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Photoprotection of Vitamins in Skimmed Milk by an Aqueous Soluble Lycopene–Gum Arabic Microcapsule

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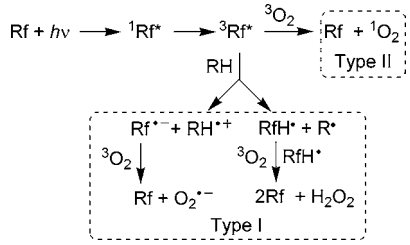
Riboflavin (Rf)-mediated photosensitized degradation of vitamins A and D_3 in skimmed milk under illumination with a white fluorescence lamp was studied by using the HPLC technique. The photosensitized degradation of both vitamins followed first-order kinetics, and the temperature effect on the observed photodegradation rate constant allowed the determination of the activation energy E_a as being 4 and 16 kcal/mol for vitamins A and D_3, respectively. The addition of lycopene microencapsulated by spray-drying with a gum arabic−sucrose (8:2) mixture (MIC) produced a reduction of ca. 45% in the photosensitized degradation rate of both vitamins. Front-face fluorescence experiments showed the same photoprotection factor in the degradation of Rf itself, indicating that the photodegradation mechanism involved Rf-mediated reactive species, such as the excited triplet state of Rf, ^{3}Rf*, and/or singlet molecular oxygen, $^1O_2$. The interaction of both ^{3}Rf* and $^1O_2$ with MIC was evaluated in aqueous solutions by using laser-induced time-resolved absorption or emission spectroscopy, and the contribution of an inner-filter effect in the presence of MIC in skimmed milk was evaluated by diffuse reflectance spectroscopy. The main operating mechanism of photoprotection is due to the deactivation of ^{3}Rf* by the proteic component of gum arabic; thus, gum arabic based microcapsules could be used to improve the photostability of milk during its storage and/or processing under light.

KEYWORDS: Photooxidation; vitamins; milk; riboflavin; triplet states; singlet oxygen; lycopene−gum arabic microcapsules

INTRODUCTION

The changes of the flavor and nutritional value of milk caused by light are well-known from the past century, as reviewed by Bradley (*1*). In particular, photoinduced degradation of vitamins (A, B_2, D, etc.), proteins, and lipids has been reported to occur in either milk or skimmed milk (*2*−*9*). The shelf life and quality of milk and dairy products are reduced by visible light in the range of 400−500 nm (*10*). The photodegradation mechanism is based on the formation of the triplet excited state of riboflavin (Rf) (*1*−*10*), also known as vitamin B_2, which is able to initiate oxidation reactions in the presence of either a biological molecule (RH) or molecular oxygen, $^3O_2$, **Scheme 1** (*11*). The riboflavin photosensitized oxidation of milk destroys vitamins and Rf itself with concomitant production of hexanal and dimethyl disulfide by oxidation of linoleic acid and methionine, which in turn are responsible for a rancid and sunlight off-flavor, respectively (*1*, *12*).

Scheme 1. Type I and Type II Photosensitized Oxidation Mechanisms Involving Rf in Fluid Aerobic Solutions



Two mechanisms have been proposed for photooxidation reactions where Rf acts as a photosensitizer, one being based on direct photooxidation of RH by either electron or hydrogen abstraction and subsequent reaction of the radical species with surrounding oxygen molecules to produce reactive oxygen species (ROS), such as anion superoxide ($O_2^{\bullet-}$) and hydrogen peroxide (H_2O_2) (type I mechanism), and the second involving electronic excitation energy transfer to molecular oxygen to generate singlet molecular oxygen ($^1O_2$) (type II mechanism). Type I reactions are expected to occur closer to the photosen-

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sitizer location considering the low diffusivity and high reactivity of the intermediate species formed. On the contrary, type II reactions are able to occur hundreds of nanometers from the photoexcitation site, due to the large diffusivity and microsecond lifetime of $^1O_2$.

Several studies reported $^1O_2$-mediated photosensitized oxidation of vitamins or related compounds in model solvents or milk (*2*−*10*, *12*−*17*), and recently, the Rf photosensitized formation of $^1O_2$ in skimmed milk has been detected using ESR spectroscopy (*18*), indicating the relevance of this species in the oxidation of target molecules in beverages and food systems. Carotenoids are naturally occurring efficient quenchers of $^1O_2$ (*19*, *20*), and therefore, their presence in food systems can avoid the $^1O_2$-mediated photooxidation of target molecules (*19*). Recently, Bradley et al. (*9*) proposed the use of 1,4-diazabicyclo-[2,2,2]octane (DABCO) and 2,5-dimethylfuran (DMF), both as efficient $^1O_2$ quenchers, for protection of milk nutrients from $^1O_2$-mediated oxidation. However, to obtain ca. 50% photoprotection, a large molar concentration (>5 mM) of either DABCO or DMF was needed, precluding their use by possible toxicological and/or flavor changes.

