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Kinetic study of the photosensitized oxygenation of the flavanone naringin and its chalcone

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Abstract

The kinetic of the $O_2(^1\Delta_g)$-photosensitized oxidation of the flavanone naringin (7-rhamnoglucosyl-4′,5-dihydroxyflavanone, FL) and its chalcone isomer (4′-rhamnoglucosyl-2′,6′,4-trihydroxychalcone, CH) in neutral and 1 mM NaOH ethanol solutions was studied using Rose Bengal as photosensitizer. The rate constants for the chemical quenching of $O_2(^1\Delta_g)$ by the flavonoids (k_r) were determined using either UV–vis absorption spectroscopy or HPLC techniques, and for the total (physical + chemical) quenching (k_t) were determined using time-resolved phosphorescence detection of $O_2(^1\Delta_g)$ at 1270 nm.

A larger reactivity towards $O_2(^1\Delta_g)$ of CH than for FL in neutral ethanol solutions was observed, due to the extra conjugated double bond in CH. However, in alkaline media the reactivity of both isomers was larger than in neutral conditions. This behavior was associated to the increment of electron density by the formation of a carbanion in FL and to the presence of the extended conjugated π-system in the CH isomer by deprotonation of phenolic groups. In all cases, the formation of 4-hydroxybenzoic acid as ending product was detected by HPLC. Based on reported mechanisms of $O_2(^1\Delta_g)$-mediated oxidation of flavonoids, the formation of 7-rhamnoglucosyl-5,4′-dihydroxyflavonol as primary photo-oxidation product was proposed.

The reactivity towards $O_2(^1\Delta_g)$ of the aglycone of naringin (5,7,4′-trihydroxyflavanone or naringenin, NG) in alkaline conditions was lower than for the parent naringin, due to incapability of NG to form a carbanion species.

These results are expected to have significance in biosynthesis, antioxidant properties and stability of naturally occurring flavonoids.

Keywords: Naringin; Flavanones; Chalcones; Singlet oxygen; Photo-oxidations

1. Introduction

Flavonoids are bioactive polyphenolic compounds widely distributed in plants, where they play several physiological functions. In the last years, this family of natural products has received large attention due to their health-related properties, such as cancer and heart disease prevention treatments [1–4]. Most of these properties are related with the important role played by flavonoids in cell protection as antioxidants against free radicals and reactive oxygen species [5–8], such as singlet molecular oxygen, $O_2(^1\Delta_g)$ [9–12].

Among the flavonoids compounds, we are currently interested in the chemistry and photochemistry of the flavanone and chalcone types [13,14]. In particular, the glycosilated flavanone naringin (7-rhamnoglucosyl-4′,5-dihydroxyflavanone) and its aglycone naringenin (5,7,4′-trihydroxyflavanone), Scheme 1, are commonly found in the peel and juice of grapefruit and certain types of orange, and are responsible of the its characteristic bitter taste. In fact, naringin is commercially used as bitter additive in some food preparations [15].

Flavanones are stable in acid and neutral solutions of protic solvents, but some of them in alkaline conditions are equilibrated with their chalcone isomers, which contains an extended conjugated π-system, Scheme 1 [13,16–18]. In the case of naringin in alkaline ethanol–water mixtures, the isomerization equilibrium is shifted to its chalcone, and it is mediated by a carbanion species produced by deprotonation of the hydrogen at the C3 of the flavanone (*conjugate base unimolecular elimination*, E1cb)

Abbreviations: DMA, 9,10-dimethylanthracene; CH, chalcone isomer; FL, flavanone or naringin; NG, naringenin; RB, Rose Bengal; S, sensitizer molecule; TRPD, time-resolved phosphorescence detection

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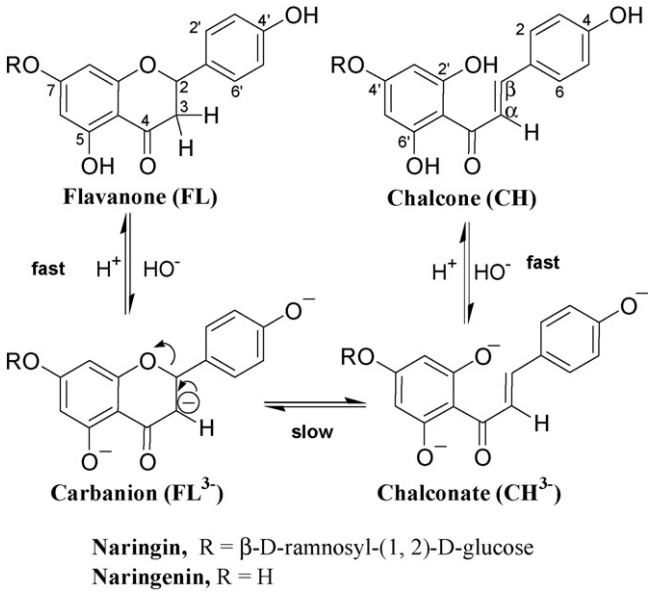


Scheme 1. Carbanion-mediated mechanism for the flavanone–chalcone isomerization of naringin in basic media [13–17].

[13,17]. The formation of the carbanion has been regarded as an acid–base equilibrium, characterized by $pK_{1/2}$ values of 11 [13] and 14 [17] for naringin and naringenin in aqueous media, respectively, indicating a much less acid C3 in the aglycone. The mechanism of the flavanone–chalcone equilibrium has received large attention, and it has been reported that the carbanion intermediate mechanism prevails at pH > 10 [13,17], while in less alkaline solutions the isomerization is produced *via* enolization equilibrium [16–18].

In addition, chalcones are considered to be the precursors in the biosynthesis of flavonoids by enzymatic reactions, such as dioxygenase-catalyzed oxygenations, where the fixation of O_2 into the substrate is produced [19]. Several authors have been used photosensitized $O_2(^1\Delta_g)$-mediated oxygenation of chalcones [20–22] and flavonols [23] to mimic dioxygenase-catalyzed reactions.

On the other hand, flavonoids are being reported as efficient quenchers of $O_2(^1\Delta_g)$ in solution [9–12]. Consequently, it would be expected that flavonoids are able to quench $O_2(^1\Delta_g)$ in biological environments decreasing the extension of harmful oxidation reactions (*antioxidant effect*). Therefore, the $O_2(^1\Delta_g)$ interaction with flavonoids is a relevant issue from both antioxidant and biosynthetic approaches.

The aim of this work is to examine the kinetic for the interaction of dye-sensitized $O_2(^1\Delta_g)$ with naringin (FL) and its derivatives, such as its chalcone (CH) and its aglycone naringenin (NG), in both neutral and alkaline ethanol solutions. The physical and chemical quenching rate constants of $O_2(^1\Delta_g)$ by the flavonoids were determined. The effect of the slower isomerization flavanone–chalcone equilibrium in basic media and the carbanion reactivity towards $O_2(^1\Delta_g)$ was also analyzed. A mechanism for the formation of the primary and secondary photo-oxidation products is suggested.

