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Stability of carotenoids toward UV-irradiation in hexane solution

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Abstract: The stabilities of four selected carotenoids dissolved in hexane, two carotenes and two xanthophylls, toward UV-irradiation of three different ranges (UV-A, UV-B and UV-C) were studied in this work. The carotenoids underwent bleaching *via* a probable free radical mediated mechanism following first-order kinetics. The bleaching rates were highly dependent on the input of the involved photons and, although not consistently, on the chemical structures of the investigated compounds. For the two xanthophylls, a possible role of oxygen associated with their bleaching cannot be neglected.

Keywords: carotenoids; UV-irradiation; free radicals; bleaching; kinetics.

INTRODUCTION

Depletion of the stratospheric ozone has led to an increase of biologically damaging UV-light at ambient levels (mainly UV-B light, 280–320 nm). The induced consequences affect many crucial biologically important processes of global importance, such as DNA replication,[1,2] photosynthesis,[3,4] *etc*.

Although UV-light can generally influence the whole human immune system,[5,6] it has been especially recognized as one of the major agents leading to melanoma skin cancer,[7] playing a triggering role in the initiation of very complex process leading finally to cancer.[8] Many cosmetics and pharmaceutical formulations have recently been employed for skin protection from UV-light. Some of them, in a form of a filter, employ plant protection pigments, such as flavonoids, or plant photosynthetic pigments, such as chlorophylls and carotenoids, despite the fact that their interaction with UV-light has not yet been sufficiently well elucidated at the basic level. The use of flavonoids for skin protection against UV-light is more understandable, since they are excellent UV-absorbers.[9] On the other hand, chlorophyll and carotenoids predominantly absorb in the visible region,[10] and, as is known from experiments performed *in vivo* on leaves or on isolated photosynthetic organelles, their composition is significantly altered when exposed to UV-light.[3]

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Hence, to understand the basic mechanisms of interaction of photosynthetic pigments with UV-light in oil/water emulsions, a fundamental component of most pharmaceutical and cosmetics formulations[11−13] and related technologies, inherent knowledge must first be obtained in the simplest possible feasible system: in solution.

For such a purpose, the stability of four chosen carotenoids, two carotenes (β-carotene and lycopene) and two xanthophylls (lutein and neoxanthin) toward UV-irradiation of three different ranges (UV-A, UV-B and UV-C) has been studied in this work. The irradiation was performed in hexane solution for different irradiation periods, providing possibilities for kinetics analysis.

EXPERIMENTAL

Pigments were isolated from plant material (β-carotene, lutein and neoxanthin from spinach, and lycopene from tomato fruits) purchased at the local market. All experiments and experimental procedures, beginning with extraction, were performed under dim light as much as possible and inside vessels and equipment covered with aluminum foil or black cloth, preventing possible pigment photo-oxidation.

*Pigment extraction from spinach (*Spinacia oleracea*)*

Plant pigments were extracted from spinach leaves using a modified method proposed by Swec.[14] Fresh spinach leaves free of midribs (0.030 kg) were dropped into boiling water, which was quickly replaced (after 1−2 min) with cooled water. Hot water inactivates enzymes thus preventing alteration of the pigments and permits coagulation of proteins and extracts water-soluble substances. After drying between paper towels, the leaves were separated and placed in a mixture of methanol (60 cm^3) and 40–75 °C petroleum ether (30 cm^3); the mixture was occasionally agitated over the next 30 min. Methanol removes water from the plant material and the petroleum ether extracts the pigments before they undergo secondary reactions. The deep-green extract was decanted through a cotton pad. The leaves were re-extracted twice with same quantities of methanol and 40–75 °C petroleum ether (2:1). The extracts were mixed with 120 cm^3 of saturated NaCl solution, whereby most of the pigments remained in the petroleum ether layer. The remaining aqueous methanol layer was re-extracted with 40 cm^3 of a mixture containing 40–75 °C petroleum ether and diethyl ether (1:1), ensuring solubility of the pigments in the organic phase. The successive extracts were treated by the same procedure. The final extract was a mixture of pigments and contained various forms of chlorophyll, as well as accessory pigments – carotenoids (carotenes and xanthophylls).