On the other hand, in the case of Rf-mediated photooxidations, both type I and type II mechanisms can be abated, avoiding the formation of the precursor ^{3}Rf* by an external- or inner-filter effect (*21*) or by efficient deactivation of ^{3}Rf* by the presence of quencher molecules (*22*), in particular by using nontoxic or edible compounds. Thus, the objective of this research was to determine the protective effect of aqueous soluble microcapsules of lycopene in a gum arabic−sucrose matrix (MIC), prepared using a spray-dried technique (*23*), on the Rf-mediated photooxidation of vitamins A, D_3, and Rf itself in skimmed milk. Detailed studies on the ^{3}Rf* deactivation, inner-filter effect, and $^1O_2$ quenching efficiency induced by MIC were also carried out.

MATERIALS AND METHODS

Materials. Lycopene crystals (96% purity) were obtained from fresh tomatoes following extraction and purification methods reported elsewhere (*24*). Rf (Fluka), vitamins A (Sigma, St. Louis, MO) and D_3 (Fluka), deuterium oxide (Aldrich, 99.9%), and Rose Bengal as a sodium salt (RB; Sigma) were used as received. The organic solvents were either HPLC (EM Science, Darmstadt, Germany) or analysis grade (Synth, Diadema, Brazil). Gum arabic, food grade, was obtained from Colloides Naturels Brasil (São Paulo, Brazil), and sucrose of analytical grade was from Synth. Distilled water was purified in a Milli-Q system (Millipore, Bedford, MA), and the samples and solvents were filtered through Millipore membranes (0.22 and 0.45 μm). The skimmed milk samples were prepared using a well-recognized Brazilian commercial brand, following the factory instructions.

Methods. Lycopene microencapsules (*25*) were prepared by spray-drying using a laboratory-scale spray-drier system (Lab Plant SD-04, Huddersfield, U.K.) operating at an air pressure of 5 kgf/cm^2 and an air flow rate of 30 mL/min (entrance and exit air temperatures of 170 and 113 °C, respectively), with an aspersion nozzle diameter of 0.7 mm. An emulsion of gum arabic−sucrose (8:2) was prepared in distilled water at 45 °C and kept under continuous stirring until the temperature reached 30 °C. Lycopene crystals (15 mg) were dissolved in dichloromethane and added to the above emulsion, maintaining vigorous mechanical stirring (7000 rpm). Afterward, distilled water was added to obtain a final concentration of a 20% (w/v) soluble solid solution. The emulsion was maintained under slow agitation during the spray-drying process. The microcapsules (MIC) were immediately stored, under N_2, in a glass bottle at −20 °C. The average microencapsulation efficiency of lycopene was ca. 95%, as determined by total carotenoid extraction with methanol followed by exhaustive extraction with dichloromethane from an aqueous solution of MIC. Superficial lycopene was directly extracted with dichloromethane. The organic phase was separated and dried with anhydrous Na_2SO_4. The dichloromethane was removed under vacuum at room temperature, and lycopene was redissolved in petroleum ether for quantification by UV−vis absorption spectroscopy at 470 nm ($\epsilon^{470} = 1.85 \times 10^5$ M^{-1} cm^{-1}) (*26*). The final lycopene concentration was 230 μg/g of microcapsules.

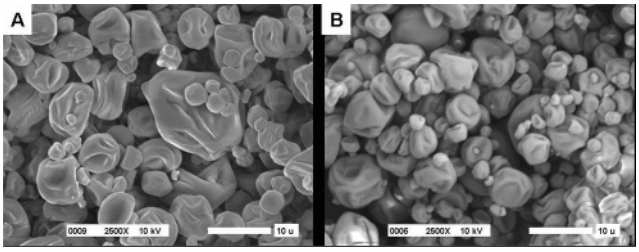

Figure 1. SEM micrographs (magnified 2500×) of spray-dried microcapsules: (**A**) gum arabic–sucrose without lycopene (empty microcapsules), (**B**) gum arabic–sucrose with lycopene (filled microcapsules).

The morphology of the microcapsules was evaluated by scanning electronic microscopy (SEM) (JEOL, T-30 model, Tokyo, Japan) using an acceleration voltage of 10 kV. The microcapsules were fixed in stubs containing a double-faced adhesive metallic tape and coated with gold in a Balzers evaporator (SCD 050, Lichtenstein, Austria) for 75 s, with a current of 40 mA. Irregularly shaped forms with external surface containing characteristic dents **(Figure 1**) were obtained for the microcapsules in either the absence ("empty") or the presence ("filled") of lycopene, as observed previously for bixin−gum arabic (*23*) and monoterpenes−gum arabic (*27*) microcapsules. The absence of apparent cracks or porosity indicated a good covering protection of the core material.

