate consumption of solutions of Fis, Fis-Cu(II) and a reference (furfuryl acetate, FFAc) using water:ethanol 70:30 v/w photoirradiated with 480 nm cutoff filter. Numbers next to the arrows represent time in minutes. $Rf$ vs solvent spectrum is included for comparative purposes. Inset: spectral evolution of an aerated solution of 0.04 mmol L⁻¹ $Rf$ + 0.08 mmol L⁻¹ Fis-Cu(II) in H2O:EtOH 70:30 v/v photoirradiated with 480 nm cutoff filter. Numbers next to the arrows represent time in minutes. $Rf$ vs solvent spectrum is included for comparative purposes.

![Figure 6](image6.png)

**Figure 6.** Spectral evolution of an aerated solution of 0.04 mmol L⁻¹ $Rf$ + 0.08 mmol L⁻¹ Fis, in H2O/EtOH 70:30 v/v taken vs. 0.04 mM $Rf$, photoirradiated with 480 nm cutoff filter. Numbers next to the arrows represent time in minutes. $Rf$ vs solvent spectrum is included for comparative purposes. Inset: spectral evolution of an aerated solution of 0.04 mmol L⁻¹ $Rf$ + 0.08 mmol L⁻¹ Fis-Cu(II) in H2O/EtOH 70:30 v/v photoirradiated with 480 nm cutoff filter. Numbers next to the arrow represent time in minutes. $Rf$ vs solvent spectrum is included for comparative purposes.

![Figure 7](image7.png)

**Figure 7.** First-order representation for substrate consumption by photoirradiated ethanolic solutions of (a) RB ($\lambda_{max} = 0.5$) + Fis (7.8 × 10⁻⁵ mol L⁻¹); (b) RB ($\lambda_{max} = 0.5$) + Fis-Cu(II) (7.8 × 10⁻⁵ mol L⁻¹); (c) RB ($\lambda_{max} = 0.5$) + FFAc (8 × 10⁻⁵ mol L⁻¹), in H2O:EtoH 70:30 v/v. Inset: Stern-Volmer treatment for the determination of the total rate constant for the quenching of O2(1Δg), $k_q$ by (a) Fis and (b) Fis-Cu(II) in D2O/EtOD 70:30 v/v.

Quenching of Rf electronically excited states

Rf fluorescence spectrum shows a band centered at 517 nm when excited at 445 nm, with a quantum yield of 0.25 (53). In the presence of increasing concentrations of free flavonoid or complex, a decrease in the fluorescence intensity of the band is observed (see Supporting Information), evidencing the interaction with $^1Rf^*$ (process 4, Scheme 12). Fis and Fis-Cu(II) can interact with $^1Rf^*$ with $^1Rf^*$ scavenging ability was improved with complexation (65). A different case is presented by 3,3'-dihydroxyflavone and its La(III) complex: the calculated $k_i/k_q$ ratios for the flavonoid and for the complex were, respectively, ~0 and 0.1, indicating both compounds interact in a similar way with O2(1Δg) (66). These results make evident that to predict whether a given flavonoid complex will improve or decrease the chemical contribution of the O2(1Δg) interaction compared with the free ligand is not simple.
calculated from the apparent stability constant of the complex is \( \approx 0.6 \) \( \mu \text{mol L}^{-1} \), not enough to effectively quench \( \cdot \text{O}_{2} \cdot \).

Fis and Fis-Cu(II) also interact with \( \cdot \text{O}_{2} \cdot \), effect that is inferred from the decrease in the anaerobic photodecomposition of Rf when Fis or Fis-Cu(II) concentrations increase (Figs. 8 or 9). The \( k_{q}\_\text{app} \) values (process 7, Scheme 12) can be determined monitoring the 445 nm absorption band of oxygen-free solutions of Rf in ethanolic medium, irradiated in the absence and in the presence of different concentrations of free flavonoid or complex (Table 2). Both studied compounds exhibit high \( k_{q} \) deactivation constant values, which means stationary concentration of \( \cdot \text{O}_{2} \cdot \) could be diminished through this via, and in consequence, have a protective effect against this species.

