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Original scientific paper

UV-effects on antioxidant activity of selected carotenoids in the presence of lecithin estimated by DPPH test

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Abstract: The effects of ultraviolet radiation (UV) on the antioxidant action of three selected carotenoids (*β*-carotene, lycopene and lutein) in the presence of a lipoidal lecithin mixture were studied by the DPPH (1,1-diphenyl-2-picrylhydrazyl) test. The test is based on the measurement of the decrease of the free DPPH radical absorbance at 517 nm caused by the antioxidant action of carotenoids, which appeared to be strongly affected by UV-action. The high-energy input of the involved UV-photons plays a major governing role.

Keywords: carotenoids; UV-light; lipids; antioxidants.

INTRODUCTION

The destruction of stratospheric ozone has led to an increase of biologically damaging UV radiation at ambient levels (mainly UV-B, 280–320 nm). As a consequence, many crucial, biologically important, processes of global importance have been affected, such as DNA replication[1] and photosynthesis,[2] among others. UV radiation can generally initiate many harmful free radical mediated processes, lipid peroxidation (LP) being one of most important among them. Lipid peroxidation appears as a precursor of many pathological processes which finish in some form of cancer, such as skin melanoma.[3,4]

Reactive oxygen species (ROS), such as hydroxy (•OH) or peroxy (ROO•) radicals, are known as typical lipid peroxidation initiators. They can be created either through a variety of chemical reactions, by typical lipid radicals producers,[5] or by external stresses, implying very commonly external radiation and UV light.[6–9]

Lipid peroxidation is partly controlled *in vivo* by antioxidants action.[10] In recent years, carotenoids have attracted wide research interest as potential antioxidants. Numerous studies report that higher consumption of carotenoids and lower risk of cancer and cardiovascular diseases are mutually connected; the antioxidant action of carotenoids is attributed to their conjugated chemical structures, having multiple potential sites approachable for attack by ROS species.[11–13]

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It is already known that UV radiation certainly affects carotenoids antioxidant function *in vivo*,[2] although the involved mechanisms have not yet been elucidated. The aim of this paper was to study *in vitro* the effects of UV radiation on the antioxidant activities of three plants photosynthetic accessory pigments (*β*-carotene, lycopene and lutein), in the presence of a lipoidal target (lecithin). The mixture was irradiated with UV-B and UV-C light. The antioxidant activities of investigated carotenoids (and their dependence on UV radiation) were monitored by the DPPH test.

EXPERIMENTAL

The pigments were isolated from two plant species (*β*-carotene and lutein from spinach (*Spinacia oleracea*) leaves, and lycopene from tomato (*Lycopersicon esculentum*) fruits purchased at a local market.

Lecithin Epikuron 100 P, a mixture of phospholipids, was a gift from ICN Galenika, Belgrade. It was manufactured by Degusa Texturant Systems, Hamburg, Germany. The lipid content was: phosphatidylethanolamine 18.0 %, phosphatidic acid 8.3 %, phosphatidylinositol 14.1 % and phosphatidylcholin 21.7 %.* The lecithin mixture was kept in the dark to prevent the auto-oxidation process during daylight. Although dark auto-oxidation could not be eliminated, it was taken into account during the calculation of LP yield.

Pigments extraction from spinach leaves

The photosynthetic pigments were extracted using a modified method of Svec.[14,15] Leaves without midribs (FW 30 g) were dropped into boiling water, which was quickly replaced (after 1–2 min) with cold water. After drying between paper towels at 40–75 °C, the leaves were separated and placed in a methanol/petroleum ether mixture (60:30 v/v), with occasional agitation, for 30 min. The methanol removes water from the plant material and the petroleum ether picks up the pigments before they undergo secondary reactions. The deep-green extract was decanted through a cotton pad. The leaves were re-extracted twice with an equal volume of the extraction mixture. The pooled extracts were diluted with 120 ml 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 ml of a mixture containing 40–75 °C petroleum ether and diethyl ether (1:1 v/v), to ensure the solubility of pigments in the organic phase. The successive extracts were treated by the same procedure. The final pigment extract contained various forms of chlorophyll as well as the accessory pigments, carotenoids (carotenes and xanthophylls).