The skimmed milk samples were prepared by dissolving 20 g of commercial skimmed milk powder in 200 mL of sterilized Milli-Q water. According to the manufacturer information, the skimmed milk solutions contained initially 4.3 and 0.6 mg/L vitamins D_3 and A, respectively. All glass materials used were previously sterilized, and the samples were prepared in a laminar flow hood to minimize microorganism contamination. The method reported by Indyk and Woollard (*28*) was used for the extraction of vitamins A and D_3 from skimmed milk samples. Briefly, the skimmed milk was saponified with 50% aqueous KOH and 1% pyrogallol ethanol solutions overnight at room temperature, under dark and N_2 atmosphere conditions. Afterward, extraction with 30 mL of ethanol/water (1:2) and 80 mL of petroleum ether/diethyl ether (9:1) was performed three times. The ethereal phase was washed with water up to neutralization, and the ether was removed under vacuum followed by complete dryness under N_2 and storage at −20 °C until HPLC analysis (see below).

The photodegradation of vitamins A and D_3 was performed by placing several Pyrex test tubes (cutoff 310 nm) with skimmed milk solutions, in the absence and presence of 6.5 g/L MIC, between standard fluorescent white lights (GE T8 32W, General Electric, Rio de Janeiro, Brazil). The white light intensity output ($350 < \lambda$ (nm) < 800) at the sample position was ca. 8600 lx, as measured with a luxmeter (Instrutherm, model LDR-208, São Paulo, Brazil). Sample solutions were kept under dark at the same temperature as the control.

UV−vis absorption and diffuse reflectance spectra were recorded with a USB2000 Ocean Optics diode-array spectrophotometer (Dunedin, FL). The diffuse reflectance measurements in skimmed milk were performed using an R400-7-VIS-NIR reflection probe (Ocean Optics) placed at a 45° angle to the skimmed milk surface. $BaSO_4$ was used as the diffuse reflectance standard. The emission spectrum of the fluorescent lamp, GE T8 32W, was measured with the USB2000 Ocean Optics diode-array spectrophotometer coupled with a fiber-optic with an SMA connector.

Fluorescence spectra of Rf in water or skimmed milk were recorded with a Hitachi F-2500 instrument (Tokyo, Japan). In the last case, front-face excitation at 30° with a cutoff filter (Schott GG475) before the emission detection was used to avoid scattering from excitation light. The photolysis of Rf in skimmed milk was performed using the Xe lamp of the same instrument as the excitation source placed at 400 (±20) nm (ca. 4000 lx) and collecting the Rf fluorescence at 520 nm.

HPLC analysis was carried out using a Waters HPLC system (model 600, Milford, MA) equipped with a photodiode array detector (model 996, Waters). The equipment also included an on line degasser, a Rheodyne injection valve with a 20 μL loop, and an external oven. Data acquisition and processing were performed using the Millenium Waters software.

Lycopene purity was verified on C_{30} YMC (Waters) (4.6 × 250 mm, 3 μm) using MeOH (0.1% triethylamine)/*tert*-butyl methyl ether (1:1) as the mobile phase at a flow rate of 1 mL/min, the column temperature being set at 33 °C. The chromatograms were processed in maximum plot mode (λ_{max}), and the spectra were recorded between 250 and 600 nm. Vitamins A and D_3 were separated on a C_{18} Vydac 218TP54 (4.6 × 250 mm, 5 μm) column (Hesperia, MI), using acetonitrile/methanol (9:1) as the mobile phase at 1.2 mL/min and a column temperature of 23 °C. The chromatograms were processed at 265 nm (vitamin D_3) and 324 nm (vitamin A). Authentic solutions of vitamins A and D_3 in methanol were used for identification of the peaks in the chromatograms.

Time-resolved phosphorescence detection (TRPD) of $^1O_2$ was performed using a Peltier-cooled Ge photodiode (Judson J16TE2-66G, Montgomeryville, PA) placed at a right angle to the excitation laser pulse from a Q-switched Nd:YAG laser (Continuum Minilite II, Santa Clara, CA), operating at the frequency-doubled output (532 nm, 10 ns fwhm, ca. 5 mJ per pulse). Spurious light was filtered using a 1270 nm band-pass filter (Spectrogon BP-1260, Parsippany, NJ). The output of the detector was fed via amplifier stages to a Tektronix TDS3032B digital oscilloscope (Wilsonville, OR) linked to an on-line PC for data transfer and analysis. Typically, about 20−30 laser cycles with the excitation laser operating at 5 Hz were averaged to obtain the decay times with a suitable signal-to-noise ratio.