2. Experimental

2.1. Materials and methods

Naringin (7-rhamnoglucosyl-4′,5-dihydroxyflavanone, FL) 97% was from Fluka; 9,10-dimethylanthracene (DMA, Aldrich); *p*-hydroxybenzoic acid and Rose Bengal as sodium salt (RB) were from Sigma. Hydrochloric acid (HCl) and sodium hydroxide (NaOH) were analytical degree from Merck (Argentina). Absolute ethanol, acetonitrile, and acetic acid were HPLC grade from Sintorgan, Argentina. Water was triply distilled. All chemical were used as received.

The chalcone of naringin (4′-rhamnoglucosyl-2′,6′,4-trihydroxychalcone, CH) was obtained from naringin following standard procedures reported elsewhere [13,24]. Naringenin (5,7,4′-trihydroxyflavanone, NG) was obtained by acidic hydrolysis of naringin [25]. The formation of both compounds was confirmed by UV–vis spectral properties and melting point analysis [25]. HPLC analysis (see details below) yielded 98% and 95% of purity for CH and NG, respectively.

Typically, photosensitized experiments were performed with 45 μM flavonoid and 8 μM RB aerated ethanol solutions, respectively. An ethanol solution of 50 μM DMA and 8 μM RB was used as actinometer for the determination of the reactive quenching rate constant of the flavonoids by comparison of the observed first-order rate constant for the consumption of the compounds under identical photosensitization conditions [26–28]. All experiments were performed by duplicate and under controlled temperature of 27 ± 1 °C.

2.2. Instrumentation

Stationary photolysis experiments were performed with a 150 W filament lamp (Phillips) coupled with an orange cut-off filter (λ > 530 nm) as excitation source of RB, which $O_2(^1\Delta_g)$ quantum yield is ca. 0.8 in ethanol [29,30]. The excitation beam was focused into temperature controlled 1 cm cell holder, being at right angle of the analyzing light from a fibber-optic diode array UV–vis spectrophotometer (Ocean Optics USB2000). Fluorescence spectra of RB in the absence and presence of flavonoids were recorded with a Hitachi F-2500 instrument.

HPLC experiments were conducted on a Konik-500A liquid chromatograph using a Konik UV–vis 200 detector operating at 285 or 360 nm. The system was equipped with a C_{18} Spherisorb ODS-2 (5 μm, 4.6 mm × 250 mm) column, with water–acetonitrile–acetic acid (79:20:1) as mobile phase at a flow rate of 1 mL/min, the column temperature being set at 25 °C.

Time-resolved phosphorescence detection (TRPD) of $O_2(^1\Delta_g)$ was performed using a Peltier cooled Ge photodiode (Judson J16TE2-66G) placed at right angle of the excitation laser pulse from a Q-switched Nd:YAG laser (Continuum Minilite II), operating at the frequency-doubled output (532 nm, 10 ns FWHM, ca. 14 mJ/pulse) in order to excite the sensitizer RB. Spurious light was obstructed using a 1270 nm band pass filter (Spectrogon BP-1260). The output of the detector was fed *via* amplifier stages to a Tektronix TDS3032B digital oscilloscope linked to an on-line PC for data transfer and analysis. Typically,

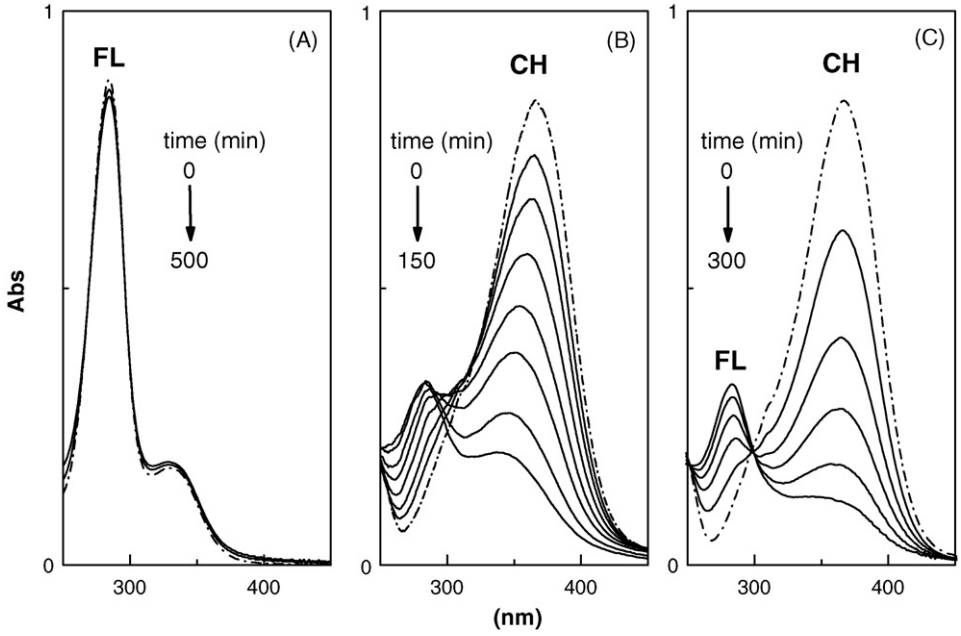


Fig. 1. UV–vis absorption changes in aerated ethanol solutions of 45 μM naringin derivatives produced during the photosensitization of 8 μM RB at $\lambda > 530$ nm: (A) FL and (B) CH. (C) CH → FL isomerization under dark conditions in the presence of RB. The dash-dot line represents the $t = 0$ spectrum.

about 20–30 laser cycles with the excitation laser operating at 5 Hz were averaged in order to obtain the decay times with suitable signal to noise ratio.

Transient absorption decay of the triplet state of RB was recorded at 480 or 620 nm with a Luzchem m-LFP 112 system, using the same laser source that for the TRPD experiments.

2.3. Isolation of the primary photo-oxidation product

The primary reaction product was purified by liquid chromatography (LC). An aliquot of 5 mL of naringin-photosensitized solutions was passed through a C_{18} Sep-Pak cartridge (Alltech Inc.). The fractions were eluted with the same mobile phase that used in the HPLC experiments. The fractions of interest were concentrated under reduced pressure, dried with N_2, and redissolved in ethanol.

3. Results and discussion

3.1. Reactive quenching of $O_2(^1\Delta_g)$

Fig. 1A and B shows the UV–vis spectral changes of 45 μM FL and CH ethanol aerated solutions, respectively, upon excitation of 8 μM RB at $\lambda > 530$ nm. Under these experimental conditions, no changes on either the absorption or emission spectra of RB were observed. In addition, no significant change in the lifetime of the triplet state of RB monitored by transient absorption spectroscopy at 450 or 620 nm N_2-saturated solutions was observed. These results indicate the absence of both ground and excite state interactions between the sensitizer and the flavonoids, in agreement with previous data [10]. Therefore, it can be assumed that the $O_2(^1\Delta_g)$ quantum yield and therefore its photo-stationary concentration was constant under these experimental conditions.

In the case of FL, small spectral changes were observed. In order to compare, under the same photosensitization conditions the reference compound DMA ($k_r^{DMA} = 5 \times 10^7\ M^{-1}\ s^{-1}$ [31]) was completely consumed after a period 100-times shorter that for the photo-oxidation of FL. Therefore, the comparison of the observed first-order rate constants for the consumption of both FL and DMA afforded $k_r^{FL} \approx (5 \pm 10) \times 10^4\ M^{-1}\ s^{-1}$ and that can be taken as upper limit value, considering the detection limit of the method employed. Thus, it can be consider that FL is almost no degraded by $O_2(^1\Delta_g)$ in ethanol solutions.