Isolation of carotenoids from spinach extract by column chromatography

The carotenoid fractions were isolated using a modified procedure of Svec[15] and Brockman[16] – column chromatography with silica gel (silica gel 60, Merck, 0.063–0.200 mm) as adsorbent and benzene/acetone mixture for the elution. The benzene/acetone ratio was changed from the initial 1:0 to the final 1:1, to permit an easier elution of the polar fractions. *β*-Carotene appears first (eluted with benzene only), followed by the chlorophylls (eluted with benzene/acetone, 7:1) and the xanthophylls fraction – lutein (eluted with benzene/acetone, 6:1–1:1). The column chromatogram is shown in the supplement (Fig. S1). The fractions were dried and resuspended in hexane. The fractions were identified by comparing their Vis spectra with standards spectra (Fig. S2).

* The acid value, peroxide number and iodine number were controlled and found to be correct.

Pigments extraction from tomato fruits

Tomato fruit (FW 8.0 g) was thoroughly mixed with 40 ml of ethanol. The slurry was stirred until the tomato material was no longer sticky (about 3 min). Ethanol was removed by vacuum filtration. The tomato residue was mixed with 60 ml of a mixture of acetone and petroleum ether (1:1). The extract was collected by vacuum filtration and the residue rewashed with the same solvent mixture (20 ml) in order to improve the yield. The filtrate was transferred to a small separating funnel and mixed with 50 ml of saturated NaCl solution. The organic layer was rewashed twice, the first one with 50 ml of 10 % K_2CO_3 and then with 50 ml of distilled water. Finally, approximately 1 g of anhydrous $MgSO_4$ was added to dry the organic layer. After 10–15 min the solution was vacuum filtered to remove the drying agent.

Isolation of carotenoids from 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 adsorbent and petroleum ether/acetone mixture for the elution. The mixture ratio was changed from an initial 10:0.1 to a final 9:1, to permit the easier elution of lycopene. β-Carotene appeared first (eluted with petroleum ether/acetone mixture of 10:0.1), followed by the lycopene fraction (eluted with a 9:1 ratio of the mixture). The column chromatogram is shown in the supplement (Fig. S3). The collected fractions were dried, resuspended and identified in hexane (Fig. S2).

HPLC analysis of carotenoid fractions

A high percentage of carotenoids in the separated fraction was evidenced by HPLC analysis. The analysis was performed on a Hewlett Packard HPLC system under isocratic 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 and 447 nm for lutein. The HPLC chromatograms are shown in the supplement (Figs. S4 and S5).

UV treatment

Continuous irradiation of the samples was performed in a cylindrical photochemical reactor "Rayonnet", with 14 symmetrically placed lamps with emission maxima in two different ranges: 254 nm (UV-C) and 300 nm (UV-B). The samples were irradiated in quartz cuvettes (1 cm×1 cm×4.5 cm) placed on a circular rotating holder. The total energy flux reaching the samples was about 25 and 21 W m^{-2} for 254 and 300 nm, respectively.

Vis spectroscopy

Vis spectra of the samples before and after UV radiation were recorded on Varian Cary-100 spectrophotometer. All spectra were recorded from 400 to 800 nm.