The transient absorption decay of the triplet state of Rf in aqueous solutions was recorded at 720 nm ($\epsilon_T^{720} = 4200\ M^{-1}\ cm^{-1}$ (*29*)) with a Luzchem m-LFP 112 system (Ontario, Canada), using the same laser source as that for the TRPD experiments, but operating at the frequency-tripled output (355 nm, 10 ns fwhm, ca. 4 mJ per pulse).

All experiments were performed in duplicate in air-equilibrated solutions and under controlled temperatures of 8 and 21 (±1) °C.

RESULTS AND DISCUSSION

Figure 2 shows the kinetic plots for the degradation of vitamins D_3 and A in skimmed milk under dark storage conditions and illumination with fluorescent light at 8 °C, obtained from HPLC analysis at 265 and 324 nm, respectively. No degradation of both vitamins was observed under dark conditions. However, both vitamins were consumed following a first-order rate law under continuous illumination, eq 1. A_0,

$$\ln\left[\frac{[\text{Vit}]_t}{[\text{Vit}]_0}\right] = \ln\left[\frac{(A_t - A_\infty)}{(A_0 - A_\infty)}\right] = -k_{obsd}t \qquad (1)$$

A_t, and A_∞ represent the initial, intermediate and final HPLC areas, respectively. The observed first-order rate constant (k_{obsd}) for the photodegradation of the vitamins was obtained by linear fitting of eq 1, **Table 1**.

Figure 3 shows the normalized absorption UV−vis spectra of vitamins A, D_3, and Rf, together with the normalized emission spectrum of the fluorescent lamp. The excitation fluorescent light only overlaps significantly at the red-shifted absorption band of Rf (>400 nm), and thus, direct photolysis of vitamins A and D_3 in skimmed milk was ruled out. The photosensitized degradation rate for vitamin A was higher than that observed for vitamin D_3, **Figure 2** and **Table 1**. The temperature effect on k_{obsd} allowed the determination of the activation energy E_a of the process by using the Arrehnius equation (2). As expected

$$E_a = R\left(\ln\frac{k_{obsd,2}}{k_{obsd,1}}\right)\left(\frac{T_2 - T_1}{T_1T_2}\right) \qquad (2)$$

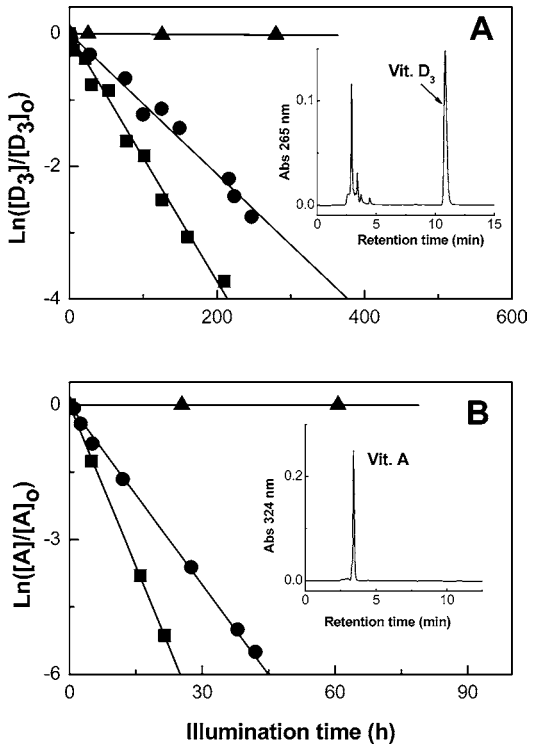

Figure 2. First-order plots for the degradation kinetic of vitamins D_3 (**A**) and A (**B**) in skimmed milk at 8 °C under dark storage conditions (▲) and under illumination with white fluorescent light of 8600 lx (350 < λ (nm) < 800) in the absence (■) and in the presence (●) of 6.5 g/L MIC. Insets: HPLC chromatograms of vitamins A and D_3 detected at 265 and 324 nm, respectively.

Table 1. Observed First-Order Rate Constant (k_{obsd}), Half-Life ($t_{1/2}$), Activation Energy (E_a), and Percentage of Photoprotection (PP) Induced by MIC for the Photosensitized Degradation of Vitamins A and D_3 in Skimmed Milk

vitamin	[MIC] (g/L)	temp (°C)	k_{obsd} (h^{-1})	$t_{1/2}$[c] (h)	E_a (kcal/mol)	PP (%)
A[a]	0.0	21	0.32 ± 0.03	2.0 ± 0.2	4 ± 1	
	0.0	8	0.24 ± 0.02	2.6 ± 0.2		46
	6.5	8	0.13 ± 0.02	4.9 ± 0.7		
D_3[a]	0.0	21	0.071 ± 0.005	8.9 ± 0.6	16 ± 3	
	0.0	8	0.020 ± 0.001	32 ± 1.6		45
	6.5	8	0.011 ± 0.001	57 ± 5.2		
Rf[b]	0.0	21	54 ± 5	0.012 ± 0.002		54
	6.5	21	25 ± 3	0.025 ± 0.005		