Isolation of carotenoids from the spinach extract by column chromatography

The carotenoid-fractions were isolated using a modified procedure of Swec[15] and Brockman[16] – column chromatography with silica gel (silica gel 60, Merck, 0.063–0.200 mm) as the adsorbent and a benzene/acetone mixture as the eluent. The benzene/acetone ratio was changed from initial 1:0 to final 1:1, to facilitate the elution of the polar fractions. β-Carotene appeared first (eluted by benzene only), followed by the chlorophylls (benzene:acetone, 7:1) and the xanthophylls fractions, lutein and neoxanthin, (benzene:acetone, 6:1−1:1). The fractions were dried and redissolved in hexane. Identification of the fractions was performed by comparing their Vis spectra with standards spectra.

Pigments extraction from tomato fruits

Ground tomato fruit (8 g) was thoroughly mixed with 40 cm^3 of ethanol. The slurry was stirred until the tomato paste material was no longer sticky (about 3 min). The ethanol was removed by vacuum filtration. The retained tomato residue was mixed with 60 cm^3 of a mixture of acetone and petroleum ether (1:1). The extract was collected by vacuum filtration and the filter residue was rewashed with the solvent mixture (20 cm^3) in order to improve the yield. The filtrate was transferred to a small separating funnel and mixed with 50 cm^3 of saturated NaCl solution. The organic layer was rewashed twice, repeatedly, first with 50 cm^3 of 10 % potassium carbonate and then with 50 cm^3 of water. Finally, approximately 1 g of anhydrous magnesium sulfate was added to dry the organic layer. After 10–15 minutes, the solution was vacuum filtered to remove the drying agent.

Isolation of carotenoids from the tomato extract by column chromatography

The lycopene fraction was isolated by column chromatography with alumina (aluminum oxide 90, Merck, 0.063–0.200 mm) as the adsorbent and petroleum ether/acetone mixture as the eluent. The mixture ratio was changed from the initial 10:0.1 to the final 9:1, to permit an easier elution of lycopene. β-Carotene appears first (eluted by the petroleum ether/acetone mixture of 10:0.1), followed by the lycopene fraction (eluted by the 9:1 mixture). The fractions were dried and redissolved in hexane.

HPLC analysis of the carotenoids fractions

HPLC analysis (Hewlet Packard) showed that there were a high percentage of carotenoids in the separated fraction. The analysis was performed under the following conditions; column: Zorbax Eclipse XDB-C18, mobile phase: acetonitrile/methanol/ethyl acetate, 60:20:20; flow rate: 0.5 ml min^{-1}. The monitoring wavelengths were: 445 nm for β-carotene and lycopene, 438 nm for lutein and 447 nm for neoxanthin.

Vis spectroscopy

The Vis spectra of the carotenoids fractions in hexane were recorded on a Varian Cary-100 Spectrophotometer. All spectra, before and after irradiation with UV-light, were recorded from 300 to 600 nm.

UV-treatment

Continuous irradiation of the pigments in hexane was performed in a cylindrical photochemical reactor "Rayonnet", with 14 symmetrically placed lamps with emission maxima in three different ranges: 254 nm (UV-C), 300 nm (UV-B) and 350 nm (UV-A). The samples were irradiated for different time periods in quartz cells (1 cm×1 cm×4.5 cm) placed on a rotating circular holder. The total measured energy flux was about 25 W m^{-2} for 254 nm, 21 W m^{-2} for 300 nm and 18 W m^{-2} for 350 nm, at a distance of 10 cm from the lamps, corresponding to light intensity values of 26.6, 26.3 and 26.4 μmol m^{-2} s^{-1}, respectively (the emission spectra of the employed lamps are given in the supplement). These very similar values were obtained from the calculated, approximately the same number of absorbed photons (belonging to the three UV-ranges), ensuring that changes in the carotenoids concentrations, if found to be caused by the UV-irradiation, were primarily related to the energy of the photons. The concentrations of β-carotene, lutein, neoxanthin and lycopene were adjusted to be about 1.3×10^{-6} mol dm^{-3}, by using the following molar extinction coefficient (ε) values: 1.39×10^5 dm^3 mol^{-1} cm^{-1} for β-carotene in hexane at 453 nm, 1.72×10^5 dm^3 mol^{-1} cm^{-1} for lycopene in hexane at 503 nm, 1.41×10^5 dm^3 mol^{-1} cm^{-1} for lutein in diethyl ether at 445 nm and 1.36×10^5 dm^3 mol^{-1} cm^{-1} for neoxanthin in ethanol at 438 nm.[17-19]

RESULTS

The structures of the carotenes, β-carotene and lycopene, changed during a continuous prolonged irradiation with UV-A light (350 nm) as evidenced by the changes in their absorption spectra in hexane. Kinetic log absorbance plots as a function of irradiation time with UV-A light are shown in Figs. 1 and 2, for β-carotene and lycopene, respectively. The pigments absorption spectra showed similar behavior during irradiation with UV-B and UV-C light (not shown). Their kinetic log plots were of a very similar shape to those presented. The plots are linear with average R values of about 0.98. The photolysis kinetics seem to obey a first-order law, $y = kx + n$ where y is the log absorbance (the pigment absorption in hexane at 448 nm for β-carotene and 470 nm for lycopene, x is the UV-irradiation time and k is the rate constant for pigments bleaching).