DPPH test

The interaction of carotenoids with the 1,1-diphenyl-2-picrylhydrazyl (DPPH) radical was estimated according to the modified method of Choi et al.[17,18] A methanolic solution of DPPH radical (1 ml, 0.3×10^{-3} mol l^{-1}) was added after irradiation to 2.5 ml of an aqueous mixture lecithin:pigment, 50:1 (v/v). The initial concentrations were 8.5×10^{-6} mol l^{-1} and 5×10^{-5} mol l^{-1} for lecithin and carotenoids, respectively. The irradiated reaction mixture was incubated at room temperature for 20 min in the dark. Vis spectra were recorded from 400 to 800 nm. The absorbance was read at 517 nm, being the wavelength of maximal absorption of the DPPH radical. Aliquots of the new mixtures (lecithin and the pigments) were irradiated with UV-C and UV-B light for various time intervals and the DPPH test was repeated. The following formula was used to calculate the scavenging capacity of DPPH in the presence of carotenoids:[17]

$$\text{Scavenging capacity (\%)} = 100 - (A_{\text{sample}} - A_{\text{blank}}) \times 100 / A_{\text{control}}$$

where A_{sample} is the absorbance at 517 nm of an irradiated lecithin/pigments mixture, 50:1, treated afterwards with a solution of DPPH radicals, A_{blank} is the absorbance at 517 nm of the unirradiated lecithin/pigments mixture and A_{control} is the absorbance at 517 nm of a methanolic solution of the DPPH radical.

RESULTS

The structures of the investigated carotenoids (lycopene, β-carotene, and lutein) are shown in Fig.1.

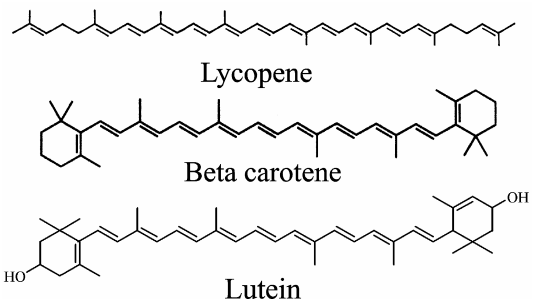

Fig. 1. Structures of the investigated carotenoids.

The DPPH scavenging capacity in presence of the investigated carotenoids as a function of time of irradiation with UV-B light is shown in Fig. 2.

The DPPH scavenging capacity in presence of the investigated carotenoids as a function of time of irradiation with UV-C light is shown in Fig. 3.

The calculated slopes from the corresponding DPPH scavenging capacity plots are given in Table I. The calculated average declinations (*i.e.*, relative errors), based on 4 other repeated experiments, for each point on the plots (Figs. 2 and 3), are 10.65 (UV-C) and 6.70 % (UV-B) for β-carotene; 10.25 (UV-C) and 4.3 % (UV-B) for lycopene; 10.2 (UV-C) and 4.9 % (UV-B) for lutein.

DISCUSSION

The influence of UV radiation on the antioxidant activities of carotenoids was spectrophotometrically analyzed by the DPPH test. DPPH is a stable free radical that produces a violet color in methanolic solution. The DPPH radical (containing a lone electron) is characterized by a strong absorption at 517 nm. As the electron is paired off in the presence of another free radical scavenger, the absorption decreases and the resulting discolorations are stoichiometric with respect to the number of electrons taken up.[18,19] Hence, the concentration of stable free DPPH radical is reduced in the presence of an antioxidant molecule. This fact was employed to evaluate its antioxidant activity.

Specifically, DPPH test was performed in this study in the presence of a mixture of lipoidal components (lecithin) in the reaction system, exposed to a long continuous UV-radiation, resulting in the formation of lipid and lipoperoxy radicals.[20] DPPH test is not specific for any particular radical species present in the