On the contrary, in the photosensitization reaction of CH the absorption band at 366 nm was depleted and blue-shifted together with absorbance increment at 280 nm. However, the decay times monitored at both wavelengths were largely different and not clear isosbestic points were observed, indicating that the consumption and formation of absorbing species not followed a simple kinetic scheme. In fact, a control experiment under dark conditions, Fig. 1C, showed the simultaneous depletion and growth at 366 and 285 nm, respectively, with a clear isosbestic point at 300 nm. These spectral features were assigned to the CH → FL irreversible isomerization process [13–18]. Therefore, the photosensitized oxygenation reaction of CH is accomplished by dark isomerization process.

The HPLC chromatograms during the dark reaction only showed the peaks of FL ($t_R = 6.6$ min) and CH ($t_R = 14.5$ min), and the kinetic analysis indicated the same rate constant, confirming the isomerization reaction, Fig. 2A. Instead, under photosensitization conditions the HPLC analysis confirmed the simultaneous formation of FL and photo-oxidation products, Fig. 2B. The three new products were labeled as P_1 ($t_R = 9.2$ min), P_2 ($t_R = 4.1$ min) and P_3 ($t_R = 2.8$ min), inset in

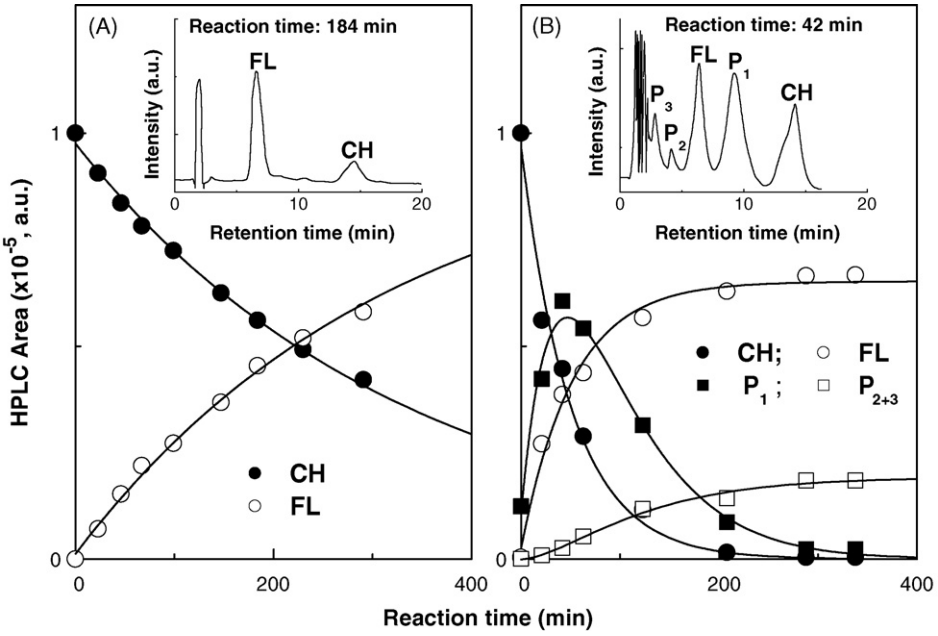


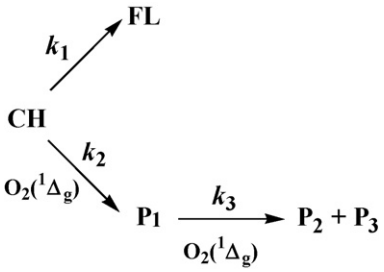
Fig. 2. Kinetic curves determined by HPLC for 45 μM chalcone isomer of naringin (CH) in aerated ethanol after: (A) dark reaction in the presence of RB and (B) photosensitized reaction upon excitation of RB at $\lambda > 530$ nm. Solid lines represent the data fitting with Eqs. (1)–(4). Insets: HPLC chromatograms observed at 285 nm after different reaction times (see Section 2.2 for HPLC conditions).

Fig. 2B. However, in the presence of 50 mM sodium azide (N_3Na), a well-known $O_2(^1\Delta_g)$ quencher [31], or in N_2-saturated solutions, the formation of $P_{1–3}$ was not observed indicating that they were $O_2(^1\Delta_g)$-mediated photoproducts. Instead, under these conditions the isomerization CH → FL occurred with similar rate constant than in dark conditions. These results also indicate the absence of chemical reaction between the excited state of RB and the flavonoids.

The HPLC kinetic profiles of these species in Fig. 2B indicated the simultaneous formation of FL and P_1, being the latter an intermediate in the formation of P_2 and P_3, Scheme 2. According to this mechanism, the integrated Eqs. (1)–(4) describe the concentration of the species assuming that the boundary conditions at $t = 0$ were $[CH] = [CH]_0$ and $[FL]_0 = [P_1]_0 = [P_2]_0 = [P_3]_0 = 0$.

$$[CH] = [CH]_0 \exp(-k_{12}t) \quad \text{with} \quad k_{12} = k_1 + k_2 \tag{1}$$

$$[FL] = [CH]_0 \left(\frac{k_1}{k_{12}}\right) [1 - \exp(-k_{12}t)] \tag{2}$$



Scheme 2. Kinetic model of the dye-sensitized photo-oxidation of the chalcone isomer of aringin in neutral ethanol.

$$[P_1] = [CH]_0 \left[\frac{k_2}{k_3 - k_{12}}\right] [\exp(-k_{12}t) - \exp(-k_3 t)] \tag{3}$$

$$[P_{2,3}] = [CH]_0 \left[1 - \exp(-k_{12}t) - \left[\frac{k_2}{k_3 - k_{12}}\right] \exp(-k_3 t)\right] \tag{4}$$

These equations confirm a first-order kinetic law for the decay and formation of CH and FL, respectively, with the same observed rate constant $k_{12} = (k_1 + k_2)$. In addition, for the photodegradation products $P_{1–3}$, bi-exponential kinetic behavior is expected. The solid lines in Fig. 2 represent the mathematical fitting of the experimental data with Eqs. (1)–(4), allowing the determination of the observed rate constants, e.g. $k_{12} = (3.3 \pm 0.3) \times 10^{-4}\ s^{-1}$ and $k_3 = (2.0 \pm 1.0) \times 10^{-4}\ s^{-1}$. The CH → FL isomerization rate constant $k_1 = (5.1 \pm 0.1) \times 10^{-5}\ s^{-1}$, was determined from the first-order fitting of the data obtained under dark conditions. Thus, $k_2 = (k_{12} - k_1) = (2.8 \pm 0.4) \times 10^{-4}\ s^{-1}$. In turn, $k_2 = k_r^{CH}[\Delta]_{ps}$ and $k_3 = k_r^{P_1}[\Delta]_{ps}$, being $[\Delta]_{ps}$ the photo-stationary $O_2(^1\Delta_g)$ concentration that is constant under our experimental conditions, and k_r^{CH} and $k_r^{P_1}$ are the bimolecular reactive quenching constant of $O_2(^1\Delta_g)$ by CH and P_1, respectively. Their values can be calculated by comparison of k_2 and k_3 with the observed rate constant for the reaction of the reference compound DMA with $O_2(^1\Delta_g)$, $k_{r,ob}^{DMA}$, obtained under identical photo-stationary conditions, Eq. (5) [26–28]:

$$k_r^F = k_r^{DMA} \left(\frac{k_{r,ob}^F}{k_{r,ob}^{DMA}}\right) \tag{5}$$

where $k_{r,ob}^{DMA} = (5.7 \pm 0.3) \times 10^{-3}\ s^{-1}$ as determined by UV–vis absorption experiments (data not shown). In organic sol-