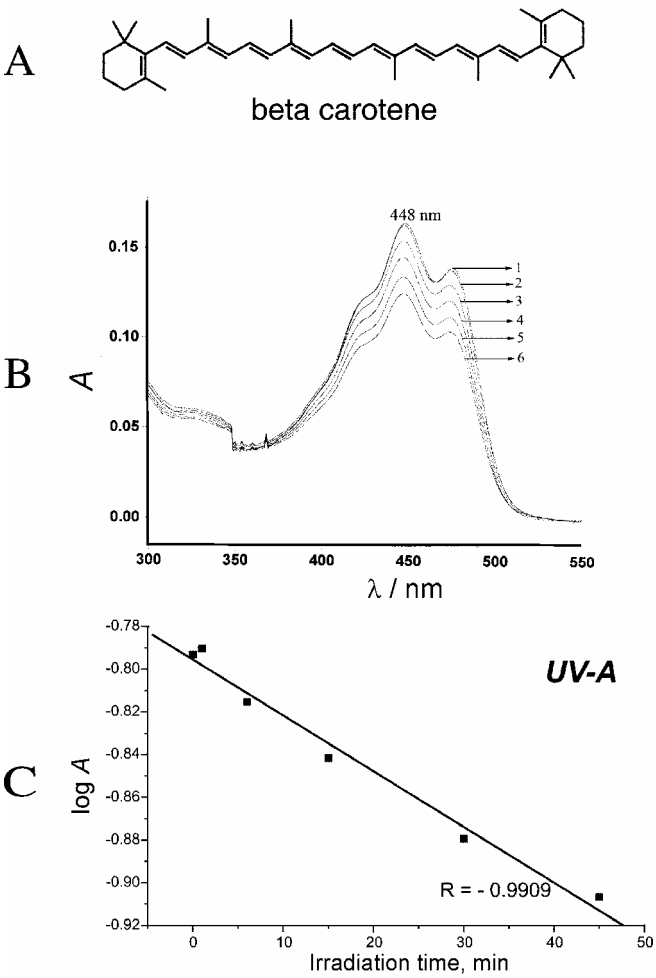

Fig 1. (A) Structure of β-carotene; (B) changes in the absorption spectra of β-carotene exposed to UV-A radiation (350 nm) in hexane. The exposure time periods were: (1) 0 min; (2) 1 min (3) 6 min; (4) 15 min; (5) 30 min; (6) 45 min. The approximate concentration of β-carotene was 1.3×10^{-6} mol dm^{-3}; (C) the kinetic log absorbance plot of the bleaching of β-carotene in hexane against the time of UV-A irradiation. The absorbance of β-carotene was followed at 448 nm.