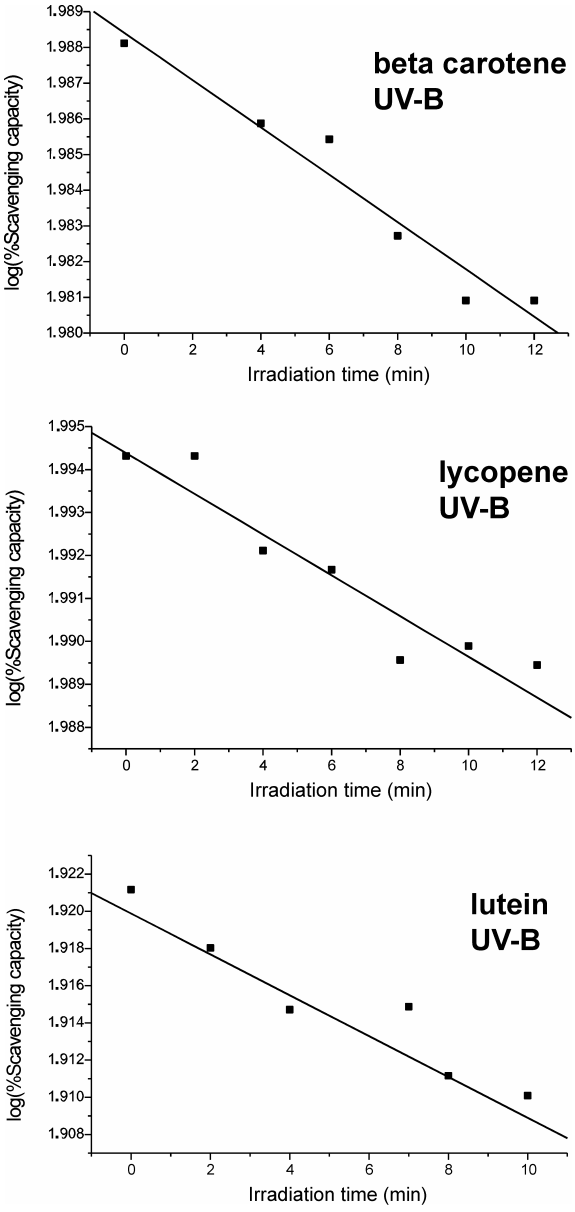

Fig. 2. Decrease of the scavenging capacity of DPPH toward β-carotene, lycopene and lutein with increasing time of UV-B irradiation (300 nm), in the presence of lecithin.

reaction mixture, *i.e.*, a DPPH radical may potentially react with carotenoid radicals (CAR), as well as with lipid radicals, or with any other radicals species potentially present in solution (at the same time, CAR radicals, obviously present in the system, certainly react with lipid radicals, thereby performing anti-oxidant lipid protection, but this cannot be followed by the DPPH test). Nevertheless, the relative linearity of the plots of scavenging capacity *vs.* irradiation time for all three

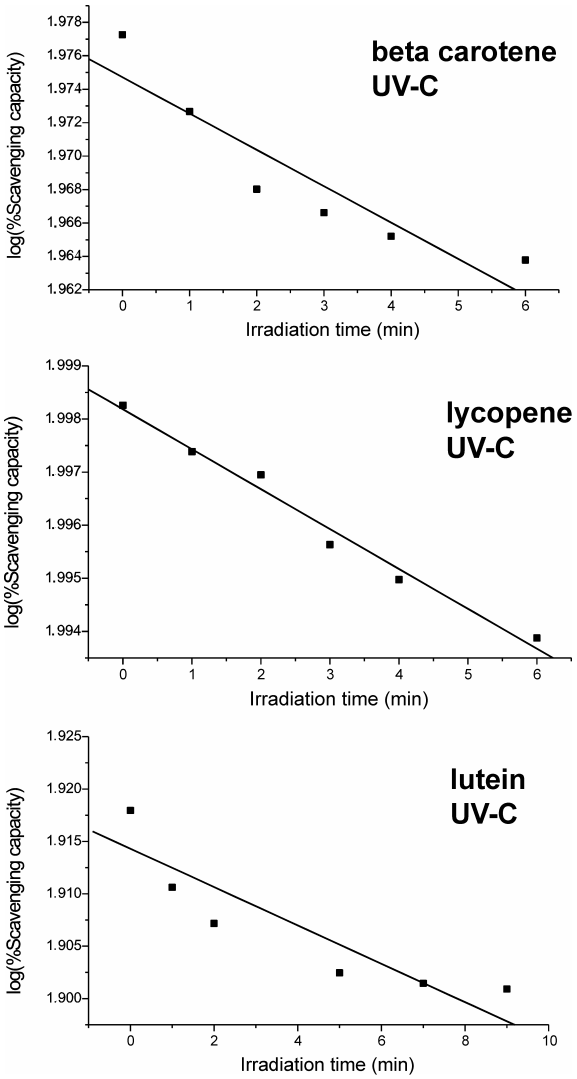

Fig. 3. Decrease of scavenging capacity of DPPH toward β-carotene, lycopene and lutein, with increasing time of UV-C irradiation (254 nm), in the presence of lecithin.