[a] Excited with a white fluorescent lamp of 8600 lx. [b] Excited with 400 (±20) nm light of 4000 lx. [c] Calculated as $t_{1/2} = 0.63/k_{obsd}$.

from the k_{obsd} values, a higher E_a value for vitamin D_3 was observed compared to that for vitamin A, **Table 1**. It has been reported that in Rf photosensitized $^1O_2$ oxidation of vitamin A several oxidation products were generated, such as retinal, retinoic acid, ethyl 2,6,6-trimethylcyclohex-1-enecarboxylate, and 5,8-peroxide derivatives (*13*). On the contrary, Rf-mediated photooxidation of vitamin D_2 yielded the 5,6-epoxide of vitamin D_2 as a single product (*30*). This different reaction pattern and the E_a difference observed in this study, **Table 1**, can be assigned to the longest electron-rich conjugated double bond system of vitamin A, which allows multiple positions and a lower energy barrier for the attack of the electrophilic $^1O_2$.

In the presence of 6.5 g/L lycopene−gum arabic−sucrose microcapsules (MIC) in skimmed milk, the photosensitized

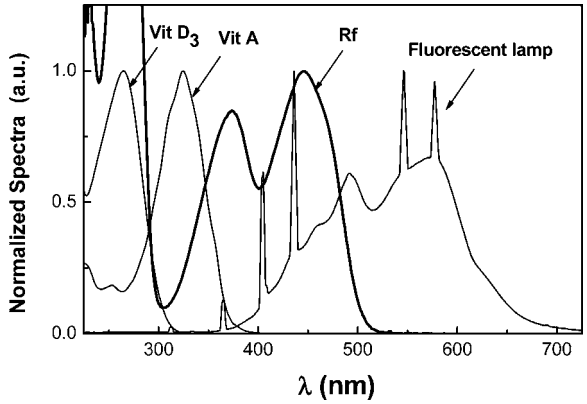

Figure 3. Normalized absorption spectra of vitamins A and D_3 and Rf in ethanol solution, together with the normalized emission spectrum of the commercial white fluorescent lamp GE T8 32W.

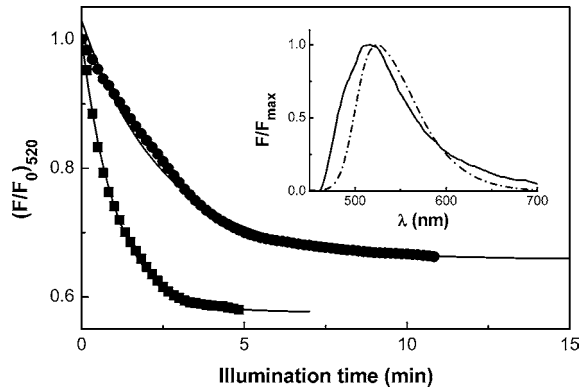

Figure 4. Photodegradation kinetics of Rf by light excitation of 400 (±20) nm (4000 lx) in skimmed milk in the absence (■) and in the presence (●) of 6.5 g/L MIC, monitored by Rf fluorescence at 520 nm using front-face excitation. Inset: fluorescence spectrum of Rf in skimmed milk (—) and in water (-••-) obtained by light excitation at 400 nm (excitation and emission slits of 2.5 nm).

degradation rate constants of both vitamins decreased, **Figure 2** and **Table 1**. The percentage of vitamin photoprotected (PP) by the presence of MIC was calculated by eq 3. where

$$\mathrm{PP} = 100\left(1 - \frac{k_{\mathrm{obsd}}^{\mathrm{MIC}}}{k_{\mathrm{obsd}}}\right) \qquad (3)$$

$k_{\mathrm{obsd}}^{\mathrm{MIC}}$ is the observed first-order rate constant for the photodegradation of vitamins A and D_3 in the presence of the microcapsule. In both cases, similar PP values were obtained, indicating that the same photosensitized oxidation mechanism was operating for both vitamins. To confirm this assumption, the self-photosensitized oxidation of Rf in skimmed milk was monitored by front-face fluorescence, **Figure 4**. In this case, the initial fluorescence decay for the photolysis of Rf in skimmed milk was fitted by an exponential decay function (solid lines in **Figure 4**), indicating a first-order decay behavior, as reported previously (*17, 31*). The inset of **Figure 4** shows that the fluorescence spectrum of Rf is blue-shifted and broader in skimmed milk than in water, indicating multiple solubilization sites of Rf in skimmed milk, which can be considered as a complex system with several microphases with different microenvironments and solubilization properties. On the other hand, the k_{obsd} value was reduced in the presence of MIC, and the PP values for the Rf photodegradation were closer to that observed for vitamins A and D_3 (**Table 1**), considering the