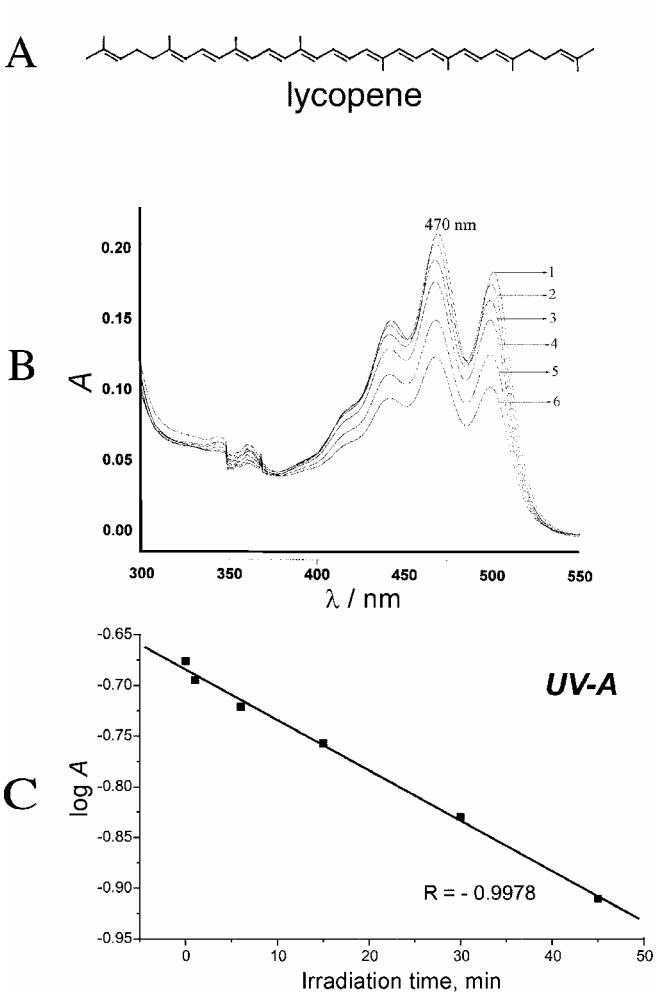

Fig. 2. (A) Structure of lycopene; (B) changes in the absorption spectra of lycopene exposed to UV-A radiation (350 nm) in hexane. The exposure time periods were: (1) 0 min; (2) 1 min; (3) 6 min; (4) 15 min; (5) 30 min; (6) 45 min. The approximate concentration of lycopene was 1.3×10^{-6} mol dm^{-3}; (C) the kinetic log absorbance plot of the bleaching of lycopene in hexane against the time of UV-A irradiation. The absorbance of lycopene was followed at 470 nm.

The structures of the xanthophylls (lutein and neoxanthin) also changed during continuous prolonged irradiation with UV-B light (300 nm). The kinetic log absorbance as a function of irradiation time with UV-B light plots are shown in Figs. 3 and 4 for lutein and neoxanthin, respectively. The changes in the absorption spectra of lutein in hexane after a continuous prolonged irradiation with UV-C light (254 nm) and the kinetic log absorbance *vs*. irradiation time with UV-C light plot are shown in Fig. 5. The pigments absorption spectra show very similar responses during the same time regime of irradiation with UV-A light (lutein and neoxanthin) and UV-C light (neoxanthin (not shown)). The not-presented absorption spectra of all the pigments for all three irradiation regimes are given in the supplementary material. The kinetics log absorbance plots are of very similar shape to those presented. The plots again show an acceptable linear fitting, with

average R values about 0.98, and photolysis kinetics seems again to obey a first-order law $y = kx + n$ where y is log absorbance (the pigments absorption in hexane at 444 nm for lutein and 436 nm for neoxanthin), x is the UV-irradiation time and k is the rate constant for pigments bleaching.