studied carotenoids with both UV-B and UV-C radiation, which was also found in the presence of the same carotenoids using the other, more specific TBA–MDA test,[21] offers arguments in favor of DPPH–CAR combination. Therefore, the results presented in Table I give a comparative view of the three "pigment slopes" calculated from kinetic measurements and represent decreasing rates of the radical scavenging capacities of DPPH toward the three carotenoids (k) during prolonged UV-irradiation in both ranges (UV-C and UV-B). Clearly, there is noticeable drop in the k values following a change from UV-C (254 nm) to UV-B (300 nm) photons. This fact confirms that the decreasing rates of the scavenging capacities of carotenoids toward the DPPH radical during prolonged UV-irradi-

ation depend on the energy input of the UV-photons (the $k_{UV\text{-}C}/k_{UV\text{-}B}$ ratio was 3.3 for β-carotene, 2.1 for lycopene and 1.7 for lutein). This type of dependence of the antioxidant activity of carotenoids on the energy input of the UV photons was also observed in a previous work, in which the antioxidant activities of carotenoids were studied by the TBA–MDA test.[21]

TABLE I. The values of rate constants (k / 10^{-3} min^{-1}) of the decreasing anti-oxidant activities of the investigated carotenoids (β-carotene, lycopene and lutein) monitored by the DPPH test in a solution containing the carotenoids (1.0×10^{-6} mol l^{-1}) and soybean lecithin (8.5×10^{-6} mol l^{-1}) mixture, during UV-irradiation with emission maxima in two different ranges: 254 (UV-C) and 300 nm (UV-B). Concentration of DPPH radicals was 3×10^{-4} mol l^{-1}

λ / nm	254	300
β-Carotene	2.17	0.66
Lycopene	1.05	0.49
Lutein	1.83	1.10

It is necessary to underline that the DPPH test is an indirect and partial measure of the antioxidant activities of carotenoids. As it is already known, the antioxidant activities of carotenoids in a free radicals rich medium (chain-breaking antioxidant activities) may be expressed through at least three possible pathways.[11–13] These actions are performed *via* the participation of carotenoid radical cations ($CAR^{+\bullet}$), radical anions ($CAR^{-\bullet}$), or neutral radicals ($CAR^{\bullet}$). These radicals in complex systems, as studied in this work, are short-lived and therefore undetectable by the techniques used in this research. Thus, possibly, only a part of the antioxidant activities of carotenoids (through recombination with lipid and lipoxy radicals, thus preventing lipid oxidation) was able to be followed through recombination with DPPH radicals. The fact that the scavenging capacity of DPPH decreased linearly in the presence of all carotenoids when irradiated with UV-B and UV-C radiation (Figs. 2 and 3) is not a direct implication, *per se*, that DPPH and CAR radicals underwent mutual recombination. However, since the same type of kinetics (linear, 1st order) was found for all carotenoids in the same medium (Figs. 2 and 3) not only by this method, but by the much more selective TBA–MDA test,[21] where decreases of the carotenoids antioxidant activities were clearly connected with some (though slight) suppression of the lipid peroxidation chain mechanism, implies most probably DPPH–CAR combination, rather than the possible scavenging by DPPH of other radicals present in the system. This does not absolutely negate the latter possibility but suggests its marginal character compared to the main presumption. Hence, the effects of UV radiation on at least a part of the antioxidant activities of carotenoids can be followed by this method.