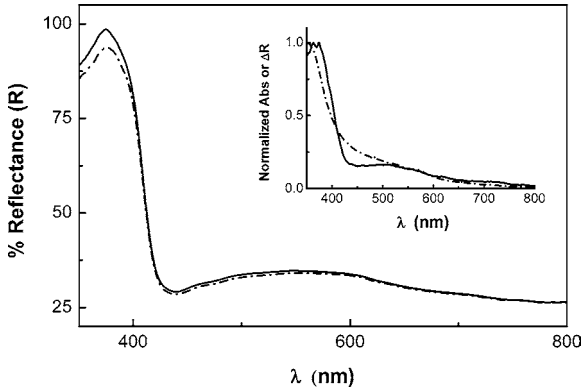

Figure 5. Diffuse reflectance spectrum of skimmed milk in the absence (—) and presence (-••-) of 6.5 g/L MIC. Inset: normalized reflectance difference (ΔR; —) and absorption (Abs; -••-) spectra of MIC in skimmed milk and water solutions, respectively.

temperature difference between both sets of experiments. Therefore, the observed photodegradation of both vitamins can be assigned to Rf-mediated photosensitized processes, **Scheme 1** (*13, 14*).

To explain the whole vitamin photoprotection mechanism introduced by MIC, the reflectance spectra (above 350 nm) of skimmed milk in the absence and presence of 6.5 g/L MIC were analyzed, **Figure 5**. The percentage of reflectance reduction in skimmed milk by the presence of MIC represented only ca. <2.2%, as calculated from the integrated spectral areas, suggesting that the contribution of the inner-filter effect introduced by superficial light absorption of MIC cannot explain by itself the observed total PP, and therefore, the trivial inner-filter photoprotective mechanism was ruled out. In addition, the difference reflectance spectrum (ΔR) in skimmed milk, obtained by the subtraction between the reflectance spectra in the presence and absence of MIC, was similar to the UV−vis absorption spectrum of 6.5 g/L MIC in aqueous solution, inset of **Figure 5**, indicating that the small reflectance changes were effectively produced by the addition of MIC.

It has been reported that singlet molecular oxygen $^1O_2$ is the main reactive oxidant species involved in the self-photosensitized degradation of Rf with a diffusion-controlled reaction rate constant of 1×10^{10} M^{-1} s^{-1} in aqueous solution (*17*). Direct evidence of the interaction of MIC with $^1O_2$ was observed by using time-resolved phosphorescence detection of $^1O_2$ in D_2O solutions after 532 nm laser excitation of the sensitizer RB, **Figure 6**. D_2O instead of H_2O solution was used with the purpose of improving the detection of the weak phosphorescence signal of $^1O_2$ at 1270 nm, since both the decay time and intensity of the $^1O_2$ signal are higher in deuterated solvents, without significant changes in the quenching reactivity relative to that of the respective hydrogenated solvent (*32*). In both cases, the involved reactions are

$$O_2(^1\Delta_g) \xrightarrow{k_\Delta^\circ} O_2(^3\Sigma_g^-) + h\nu + \text{heat} \qquad (4)$$

$$O_2(^1\Delta_g) + \text{MIC} \xrightarrow{k_q^\Delta} O_2(^3\Sigma_g^-) + \text{MIC} + \text{oxidation products} \qquad (5)$$

where eq 4 represents the unimolecular deactivation of $^1O_2$, where its decay rate constant k_Δ° depends on the solvent (*32*), and eq 5 represents the bimolecular total (physical + chemical) quenching reaction, with rate constant k_q^Δ (*11, 32*). Thus, the observed first-order decay rate constant of $^1O_2$, k_Δ, was obtained by exponential fitting of the decay portion of the $^1O_2$ phospho-

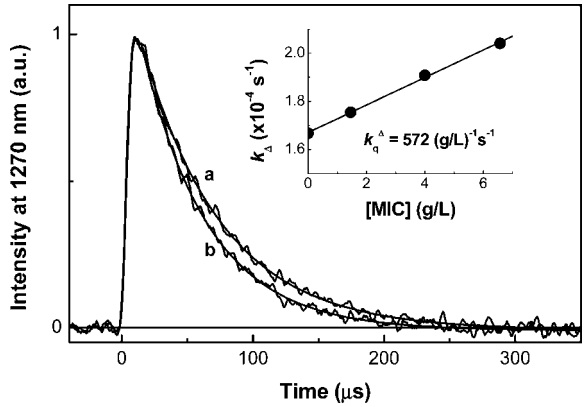

Figure 6. Time-resolved phosphorescence signals of $^1O_2$ at 1270 nm observed after laser excitation at 532 nm of RB in D_2O solutions in the absence (**a**) and in the presence (**b**) of 6.5 g/L MIC solutions. Inset: linear dependence of k_Δ on the MIC concentration, eq 6.