Table 1
Rate constants for the chemical (k_r) and total (k_t) quenching of $O_2(^1\Delta_g)$ by naringin and its derivatives in neutral and 1 mM NaOH ethanol solutions at 27 °C

	Compound	k_r ($\times 10^5$ M^{-1} s^{-1})	k_t ($\times 10^{-7}$ M^{-1} s^{-1})[a]	k_q/k_t (%)
Flavanone	Neutral	$<0.5 \pm 1.0$[b]	0.6 ± 0.1	>99
	1 mM NaOH	130 ± 30[c]	14.3 ± 0.7	91
Chalcone	Neutral	25 ± 5[c]	5.3 ± 0.1	95
	1 mM NaOH	590 ± 50[c]	21.3 ± 2.0	86
Naringenin	Neutral	$<0.5 \pm 1.0$[b]	0.1 ± 0.2	>95
	1 mM NaOH	43 ± 8[b]	3.6 ± 0.5	88
P_1	Neutral	20 ± 5[b], 18 ± 10[c]	n.d.	–
	1 mM NaOH	61 ± 10[b]	n.d.	–

n.d.: not determined.
[a] From TRPD experiments.
[b] From UV–vis experiments.
[c] From HPLC experiments.

vents, the bimolecular reactive quenching constant of $O_2(^1\Delta_g)$ by DMA, $k_r^{DMA} = (5 \pm 1) \times 10^7$ M^{-1} s^{-1} [31]. Therefore, Eq. (5) yields $k_r^{CH} = (2.5 \pm 0.5) \times 10^6$ M^{-1} s^{-1} and $k_r^{P_1} = (1.8 \pm 1.0) \times 10^6$ M^{-1} s^{-1}, respectively, Table 1.

Fig. 3A and B shows the UV–vis spectral changes produced for both 45 μM FL and CH in 1 mM NaOH ethanol aerated solutions during the photosensitization of 8 μM RB at $\lambda > 530$ nm. Fig. 3C shows the spectral changes of FL produced in the presence of 50 mM NaN_3 under the same experimental conditions.

The dashed lines in Fig. 3 represent the initial UV–vis spectra of FL and CH in ethanol solution before the addition of the base. After the addition of NaOH, instantaneous bathochromic shifts on the flavonoid spectra were observed (dash-dot spectra), e.g. new bands at 245 and 365 nm were observed for FL, and broad absorption band at 430 nm for CH. These new bands were associated with the fully anionic form of the flavonoids, e.g. FL^{3-} (carbanion) and CH^{3-} (chalconate anion) [13], Scheme 1.

The UV–vis spectra of both FL and CH-photosensitized solutions showed the formation of a new absorption band at 403 nm. In contrast, photosensitization of FL solution in the presence of 50 mM NaN_3 produced the slower growth of the absorption band at 430 nm, which was associated with the simple formation of CH in basic media [13]. These results suggest that the band at 403 nm corresponds to an $O_2(^1\Delta_g)$ photoproduct.

Fig. 4A and B shows the HPLC kinetic analysis of the photosensitized reaction of FL^{3-} and CH^{3-}, respectively. In both

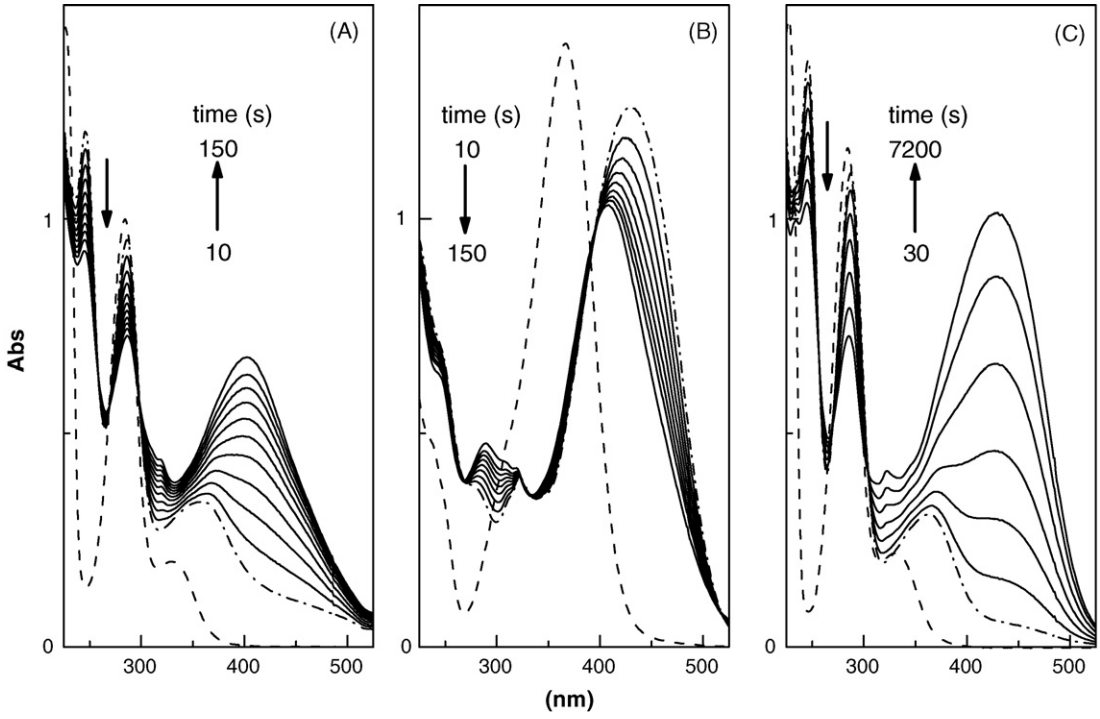


Fig. 3. UV–vis absorption changes of 45 μM naringin derivatives in aerated 1 mM NaOH ethanolic solutions produced during the photosensitization of 8 μM RB at $\lambda > 530$ nm for: (A) FL^{3-}, (B) CH^{3-}, and (C) FL^{3-} in the presence of 50 mM of sodium azide (NaN_3). The dash and dash-dot line symbolizes the spectrum of the flavonoid in ethanol and after addition of NaOH, respectively.