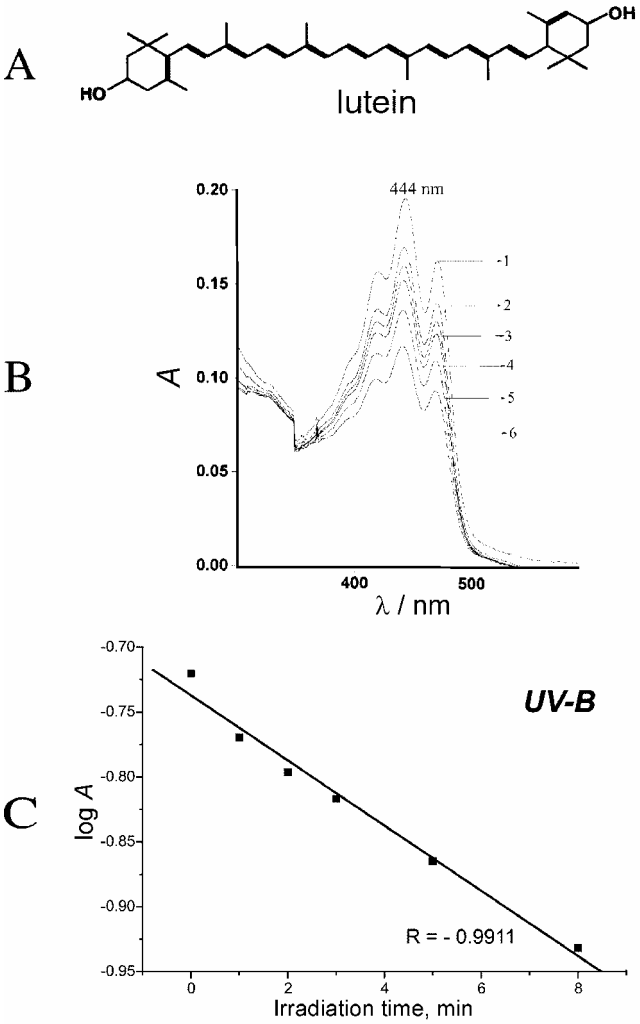

Fig. 3. (A) Structure of lutein; (B) changes in the absorption spectra of lutein exposed to UV-B radiation (300 nm) in hexane. The exposure time periods were: (1) 0 min; (2) 1 min; (3) 2 min; (4) 3 min; (5) 5 min; (6) 8 min. The approximate concentration of lutein was 1.3×10^{-6} mol dm^{-3}; (C) the kinetic log absorbance plot of the bleaching of lutein in hexane against the time of UV-B irradiation. The absorbance of lutein was followed at 444 nm.

The calculated slopes (k) for each carotenoid and each radiation type are presented in Table I. Such a presentation provides for a comparison of the slopes, which reflect differences in the kinetics of pigments bleaching for all three UV-irradiation ranges. It therefore allows an insight into the pigments resistance toward UV-light.

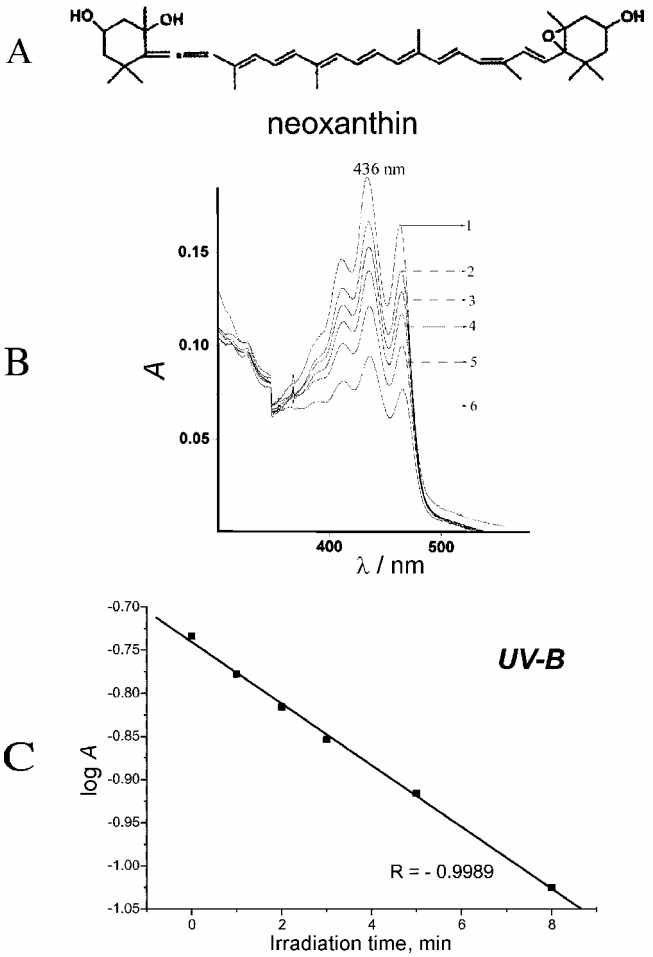

Fig. 4. (A) Structure of neoxanthin; (B) changes of the absorption spectra of neoxanthin exposed to UV-B radiation (300 nm) in hexane. The exposure time periods were: (1) 0 min; (2) 1 min; (3) 2 min; (4) 3 min; (5) 5 min; (6) 8 min. The approximate concentration of neoxanthin was 1.3×10^{-6} mol dm^{-3}; (C) the kinetic log absorbance plot of the bleaching of neoxanthin in hexane against the time of UV-B irradiation. The absorbance of neoxanthin was followed at 436 nm.

DISCUSSION

Carotenoids are usually C_{40} tetraterpenoids built up from eight C_5 isoprenoid units. The basic linear and symmetrical skeleton can be cyclized at one or both ends. A significant characteristic is a long conjugated double-bond system, providing an extended π-delocalization, leading to a substantial bathochromic shift in the Vis region. The shift is responsible for the yellow, orange or red color of these compounds. Carotenoids consisting of only carbon and hydrogen are called carotenes, whereas those containing oxygen are called xanthophylls.

With an absorption maximum in the Vis region, carotenoids are obviously not efficient UV-absorbers but still are able to perform a protective function against UV-light in plants.[3,4] The explanation for such a behavior should be searched for not only in *in vivo* studies,[20,21] but also in basic studies, in very simple homogeneous solution media.