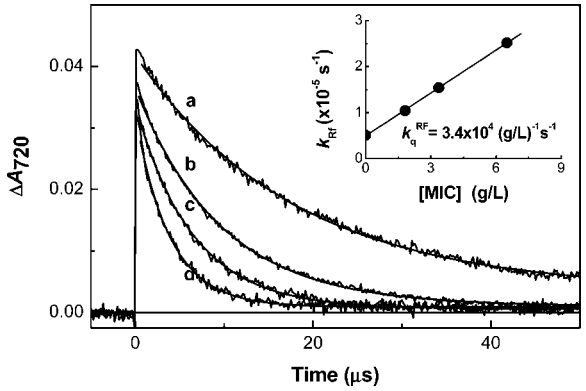

Figure 7. Transient absorption decay at 720 nm of ^{3}Rf* after laser pulsed excitation at 355 nm of Rf in aqueous solutions as a function of the MIC concentration: (**a**) 0 g/L, (**b**) 1.8 g/L (**c**) 3.4 g/L, (**d**) 6.5 g/L. Inset: linear dependence of k_{Rf} on the MIC concentration, eq 9.

rescence signals, which linearly depended on the MIC concentration (g/L), eq 6 (inset of **Figure 6**), where $k_\Delta°$ is the $^1O_2$

$$k_\Delta = k_\Delta° + k_q^\Delta[\text{MIC}] \quad (6)$$

decay rate constant in the absence of MIC ($1/k_\Delta° = 61\ \mu$s) and $k_q^\Delta = (572 \pm 20)$ L g^{-1} s^{-1} is the apparent bimolecular quenching rate constant of $^1O_2$ by MIC. This quenching rate constant can be assigned to the presence of lycopene in the microcapsules since it is known that lycopene is the most efficient natural carotenoid quencher of $^1O_2$ in either in vivo or in vitro conditions (*33*), with diffusion-controlled quenching rate constants of ca. 2×10^{10} M^{-1} s^{-1} in organic solvents and 2×10^9 M^{-1} s^{-1} in aqueous micelle solutions (*34*). In the present study, a gum arabic−sucrose (8:2) film coats lycopene molecules, allowing their solubilization in aqueous media. Although gum arabic is widely used due to its excellent emulsifying properties, its capsules act as semipermeable membranes for oxygen (*27*), allowing $^1O_2$ diffusion inside the microcapsules to be further deactivated by the microencapsulated lycopene molecules. In this case, and taking into account the bulk molar concentration of lycopene incorporated into the MIC solutions (~2 μM), a value of 1.8×10^9 M^{-1} s^{-1} was obtained for the bimolecular quenching rate constant of $^1O_2$ by microencapsulated lycopene. This value is very close to 1.6×10^9 M^{-1} s^{-1} obtained for $^1O_2$ quenching by lycopene dispersed in 30 mM Triton X-100 micelles in D_2O solutions (data not shown), indicating that the microencapsulated carotenoid retained its $^1O_2$-quenching efficiency.

The percentage of $^1O_2$ quenched by MIC in skimmed milk, Q_Δ, can be calculated with eq 7, using $k_q^\Delta = 572$ L g^{-1} s^{-1} and taking into account that the $^1O_2$ lifetime in skimmed milk

$$Q_\Delta = 100\left[\frac{k_q^\Delta[\text{MIC}]}{k_\Delta° + k_q^\Delta[\text{MIC}]}\right] \quad (7)$$

solution should be similar to the value in aqueous media ($\tau_\Delta° = 1/k_\Delta° \approx 3.5\ \mu$s (*32*)). Under these assumptions, the percentage of $^1O_2$ deactivated by 6.5 g/L MIC in skimmed milk should represent ~1.5%, which is a small value to explain the total observed PP for the vitamins, **Table 1**. This result can be expected considering the low bulk concentration of lycopene (see above). A larger lycopene concentration was avoided so the milk color properties would not be modified. Therefore, the photoprotection mechanism of MIC in skimmed milk should involve an extra effect rather than efficient $^1O_2$ quenching or inner-filter effects.