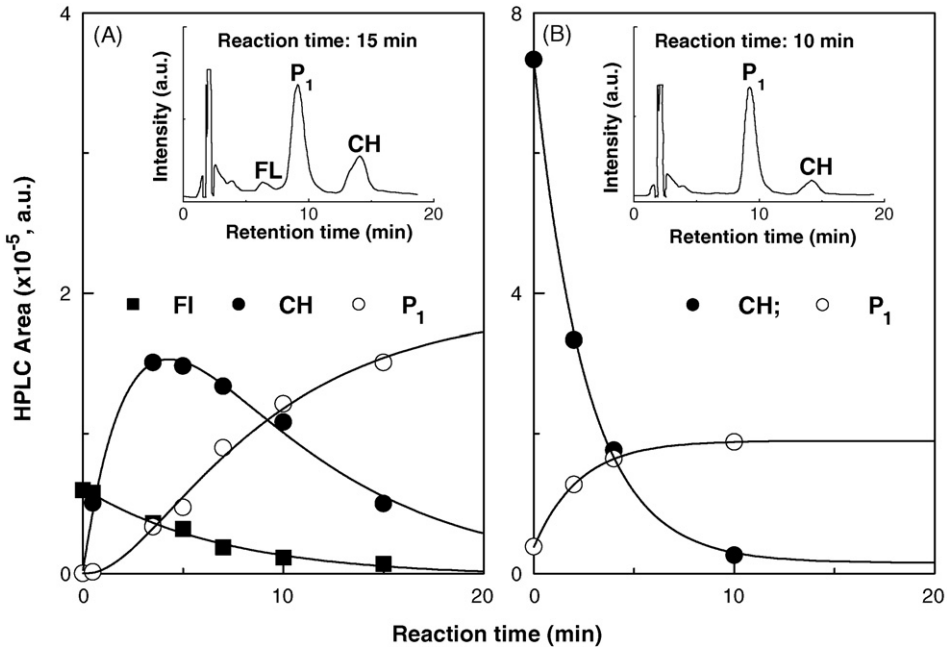


Fig. 4. Kinetic curves determined by HPLC for 45 μM naringin derivatives observed upon photosensitization of 8 μM RB at $\lambda > 530$ nm in aerated 1 mM NaOH ethanol solution: (A) FL^{3-} and (B) CH^{3-}. Solid lines represent the data fitting with Eqs. (6)–(10). Insets: HPLC chromatograms observed at 360 nm after different reaction times (see Section 2.2 for HPLC conditions).

cases, the formation of a product with identical retention time (9.2 min) was observed, insets Fig. 4. This retention time was coincident with that observed for P_1 in the photosensitized reaction of CH in ethanol solutions (see above). Taking into account the acid content of the mobile phase in the HPLC determinations (see Section 2), the flavonoids were detected in its protonated form. Therefore, it can be assumed that the absorption band at 403 nm corresponded to the fully anionic form of P_1, e.g. P_1^{p-}.

The kinetic curves in Fig. 4A showed that FL^{3-} was consumed following first-order decay, while both CH^{3-} and P_1^{p-} displayed bi-exponential behavior, with CH^{3-} being intermediate for the formation of P_1^{p-}. However, in the photosensitized reaction of CH^{3-}, only the simultaneous first-order decay and formation of CH^{3-} and P_1^{p-} was observed, Fig. 4B. All these experimental features for the photosensitized degradation of FL and CH in alkaline ethanol can be summarized in Scheme 3.

In the case of FL^{3-} as initial reactant, the initial boundary conditions at $t = 0$ were $[FL^{3-}] = [FL]_0$ and $[CH^{3-}]_0 = [P_1^{p-}]_0 = 0$, and the integrated equations for the concentration of the species are given by Eqs. (6)–(8).

$$[FL^{3-}] = [FL]_0 \exp(-k_{45}t) \quad \text{with} \quad k_{45} = k_4 + k_5 \tag{6}$$

$$[CH^{3-}] = [FL]_0 \left[\frac{k_4}{k_6 - k_{45}}\right] [\exp(-k_{45}t) - \exp(-k_6 t)] \tag{7}$$

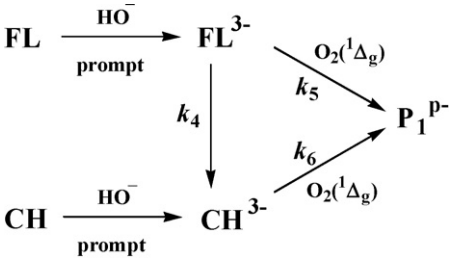
$$[P_1^{p-}] = [FL]_0 \left[1 - \left[\frac{k_6 - k_5}{k_6 - k_{45}}\right] \exp(-k_{45}t) - \left[\frac{k_5}{k_6 - k_{45}}\right] \exp(-k_6 t)\right] \tag{8}$$

Otherwise, for CH^{3-} as starting material, the integrated Eqs. (9) and (10) with the boundary conditions at $t = 0$, $[CH^{3-}]_0 = [CH]_0$, and $[P_1^{p-}] = 0$ were obtained.

$$[CH^{-3}] = [CH]_0 \exp(-k_6 t) \tag{9}$$

$$[P_1^{p-}] = [CH]_0[1 - \exp(-k_6 t)] \tag{10}$$

The values of $k_{45} = (2.3 \pm 0.5) \times 10^{-3}\ s^{-1}$ and $k_6 = (6.7 \pm 1.3) \times 10^{-3}\ s^{-1}$ were obtained from the exponential or bi-exponential fitting using Eqs. (6)–(10), as showed by the solid lines in Fig. 4. The rate constant for the $FL^{3-} \rightarrow CH^{3-}$ reaction, $k_4 = (8.2 \pm 0.5) \times 10^{-4}\ s^{-1}$, was obtained by exponential fitting of the 430 nm absorption band of CH^{3-} either in the presence of 50 mM NaN_3 or under dark conditions, Fig. 3C, and therefore $k_5 = (k_{45} - k_4) = (1.5 \pm 0.5) \times 10^{-3}\ s^{-1}$. In turn, $k_5 = k_r^{FL^{3-}}[\Delta]_{ps}$ and $k_6 = k_r^{CH^{3-}}[\Delta]_{ps}$, where $k_r^{FL^{3-}}$ and $k_r^{CH^{3-}}$ are the bimolecular reactive quenching constant of $O_2(^1\Delta_g)$ by FL^{3-} and CH^{3-}, respectively. Again, neither ground nor excited state interactions between sensitizer and the flavonoids in alka-



Scheme 3. Kinetic model of the dye-sensitized photo-oxidation of naringin (flavanone) and its chalcone isomer in 1 mM NaOH ethanol solutions.

line media were observed by fluorescence or transient absorption spectroscopy of RB, and therefore it can be expected that $[\Delta]_{ps}$ is almost constant. Thus, $k_r^{FL^{3-}} = (1.3 \pm 0.3) \times 10^7\ M^{-1}\ s^{-1}$ and $k_r^{CH^{3-}} = (5.9 \pm 0.5) \times 10^7\ M^{-1}\ s^{-1}$ were calculated using Eq (5).

The primary photo-oxidation product P_1 obtained from the photosensitization experiments of FL or CH in basic media was isolated by LC procedures (see Section 2.3). The absorption maxima were 359 and 403 nm in neutral and basic ethanol, respectively, being the spectral changes reversibles by the addition of HCl. These pH-depending prompt absorption changes confirmed their assignment to the acid–base equilibria of the phenolic groups of P_1, and therefore the absorption band at 403 nm corresponded to the deprotonated species P_1^{p-}, as mentioned before. The photosensitized degradation kinetics of both P_1 and P_1^{p-} were monitored by UV–vis spectroscopy (data not shown), and followed first-order kinetics. In both cases, clear isosbestic points were observed during the time window studied, indicating that the degradation kinetic followed a simple mechanism. The photosensitized reaction was abated by the presence of NaN_3, indicating that the photodegradation of both P_1 and P_1^{p-} was also $O_2(^1\Delta_g)$-mediated. By using DMA as reference compound and Eq. (5), the values of $k_r^{P_1} = (2.0 \pm 0.5) \times 10^6\ M^{-1}\ s^{-1}$ and $k_r^{P_1^{p-}} = (6.1 \pm 1.0) \times 10^7\ M^{-1}\ s^{-1}$ in neutral and alkaline solutions were obtained, respectively, Table 1.