**Fis and Fis-Cu(II) as ROS scavengers**

Oxygen consumption experiments in the presence and in the absence of specific ROS quenchers allowed to evaluate the possible deactivation of ROS photogenerated from Rf + Fis or Rf + Fis-Cu(II) systems. The specific ROS quenchers used were as follows: NaN\(_{3}\), a physical quencher of \( \cdot \text{O}_{2} \cdot \), SOD, which catalyzes dismutation of \( \text{O}_2 \cdot \), and CAT, which catalyzes the decomposition of \( \text{H}_2\text{O}_2 \). Although \( \cdot \text{O}_{2} \cdot \) is reactive toward proteins, employing the concentrations described in these experiments, the lifetime of this species is not affected. Moreover, all experiments were carried out keeping the concentrations of Rf and the corresponding substrate constant. The oxygen consumption profiles obtained by irradiating the above-described solutions were compared (Fig. 10).

In the presence of NaN\(_{3}\), no inhibition effect was observed (data not shown), while Fig. 10 shows oxygen uptake profiles for Rf + (free flavonoid or complex) and Rf + (free flavonoid or complex) in the presence of quenchers CAT and SOD. The first set of experiments involves Rf + Fis system and shows that the higher inhibition in the rate of oxygen consumption occurs in the presence of CAT. This inhibition indicates a reaction between Fis and H\(_2\)O\(_2\).

### Table 2: Rate constant values for total \( (k_t) \) and reactive quenching \( (k_r) \) of \( \cdot \text{O}_{2} \cdot \), \( k_r/k_t \) and quenching of \( \cdot \text{Rf}^\bullet \) \( (k_q) \) values for Fis and Fis-Cu(II).

| Compound     | \( k_t \) (10\(^7\) L mol\(^{-1}\) s\(^{-1}\)) | \( k_r \) (10\(^6\) L mol\(^{-1}\) s\(^{-1}\)) | \( k_r/k_t \) | \( k_q \) (10\(^7\) L mol\(^{-1}\) s\(^{-1}\)) |
|--------------|---------------------------------|---------------------------------|----------------|---------------------------------|
| Fis          | 14.4                            | 2.6                             | 0.02           | 1.42                            |
| Fis-Cu(II)   | 7.25                            | 26.6                            | 0.36           | 1.38                            |

### Figure 8

Left: Degradation of Rf as a function of photoirradiation time (cutoff 480 nm), monitored through the decrease in the 445 nm absorbance peak, in the absence (Rf) and in the presence of Fis: (a) 20.8 \( \mu \text{mol L}^{-1} \), (b) 15.6 \( \mu \text{mol L}^{-1} \) and (c) 15.6 \( \mu \text{mol L}^{-1} \). Right: Stern-Volmer treatment for the evaluation of the rate constant for the quenching of \( \cdot \text{Rf}^\bullet \) by Fis in H\(_2\)O:EtOH 70:30 v/v deaerated solutions. \( V_0 \) and \( V \) represent the respective rates of Rf degradation, upon photoirradiation (cutoff 480 nm), in the absence and in the presence of Fis.

### Figure 9

Left: Degradation of Rf as a function of photoirradiation time (cutoff 480 nm), monitored through the decrease in the 445 nm absorbance peak, in the absence (Rf) and in the presence of Fis-Cu(II): (a) 24.8 \( \mu \text{mol L}^{-1} \), (b) 15.5 \( \mu \text{mol L}^{-1} \) and (c) 9.3 \( \mu \text{mol L}^{-1} \). Right: Stern-Volmer treatment for the evaluation of the rate constant for the quenching of \( \cdot \text{Rf}^\bullet \) by Fis-Cu(II) in H\(_2\)O:EtOH 70:30 v/v deaerated solutions. \( V_0 \) and \( V \) represent the respective rates of Rf degradation, upon photoirradiation (cutoff 480 nm), in the absence and in the presence of Fis-Cu(II).
generated from Rf (step 11, Scheme 12) could be occurring. In the presence of the inhibitor, H$_2$O$_2$ is deactivated and oxygen consumption decreases. In contrast, the rate of oxygen consumption is relatively increased in the presence of SOD compared to Rf + Fis. This corroborates the importance of the reaction between Fis and Trp, because it is an oxidizable amino acid involved in photodynamic processes of proteins (68). Trp interacts with 3Rf* with a 3-4 kq constant rate value of 2.5 × 10$^9$ L mol$^{-1}$ s$^{-1}$ (69), and it interacts with O$_2$(1$^g$) with a $k_1$ = 7.2 × 10$^7$ L mol$^{-1}$ s$^{-1}$ and $k_2$ = 4.7 × 10$^7$ L mol$^{-1}$ s$^{-1}$ (67, 70).