As denoted in **Scheme 1**, an extra photoprotection mechanism can be related to the quenching process of ^{3}Rf* by MIC, competing with the formation of harmful species, eq 8.

$$^3\text{Rf}^* + \text{MIC} \xrightarrow{k_q^{Rf}} \text{Rf} + \text{MIC and/or products} \quad (8)$$

Direct evidence of ^{3}Rf* quenching by MIC in N_2-satured aqueous solution was obtained by monitoring the typical transient absorption of ^{3}Rf* at 720 nm after laser excitation at 355 nm, using transient absorption spectroscopy, **Figure 7**. In the presence of MIC, the rate constant for the observed decay of ^{3}Rf* was increased according to eq 9, where $k_{Rf}°$ is the ^{3}Rf*

$$k_{Rf} = k_{Rf}° + k_q^{Rf}[\text{MIC}] \quad (9)$$

decay rate constant in the absence of MIC ($1/k_{Rf}° = 20\ \mu$s) and $k_q^{Rf} = 3.4 \times 10^4$ L g^{-1}s^{-1} is the apparent bimolecular quenching rate constant of ^{3}Rf* by MIC. This value is almost 60 times larger than k_q^Δ, indicating that the main photoprotection pathway is due to the efficient deactivation of ^{3}Rf* by MIC. In fact, the percentage of ^{3}Rf* quenched by MIC in skimmed milk, Q_T, can be estimated with eq 10.

$$Q_T = 100\left[\frac{k_q^{Rf}[\text{MIC}]}{k_{Rf}° + k_q^{Rf}[\text{MIC}]}\right] \quad (10)$$

In this condition, and considering a shorter decay time of ^{3}Rf* in aerated aqueous solutions ($1/k_{Rf}° = 3\ \mu$s), a value of $Q_T = 40\%$ is obtained in 6.5 g/L MIC aqueous solutions. This value is much closer to the PP of the vitamins calculated with eq 3.

An interesting point is that the same quenching rate constant was obtained by the interaction of ^{3}Rf* with gum arabic−sucrose spray-dried microencapsules without lycopene ("empty" microcapsules). In addition, ^{3}Rf* was not quenched by lycopene in 30 mM Triton X-100 aqueous micelle solutions (data not shown). These combined results strongly suggested that the microcapsule wall material is the only material responsible for ^{3}Rf* quenching. The main component of the wall material is gum arabic, since sucrose is added to form a glassy layer on the microcapsules, preventing the loss of core material from cracks and holes on the surface (*35*, *36*). Gum arabic is a complex and variable mixture of arabinogalactan oligosaccharides, polysaccharides, and glycoproteins. Depending on the

source, the glycan components contain a higher proportion of L-arabinose compared to D-galactose (*Acacia seyal*) or the opposite (*Acacia senegal*) (*37*−*39*). In both cases, the gums consist of two components, the main one representing ∼90% of the total with a molecular mass of a few hundred thousand and the other representing about 10% of the total with a molecular mass of several million. The protein-rich component of gum arabic has a molecular mass of ca. 2.5×10^5 and possesses a flexible but compact conformation (*37*, *39*), although since it is a mixture and the material varies significantly with the source, the exact molecular structures are still rather uncertain. Thus, the protein component of gum arabic can be responsible for ^{3}Rf* quenching, since this state is efficiently quenched by amino acids and peptides (*22*). Considering a diffusion-controlled quenching process of ^{3}Rf* by MIC in water at 21 °C, e.g., $k_{q,dif} = 6.5 \times 10^9$ M^{-1} s^{-1} (*40*), it is straightforward that the apparent quenching rate constant $k_q^{Rf} = 3.4 \times 10^4$ L g^{-1} s^{-1} corresponds to a molecular mass quencher of 1.91×10^5, which agrees with the average molecular mass of the proteic component of gum arabic (see above).

In summary, the photosensitized Rf-mediated degradation of vitamins A, D_3, and Rf itself in skimmed milk can be strongly reduced by the addition of small amounts of lycopene−gum arabic−sucrose microcapsules, without modification of the bulk properties of skimmed milk. The photoprotection mechanism is based on the efficient quenching of the Rf triplet state by the protein moiety of gum arabic. A small contribution (<5%) to the total photoprotection percentage is due to both an inner-filter effect and $^1O_2$ quenching by microencapsulated lycopene. Nevertheless, the bimolecular quenching rate constant of $^1O_2$ by microencapsulated lycopene was similar to that observed in neutral micelle solutions ($k_q^{\Delta} = 1.8 \times 10^9$ M^{-1} s^{-1}), indicating that the microencapsulated carotenoid remained as a very efficient $^1O_2$ quencher. These results show the functional ability of gum arabic-based microcapsules to act as antioxidant species in food systems and that the microencapsulation spray-drying process does not affect the excellent $^1O_2$ quencher lycopene ability.

ABBREVIATIONS USED

HPLC, high-performance liquid chromatography; MIC, lycopene−gum arabic−sucrose microcapsule; Rf, riboflavin; ^{3}Rf*, riboflavin triplet state; RB, Rose Bengal; $^1O_2$ (≡Δ), singlet molecular oxygen; TRPD, time-resolved phosphorescence detection.

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