In order to compare, the reactivity of the aglycone naringenin (NG) towards $O_2(^1\Delta_g)$ was also analyzed. The UV–vis absorption spectra of NG in neutral and 1 mM NaOH ethanol solutions were identical with those reported in literature [25], with absorption maxima at 290 and 330 nm, respectively. In addition, no slower spectral changes were observed in alkaline media and the absorption changes were reversible after neutralization with HCl solution. These results indicated that the flavanone–chalcone isomerization equilibrium with carbanion formation does not occur, in accord with the $pK_{1/2} = 14$ for the formation of the carbanion of NG [17]. Therefore, the bathochromic shift in 1 mM NaOH ethanol solutions was assigned to the fully deprotonated form of NG, e.g. NG^{3-}. The photosensitized reaction of both NG and NG^{3-} were monitored by UV–vis spectroscopy (data not shown). As in the case of naringin, in neutral ethanol the chemical reactivity of NG towards $O_2(^1\Delta_g)$ was close to the detection limit of our experimental set-up, and the k_r was estimated as $<(5 \pm 10) \times 10^{-4}\ M^{-1}\ s^{-1}$, in agreement with the result obtained by Tournaire et al. in methanol solutions [10]. However, in basic media, NG^{3-} species was more reactive towards $O_2(^1\Delta_g)$ with $k_r = (4.3 \pm 0.5) \times 10^{-6}\ M^{-1}\ s^{-1}$, Table 1.

3.2. Total quenching of $O_2(^1\Delta_g)$

Fig. 5 shows the Stern–Volmer plots (Eq. (11)) obtained for the $O_2(^1\Delta_g)$ quenching by both FL and CH in neutral and alkaline ethanol solutions, where k_t is the total quenching rate constant of $O_2(^1\Delta_g)$ by the flavonoids, it being the sum of the chemical and physical quenching rate constants, e.g. $k_t = k_r + k_q$

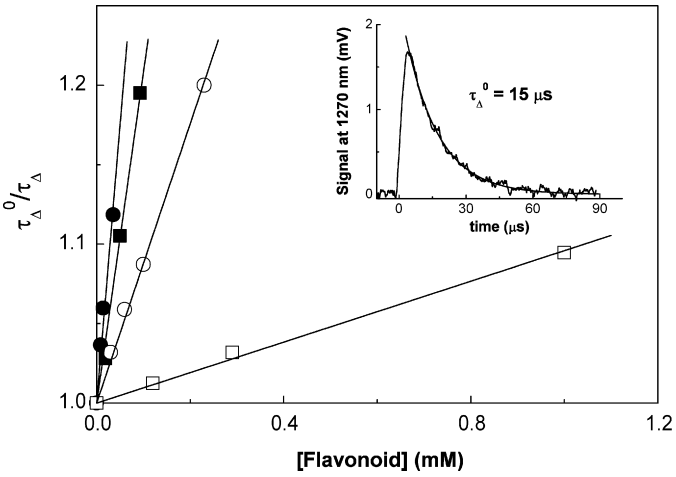


Fig. 5. Stern–Volmer plots for the 1270 nm phosphorescence quenching of $O_2(^1\Delta_g)$ by naringin derivatives in neutral and 1 mM NaOH ethanol, with RB as photosensitizer at 532 nm: (●) CH^{3-}, (■) FL^{3-}, (◯) CH, and (□) FL. Inset: typical $O_2(^1\Delta_g)$ decay in 1 mM NaOH ethanol in the absence of flavonoid.

[10,11,27,28,31].

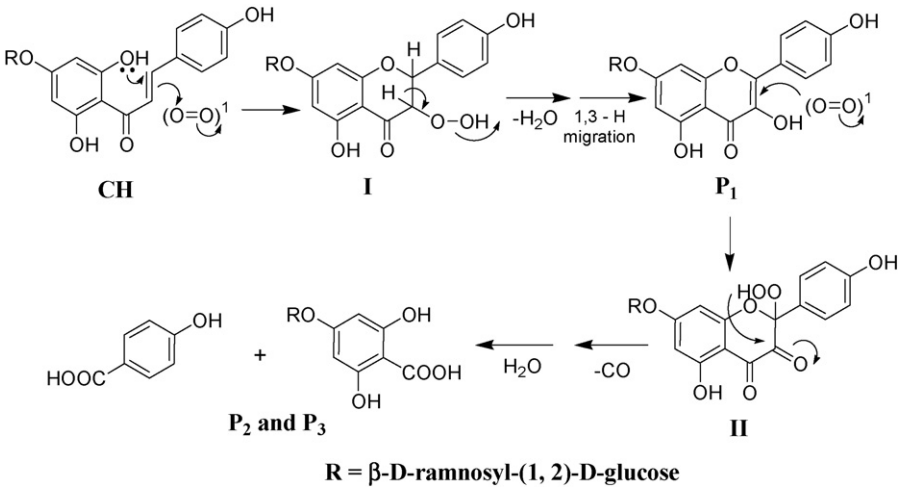
$$\frac{\tau_\Delta^0}{\tau_\Delta} = 1 + k_t \tau_\Delta^0 [F] \quad (11)$$

The decay portion of the phosphorescence signals were fitted with an exponential function in order to obtain the lifetime of $O_2(^1\Delta_g)$, τ_Δ, inset of Fig. 5. In the absence of flavonoids, the τ_Δ^0 were 16 and 15 μs in neutral and 1 mM NaOH, respectively, in good agreement with literature data [31]. A slightly shorter τ_Δ^0 in basic ethanol can be expected, due to the small amount of water (<3%, V/V) dissolved by the addition of a stock of NaOH aqueous solution. The slope analysis of the linear Stern–Volmer plots allowed the determination of k_t, giving unambiguous evidence for the interaction between $O_2(^1\Delta_g)$ and the flavonoids species, Table 1.

3.3. Reactivity and antioxidant ability towards $O_2(^1\Delta_g)$

From both k_r and k_t values reported in Table 1, it can be observed that in alkaline media all the flavonoids species were more efficient quenchers of $O_2(^1\Delta_g)$ than in neutral ethanol. It is well known that polyphenolic compounds are more efficient $O_2(^1\Delta_g)$ quenchers when their hydroxyl groups are fully ionized [27]. In those cases, the mechanism involves an excited state encounter complex with a partial charge-transfer character, in which the $O_2(^1\Delta_g)$ is the acceptor species. Therefore, the $O_2(^1\Delta_g)$ quenching efficiency should be mainly affected by the ability of the substrate to be an electron-donor, being enhanced in the anionic flavonoids, explaining the much higher values of both k_r and k_t in the alkaline media. Similar reactivity enhancement was also observed for 7-hydroxyflavanone in MeCN–D_2O mixtures, which k_t increased from $3.8 \times 10^6\ M^{-1}\ s^{-1}$ to $2.6 \times 10^7\ M^{-1}\ s^{-1}$ upon addition of 10 mM NaOH [11].