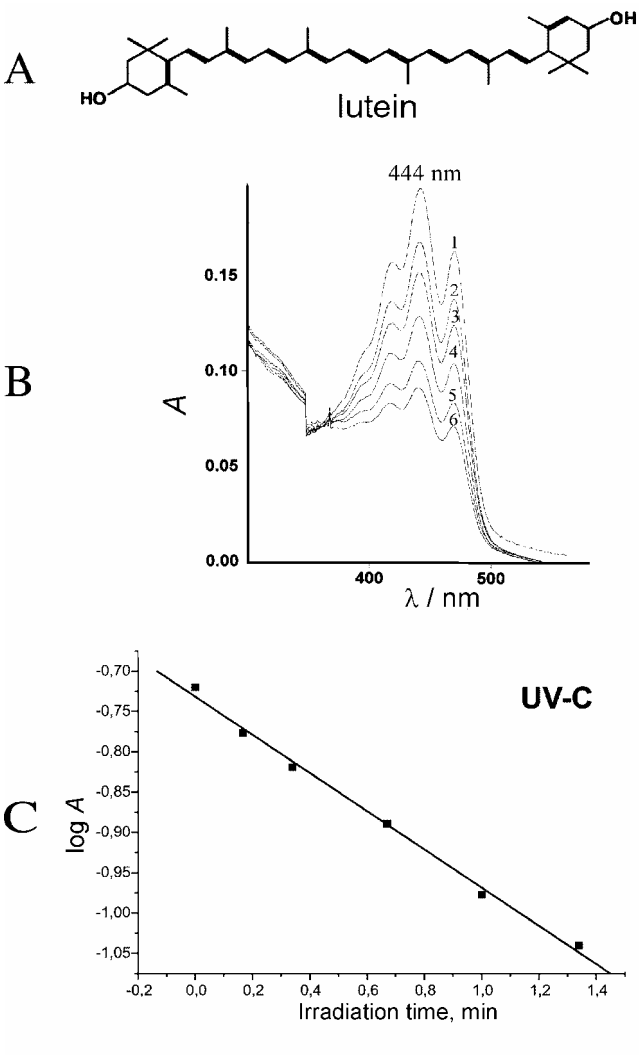

Fig 5. (A) Structure of lutein; (B) changes of the absorption spectra of lutein exposed to UV-C radiation (254 nm) in hexane. The exposure time periods were: (1) 0 min; (2) 0.17 min; (3) 0.34 min; (4) 0.67 min; (5) 1 min; (6) 1.34 min. The approximate concentration of lutein was 1.3×10^{-6} mol dm^{-3}; (C) The kinetic log absorbance plot of the bleaching of lutein in hexane against time of UV-C irradiation. The absorbance of lutein was followed at 444 nm.

The other very important function of carotenoids, of global character, is their anti-oxidant function (this is one of the reasons for the wide use of carotenoids in the food industry[22,23]). For such a purpose, carotenoids may act in a preventive manner, *i.e.*, they inhibit the formation of reactive oxygen species (ROS) by reacting directly with oxygen, or, if radicals are already present, they act as chain-breaking anti-oxidants.[23–27] There are three possible mechanisms for carotenoid (CAR)–radical ($R^{\bullet}$) interactions: (*i*) radical addition or adduct formation ($CAR–R^{\bullet}$), (*ii*) electron-transfer reaction resulting in either a cation-radical ($CAR^{\bullet+}$), anion-radical ($CAR^{\bullet-}$) or a neutral alkyl-radical ($CAR^{\bullet}$), and (*iii*) hydrogen-abstraction, mostly related to the presence of carbonyl chromophores in

the involved radicals ($CAR + ROO^{\bullet} \rightarrow CAR^{\bullet} + ROOH$).[28–31] The cation-radicals ($CAR^{\bullet+}$) absorb strongly in the near-IR, with maxima in 900–1000 nm range.[32–34] The anion-radicals ($CAR^{\bullet-}$) also absorb strongly in the near-IR.[35] On the other hand, it is very difficult to characterize neutral carotenoid-radicals ($CAR^{\bullet}$), since they have no distinctive strong absorption, as observed for $CAR^{\bullet+}$ or $CAR^{\bullet-}$.[30] The possible occurrence of any of the cited mechanisms (*i–iii*) with carotenoids in hexane solution certainly depends on the chemical structures of the involved species.