Kinetic information obtained measuring ROU provides a path to understand the protection exerted by flavonoids or their complexes on protein residues through photopromoted oxidation. The same experiments using RB instead of Rf as sensitizer facilitate interpretation on the grounds of an O$_2$(1$^g$)-mediated operation. Results are shown in Fig. 11.

As shown in Fig. 11, the photooxidation rate of the mixture Fis + Trp is lower than the sum of the photooxidation rates of Fis and Trp individually. This value added to the ability of Fis to deactivate O$_2$(1$^g$) through a physical mechanism and leads us to the conclusion that the flavonoid is able to protect Trp from the oxidative species. On the other hand, Trp + Fis-Cu(II) system shows practically no oxygen consumption, which implies a significant protection effect, even though the $k_1/k_2$ ratio has a reactive component. In order to gain insight into this reaction, a new experiment was carried out measuring rate of oxygen uptake of similar solutions of Trp, Fis-Cu(II) and Trp + Fis-Cu(II) but replacing Rf by RB, which photogenerates O$_2$(1$^g$) exclusively. The results show the protective effect against O$_2$(1$^g$) exerted by the complex toward the amino acid is not enough to justify the behavior in the presence of Rf; hence, the protection exerted by the complex may include other reactive oxygen species.

**CONCLUSIONS**

In this work, the complex fisetin-Cu(II) was studied. The stoichiometry and apparent constant of Fis-Cu(II) complex were evaluated in H$_2$O:EtOH 70:30 v/v solvent, resulting in a 1:1 ligand:metal complex stoichiometry with a log $K$ = 5.17 ± 0.12 at 25°C. The molecular structure of the complex was analyzed by molecular modeling and FTIR analysis. Two possible chelation sites were proposed for the complex; the catechol group (site ii) is the preferred site of Cu(II) ions under the adopted conditions. However, these results suggest that the coexistence of both chelation sites must be considered.

Fisetin and its Cu(II) complex in ethanolic medium deactivates O$_2$(1$^g$) generated by visible light photosensitization, with an increased reactive component for the latter. The oxygen uptake profiles of the substrates in the presence of specific inhibitors of ROS indicate an interaction between Fis-Cu(II) and O$_2$(1$^g$). It is also possible to infer the participation of H$_2$O$_2$ in the photooxidative process in the presence of Fis. A model oxidation system was employed with Rf as photosensitizer and the amino acid tryptophan. Although free flavonoid has a protective effect, the protection exerted by complex is greater, evidencing an improvement of this capacity when complexation occurs.

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**Figure 10.** Normalized rate of oxygen uptake of aerated solutions of 0.04 mmol L$^{-1}$ Rf + 0.05 mmol L$^{-1}$ Fis or Rf + 0.05 mmol L$^{-1}$ Fis-Cu(II) in the presence of the specific ROS quenchers: 1 μg/mL CAT, 1 μg/mL SOD. Solvent: H$_2$O/EtOH 70:30 v/v.

**Figure 11.** Rate of oxygen uptake of aerated solutions of Rf 0.04 mmol L$^{-1}$ in the presence of Trp and/or Fis or Fis-Cu(II) (black bars). Rate of oxygen uptake of aerated solutions of RB (λ<sub>ex690nm</sub> = 0.5) in the presence of Trp, Fis-Cu(II) and Trp + Fis-Cu(II) (gray bars). All experiments were carried out irradiating using 480 nm filter. Solvent: H$_2$O/EtOH 70:30 v/v. [Trp]=([Fis]+[Fis-Cu(II)])=0.05 mmol L$^{-1}$. [Color figure can be viewed at wileyonlinelibrary.com]
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SUPPORTING INFORMATION
Additional supporting information may be found online in the Supporting Information section at the end of the article:

Figure S1. Thermodynamic cycle employed for the calculation of the Gibbs energy change of the complex formation reaction. The first cycle correspond to the site (i) Fis-Cu complex and the second one to the site (ii) Fis-Cu complex.

Figure S2. Stern-Volmer treatment for the evaluation of the rate constant for the quenching of IR* by Fis (left) and Fis-Cu (II) (right) in H2O/ EtOH 70:30 v/v. Insets: Fluorescence spectra of Rf in the presence of increasing concentration of Fis (left) and Fis-Cu(II) (right).

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