In neutral ethanol, the CH isomer was almost one-order of magnitude more efficient quencher of $O_2(^1\Delta_g)$ that FL. A similar result was observed for the $O_2(^1\Delta_g)$ quench-



Scheme 4. Suggested mechanism and possible oxidation products for the photo-oxygenation of the chalcone isomer of naringin in neutral ethanol.

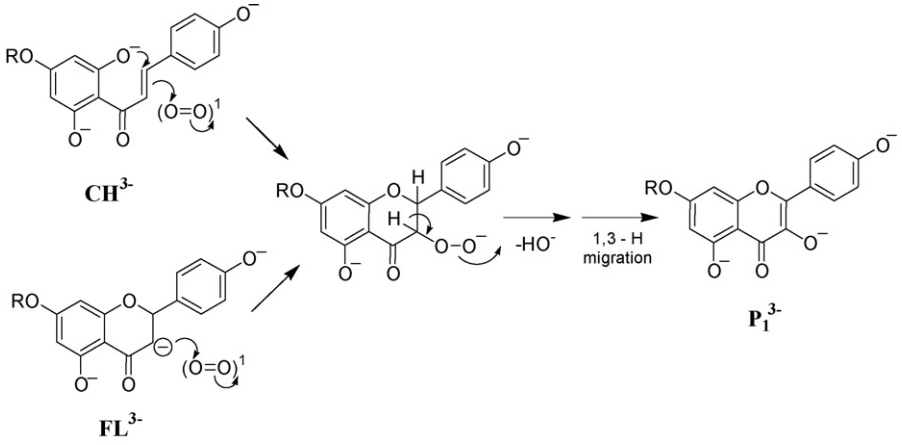
ing by 2′,4′-dihydroxy-3-methoxychalcone and 7-hydroxy-8-methoxyflavanone in benzene [11]. An explanation of this behavior is the presence of the electron rich Cα=Cβ double bond in the chalcones that increases the interaction with the electrophilic $O_2(^1\Delta_g)$. The effect of the Cα=Cβ double bond on the flavonoid skeleton on the $O_2(^1\Delta_g)$ quenching can be also noted by comparison of the k_t values in ethanol solutions of apigenin (5,7,4′-trihydroxyflavanone, $k_t = 2.8 \times 10^7\ M^{-1}\ s^{-1}$) with NG (5,7,4′-trihydroxyflavanone, $k_t = 3.7 \times 10^6\ M^{-1}\ s^{-1}$) reported by Nagai et al. [12].

On the other hand, both k_r and k_t values for naringin in basic media were about four-times larger than those for NG. This is an interesting result, since both ionized flavonoids contain the same number of anionic charges. However, a larger reactivity for the carbanion of naringin FL^{3-} than for the triphenolate NG^{3-} can be expected, due to the increment of electron density at C3 of the carbanion favouring the electrophilic attack of $O_2(^1\Delta_g)$.

In Table 1 is also shown the percentage of the ratio k_q/k_t, which represents the relative amount of physical quenching of $O_2(^1\Delta_g)$ produced by the flavonoids. This magnitude can be taken as an catalytic antioxidant index for $O_2(^1\Delta_g)$, since lower value of the ratio indicates larger degradation of the flavonoids. From this point of view, better catalytic antioxidant behavior is observed in neutral than in alkaline conditions. In spite of the its lower k_t value in neutral ethanol, naringin showed the best performance as catalytic antioxidant of $O_2(^1\Delta_g)$. This type of behavior has been reported for different flavonoids and different phenolic derivatives, where it was found that k_q is about two or three orders of magnitude larger than k_r [10,11,27].

3.4. Photo-oxygenation products

It has been reported that the photosensitized oxygenation of 2′,6′-dihydroxychalcones produces flavonols (3-hydroxyflavones) and other degradation products, such as benzoic acid and benzaldehyde derivatives [20,21]. In this mechanism, intramolecular nucleophilic attack of the 2′-OH group on the Cβ of the chalcone is envisaged, together with the 2 + 2 addition of $O_2(^1\Delta_g)$ to the Cα=Cβ double bond producing a hydroperoxide intermediate I, which after loss of water and 1,3-hydrogen migration yield the respective flavonol [20,21]. Consecutively, the addition of a second $O_2(^1\Delta_g)$ molecule to the flavonol produces a ketohydroperoxide intermediate II, which after rearrangement into a 4-membered cyclic peroxide followed



Scheme 5. Suggested mechanism of formation of the primary photo-oxidation P_1^{p-} in alkaline ethanol from both FL^{3-} and CH^{3-} species.

of cleavage and decarbonylation to give the corresponding depside, hydrolyzes to produces benzoic acid derivatives, among other products [23]. According to this reported mechanism, in the present case the formation of 7-rhamnoglucosyl-5,4′-dihydroxyflavonol as primary photo-oxygenation product P_1, and 4-hydroxybenzoic and 4-rhamnoglucosyl-2,6-dihydroxybenzoic acids as its photo-oxygenation products, e.g. P_2 and P_3, is suggested, Scheme 4. HPLC experiments of co-injection with authentic 4-hydroxybenzoic acid confirmed the identification of the peak labeled as P_2 as this compound, and the UV–vis spectral features of P_1 and P_1^{p-} were closer to those reported for kaempferol derivatives under similar experimental conditions [25], supporting the proposed mechanism. However, further MS and/or NMR experiments should be performed in order to corroborate the product structure.

Finally, the formation of the same primary photo-oxygenation product P_1 by reaction of $O_2(^1\Delta_g)$ with either CH^{3-} or FL^{3-} in alkaline ethanol, can be explained on the basis of the formation of the same hydroperoxide intermediate I, due to the increased electron density at the C3 in FL^{3-} and in the Cα=Cβ double bond in CH^{3-}, Scheme 5.

4. Conclusions

The natural flavanone naringin and its derivatives presented interesting properties of photoprotection against $O_2(^1\Delta_g)$-mediated reactions due to the high predominance of the physical quenching pathway, preventing the photodegradation of the flavonoid. The chalcone is a more efficient quencher than the flavanone isomer. However, in alkaline media the reactivity of the flavanone was increased due to formation of a carbanion species at C3. This species reacts more efficiently with $O_2(^1\Delta_g)$ than the neutral flavanone, but also isomerizes to the chalcone. For both naringin isomers, 4-hydroxybenzoic acid was identified as one of the ending oxidation products. A possible oxidation mechanism can involve the formation of a flavonol (e.g. 7-rhamnoglucosyl-5,4′-dihydroxyflavonol) as primary photo-oxidation product. These results may have significance in stability and biogenesis of naturally occurring flavonoids, and in their potential applications in food and pharmaceutical industries as $O_2(^1\Delta_g)$ antioxidants.

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References

[1] O. Benavente-García, J. Castillo, F.R. Marin, A. Ortuño, J.A. Del Río, Uses and properties of citrus flavonoids, J. Agric. Food Chem. 45 (1997) 4505–4515.