TABLE I. Kinetics of pigment bleaching in hexane during increasing times of UV-irradiation for three different UV-ranges: 254 nm (UV-C), 300 nm (UV-B) and 350 nm (UV-A). The absorbances of β-carotene, lycopene, lutein and neoxanthin were followed at 448 nm, 470 nm, 444 nm and 436 nm, respectively. The kinetics obey a linear first-order plot: $y = kx + n$, where y is log absorbance (the pigments absorption in hexane at 448 nm (β-carotene), 470 nm (lycopene), 444 nm (lutein) and 436 nm (neoxanthin)), x the UV-irradiation time and k is the first order rate constant for pigment bleaching.

λ_{UV} / nm	k / min^{-1}			
	β-Carotene (A_{max} at 448 nm)	Lycopene (A_{max} at 470 nm)	Lutein (A_{max} at 444 nm)	Neoxanthin (A_{max} at 436 nm)
254	0.24434	0.60110	0.23685	0.47786
300	0.04205	0.05951	0.02510	0.03573
350	0.00261	0.00497	0.00228	0.00229

Since carotenoids, including the four studied here, are not efficient UV-absorbers, their increasing bleaching during prolonged UV-irradiation could be radical-mediated; if this occurs then the electron-transfer mechanism (*ii*)[28,30] appears more probable than the other two (*i* and *iii*). As reported, the reactions of β-carotene in organic solvents, specifically in an ionized hexane solution, are very fast and lead to the formation of strongly absorbing intermediates, $CAR^{\bullet+}$ and $CAR^{\bullet-}$.[35] Adduct formation (*i*) and hydrogen abstraction (*iii*) appear less probable. The Vis absorbance (*i.e.* the carotenoids spectra) should remain unaltered if the former mechanism occurs, since, as reported, the CAR-adducts ($CAR–R^{\bullet}$) have similar spectra to that of CAR itself,[30,31] and this is evidently not the case here (Figs. 1B, 2B, 3B, 4B and 5B); however, this mechanism can theoretically not be excluded as a possibility, but if it occurs short-lived adducts species are formed, which could not be detected with the techniques employed in this study. The hydrogen abstraction mechanism should be neglected because there are no carbonyl moieties, the most selective H-abstractors, present in the investigated solution.[36–38]

There is another hypothetical possibility that the production of at least one of the two CAR-ion-radicals involves ROS species, specifically the superoxide anion radical, $O_2^{\bullet-}$. The reports about the production of them *in vivo* under shorter,[39] or even longer UV-light[21,40] are not comparable with the system studied in

this work, although oxygen was certainly present in the hexane solutions.[41] However, even if $O_2^{\bullet-}$ were really present in solution, it should not affect the above discussion. Since there are neither H^+ ions nor free metal ions in solution, its conversion into hydrogen peroxide and then to hydroxyl radicals ($OH^\bullet$), also possible hydrogen abstraction agents[42,43] *via* the Fenton reaction,[44] is not possible. The possibility for adduct formation with O_2[23–26] can not hypothetically be excluded but if this occurs at all, it is to a minor extent since the adduct (CAR–O_2) has the same spectrum as CAR itself[30,31] and hence, the absorbance should not change significantly during UV-irradiation. The recorded spectra (Figs. 1B, 2B, 3B, 4B and 5B) show this was not the case. The presence of short-chain, oxygen--containing derivatives, indicating instabilities of the carotenoids in the presence of oxygen, which has been already reported,[45] and which should certainly be expressed through a blue-shift of the recorded spectra, was also not detected in this study (Figs. 1B, 2B, 3B, 4B and 5B).

The kinetic plots for the carotenes (β-carotene and lycopene, Figs. 1C and 2C) and the two xanthophylls (lutein and neoxanthin, Figs. 3C, 4C and 5C) are indications for the possible proposed mechanism of the involved bleaching of the carotenoids leading to $CAR^{\bullet+}$ formation, even independent of the UV-irradiation range. The two carotenes irradiated with UV-A and the two xanthophylls irradiated with UV-B both expressed obvious first-order kinetics, implying the rate of reaction is dependent on the carotenoid concentration. In a very relevant study performed in ionized hexane solution, the solvent itself was proposed to be an acceptor, preceded by β-carotene cation-radical formation.[35] As the solvent was present in a huge excess (compared to the carotenoids concentration), it does not affect the reaction rate.

The bleaching rates of the four investigated carotenoids (expressed as the slopes of the linear plots, k, in min^{-1}) for the three UV-ranges are presented in Table I. The results suggest that the bleaching rates are dependent on two factors: the type of UV-irradiation (*i.e.*, the energy of the photons) and the chemical structure of the carotenoid.