[2] E.B. Rimm, M.B. Katan, A. Ascherio, M.J. Stampfer, W.C. Willet, Relation between intake of flavonoids and risk for coronary heart disease in male health professionals, Ann. Intern. Med. 125 (1996) 384–389.

[3] P. Knekt, R. Järvinen, R. Seppänen, M. Hellövaara, L. Teppo, E. Pukkala, E. Aromaa, Dietary flavonoids and the risk of lung cancer and other malignant neoplasm, Am. J. Epidemiol. 146 (1997) 223–230.

[4] S. Kanno, A. Tomizawa, T. Hiura, Y. Osanai, A. Shouji, M. Ujibe, T. Ohtake, K. Kimura, M. Ishikawa, Inhibitory effects of naringenin on tumour growth in human cancer cell lines and sarcoma S-180-implantedmice, Biol. Pharm. Bull. 28 (2005) 527–530.

[5] G. Igile, W. Oleszek, M. Jurzysta, S. Burda, M. Fafunso, A. Fasanmade, Flavonoids form *Vernonia amygdalina* and their antioxidant activities, J. Agric. Food Chem. 42 (1994) 2445–2448.

[6] J. Torel, J. Cillard, P. Cillard, Antioxidant activity of flavonoids and reactivity with peroxy radical, Phytochemistry 25 (1988) 383–385.

[7] Y. Miyake, K. Minato, S. Fukumoto, K. Yamamoto, T. Oya-Ito, S. Kawakishi, T. Osawa, New potent antioxidative hydroxyflavanones produced with *Aspergillus saitoi* from flavanone glycoside in citrus fruit, Biosci. Biotechnol. Biochem. 67 (2003) 1443–1450.

[8] J. Yu, L. Wang, R.L. Walzem, E.G. Miller, L.M. Pike, B.S. Patil, Antioxidnat activity of citrus limonoids, flavonoids, and coumarins, J. Agric. Food Chem. 53 (2005) 2009–2014.

[9] S. Criado, S.G. Bertolotti, A.T. Soltermann, V. Avila, N.A. García, Effect of flavonoids on the photooxidation of fats—a study on their activity as singlet molecular oxygen [$O_2(^1\Delta_g)$] generators and quenchers, Fat Sci. Technol. 97 (1995) 265–269.

[10] C. Tournaire, S. Croux, M.-T. Maurette, I. Beck, M. Hocquaux, A.M. Braun, E. Oliveros, Antioxidant activity of flavonoids: efficiency of singlet oxygen ($^1\Delta_g$) quenching, J. Photochem. Photobiol. B: Biol. 19 (1993) 205–215.

[11] V. Avila, S.G. Bertolotti, S. Criado, N. Pappano, N. Debattista, N.A. García, Antioxidant properties of natural flavonoids: quenching and generation of singlet molecular oxygen, Int. J. Food Sci. Technol. 36 (2001) 25–33.

[12] S. Nagai, K. Ohara, K. Mukai, Kinetic study of the quenching reaction of singlet oxygen by flavonoids in ethanol solution, J. Phys. Chem. B 109 (2005) 4234–4240.

[13] E.A. Gonzalez, M.A. Nazareno, C.D. Borsarelli, Enthalpy–entropy compensation effect in the chalcone formation from naringin in water–ethanol mixtures, J. Chem. Soc., Perkin Trans. 2 (2002) 2052–2056.

[14] E.A. Gonzalez, PhD Thesis. Reactividad química de flavanonas presentes en cítricos: su relación con aplicaciones industriales y nutraceúticas, Universidad Nacional de Santiago del Estero, Santiago del Estero, Argentina, 2005.

[15] R.C. Lindsay, in: O.R. Fenema (Ed.), Food Chemistry, 3th ed., Marcel Dekker, New York, 1996, p. 731.

[16] C.O. Miles, L. Main, The kinetics and mechanism, and the equilibrium position as a function of pH, of the isomerization of naringin and the 4′-rhamnoglucoside of 2′,4,4′,6′-tetrahydroxychalcone, J. Chem. Soc., Perkin Trans. II (1988) 195–198.

[17] A. Cisak, C. Mielczarek, Practical and theoretical aspects of flavanone–chalcone isomerisations, J. Chem. Soc., Perkin Trans. 2 (1992) 1603–1607.

[18] J.J.P. Furlong, N. Sbarbati Nudelman, Mechanism of cyclization of substituted 2′-hydroxychalcones to flavanones, J. Chem. Soc., Perkin Trans. II (1985) 633–639.

[19] K. Hahlbrock, H. Grisebach, in: J.B. Harborne, T.J. Mabry, H. Mabry (Eds.), The Flavonoids, Chapman and Hall, London, 1975, p. 866.

[20] H.M. Chawla, S.S. Chibber, Biologically patterned sensitized photooxygenation of chalcones, Tetrahedron Lett. 25 (1976) 2171–2172.

[21] H.M. Chawla, K. Chakrabarty, Dye-sensitized photo-oxygenation of chalcones, J. Chem. Soc., Perkin Trans. I (1984) 1511–1513.

[22] H.M. Chawla, S.K. Sharma, Regioselective synthesis of 2-arylidene coumaran-3-ones by dye-sensitized photooxygenation of 2-hydroxyphenyl-styrylketones in the presence of sodium dodecyl sulphate, Tetrahedron 46 (1990) 1611–1624.

[23] T. Matsuura, H. Matsushima, R. Nakashima, Photoinduced reactions, XXXVI. Photosensitized oxygenation of 3-hidroxyflavones as a nonenzymatic model quercetinase, Tetrahedron 26 (1970) 435–443.

[24] M. Shimokoriyama, Interconversion of chalcones and flavanones of a phloroglucinol-type structure, J. Am. Chem. Soc. 79 (1957) 4199–4202.
[25] T. Mabry, K. Markham, M. Thomas, The Systematic Identification of Flavonoids, Springler–Verlag, New York, 1970.
[26] F.E. Scully, J. Hoigné, Rate constants for reactions of singlet oxygen with phenol and other compounds in water, Chemosphere 16 (1987) 681–694.
[27] N.A. García, Singlet molecular oxygen-mediated photogeneration of aquatic phenolic pollutants. A kinetic and mechanistic overview, J. Photochem. Photobiol. B: Biol. 22 (1994) 185–196.
[28] M.A. Montenegro, M.A. Nazareno, E.N. Durantini, C.D. Borsarelli, Singlet oxygen quenching ability of carotenoids in a reverse micelle membrane mimetic system, Photochem. Photobiol. 75 (2002), 353–342.
[29] F. Wilkinson, W.P. Helman, A.B. Ross, Quantum yields for the photosensitized formation of the lowest electronically excited singlet state of molecular oxygen in solution, J. Phys. Chem. Ref. Data 22 (1993) 113–262.
[30] R. Redmond, J.N. Gamlin, A compilation of singlet oxygen yields from biologically relevant molecules, Photochem. Photobiol. 70 (1999) 391–475.
[31] F. Wilkinson, W.P. Helman, A.B. Ross, Rate constants for the decay and reactions of the lowest electronically excited state of molecular oxygen in solution. An expanded and revised compilation, J. Phys. Chem. Ref. Data 24 (1995) 663–1021.