Concerning the photon energy input, it is clear from Table I that the bleaching rates decline approximately by one order of magnitude for all investigated carotenoids on going from UV-C to UV-A irradiation. The only exception is β-carotene, for which the ratio between the bleaching rates achieved by UV-C and UV-B radiation was about 6 and the ratio of the bleaching rates for UV-B and UV-A was about 20, which is about double the value when compared to the corresponding ratios obtained with the other carotenoids (Table I). For lycopene, lutein and neoxanthin the UV-C/UV-B and UV-B/UV-A ratios of the bleaching rates are close to 10, emphasizing the crucial governing role of the involved photons. Evidently, basic structural difference between the two carotenes and the two xanthophylls (absence or presence of oxygen, respectively), where lutein and

neoxanthin mimic the structure of β-carotene rather than that of lycopene (with cyclic, oxygen-containing moieties at the ends of the hydrocarbons chains) does not counter this fact.

However, the differences between the chemical structures of the investigated compounds (Figs. 1A, 2A, 3A and 4A) certainly play an undeniable role.

The bleaching rates of lycopene were higher than the ones of β-carotene for all three UV-ranges (Table I). However, the difference was the largest for UV-C (a ratio of 2.5), then for UV-A (1.9) and UV-B (1.42). Lycopene is clearly more reactive and the difference must be somehow connected to the absence of rings at the end of the hydrocarbon chain. For the xanthophylls, the situation is a little clearer. The ratios of the bleaching rates (neoxanthin/lutein, Table I) decreased proportionally from UV-C (2.0), *via* UV-B (1.42) to UV-A (1.0). Since neoxanthin contains two more oxygen atoms than lutein, (Figs. 4A and 3A, respectively), its higher reactivity could reasonably be related to the excess of unpaired electrons (compared to lutein). However, comparison between the carotenes and xanthophylls does not support such a suggestion. The bleaching rates for β-carotene and lutein (lutein containing two hydroxyl groups added to the carotene structure, Figs. 1A and 3A) are very close (particularly for the UV-C and UV-A range) but, on the other hand, the bleaching rates for lycopene (no oxygen, Fig. 2A) are remarkably higher than those of neoxanthin, for all three UV-ranges (Table I).

CONCLUSIONS

To conclude, (1) even in this simplest possible (solution) system, carotenoids undergo degradation, *i.e.*, bleaching, when exposed to prolonged UV-irradiation; (2) their bleaching is probably free radicals-mediated and highly dependent and proportional to the energy input of the UV-photons; (3) the differences between the chemical structures of the investigated carotenoids do not counter conclusion (2), although a particular role of oxygen in the bleaching of xanthophylls, potentially, can not be excluded.

This study should be viewed as a basis for upcoming studies in more complex, microheterogeneous systems, such as oil/water emulsions (with carotenoids included), as carotenoids are raw materials for numerous cosmetic and pharmaceutical formulations for protection against UV-light[11−13] (specifically against the UV-B component which is increasingly present in the emission spectrum of sunlight) and related technologies. It will be interesting to see how a higher level of molecular organization affects the bleaching ratios established in this work.

SUPPLEMENTARY MATERIAL

The changes in the spectra and log absorbance *vs.* time plots for all the examined carotenoids not given in the paper, as well as the emission profiles of the employed lamps are available electronically from http://www.shd.org.yu/JSCS/ or from the corresponding author on request.

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И З В О Д

СТАБИЛНОСТ КАРОТЕНОИДА ПРЕМА UV-ОЗРАЧИВАЊУ У ХЕКСАНУ

ДРАГАН ЦВЕТКОВИЋ и ДЕЈАН МАРКОВИЋ

Tehnolo{ki fakul t e t, Univerzi t e t u Ni{u, Bulevar oslobo|ewa 124, 16000 Leskovac

У раду је испитивана стабилност 4 изабрана каротеноида (два каротена и два ксантофила) према ултраљубичастом зрачењу (UV) из три различита опсега (UV-A, UV-B и UV-C). Каротеноиди подлежу деструкцији, то јест обезбојавању путем вероватног слободно-радикалског механизма који се може описати кинетиком 1. реда. Константе брзине обезбојавања су врло зависне од енергија упадних фотона, и, мада не на конзистентан начин, од хемијских структура испитиваних једињења. Кад су у питању два испитивана ксантофила могућа улога кисеоника у њиховом обезбојавању не може бити негирана.

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