It is also evident that decrease of the scavenging capacity of DPPH toward the three carotenoids under UV-irradiation depends on the chemical structure of the carotenoid. Lycopene has the lowest values of k in respect to the other investtigated carotenoids. Thus lycopene (being non-cyclized and containing no oxy-

gen atoms) was the most resistant toward the effects of UV-irradiation. Contrary to this, lutein (containing two oxygen atoms and two cyclic rings, Fig. 1) was the most sensitive to UV-B irradiation, while keeping the "middle position" between β-carotene and lycopene, concerning its resistance toward UV-C irradiation (Table I). It has already been reported that the antioxidant activities of xanthophylls were more sensitive in comparison to carotenes to changes in the energy of the incident UV photons in hexane solution.[22] This reinforces the conclusion, since another conjugated dienes test was employed in that study.

CONCLUSIONS

To conclude, (*i*) although the DPPH test is not very selective, the results enable the speculation that DPPH radical preferably recombine with carotenoid radicals present in the system; (*ii*) the measured UV radiation mediated decrease in the antioxidant activities of the carotenoids through recombination with DPPH radicals highly depends on the energy of the UV photons employed and on the chemical structures of the carotenoid; (*iii*) antioxidant activity of lycopene appeared to be most resistant to UV radiation.

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SUPPLEMENT

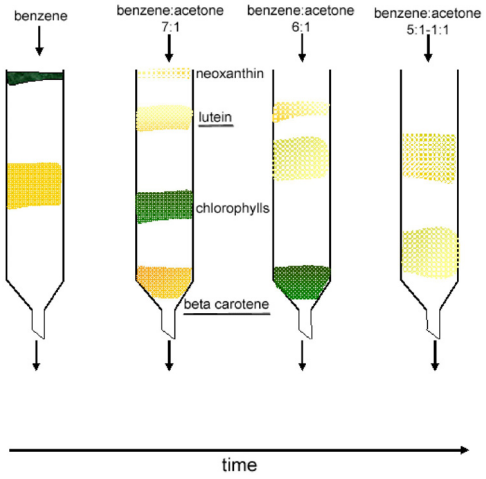

Fig. S1. Column chromatography of spinach leaves extract.

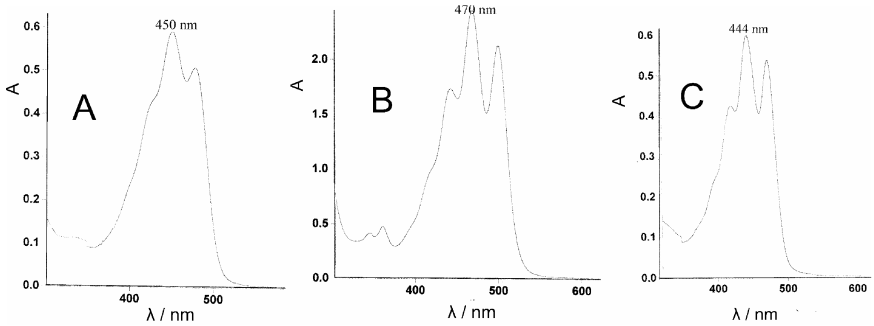

Fig. S2. Vis spectra of isolated carotenoid fractions in hexane: β-carotene (A), lycopene (B) and lutein (C).

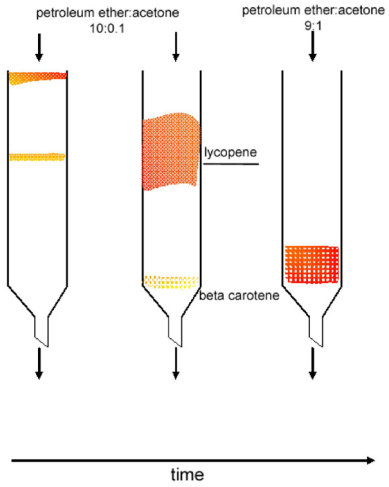

Fig. S3. Column chromatography of tomato fruit extract.

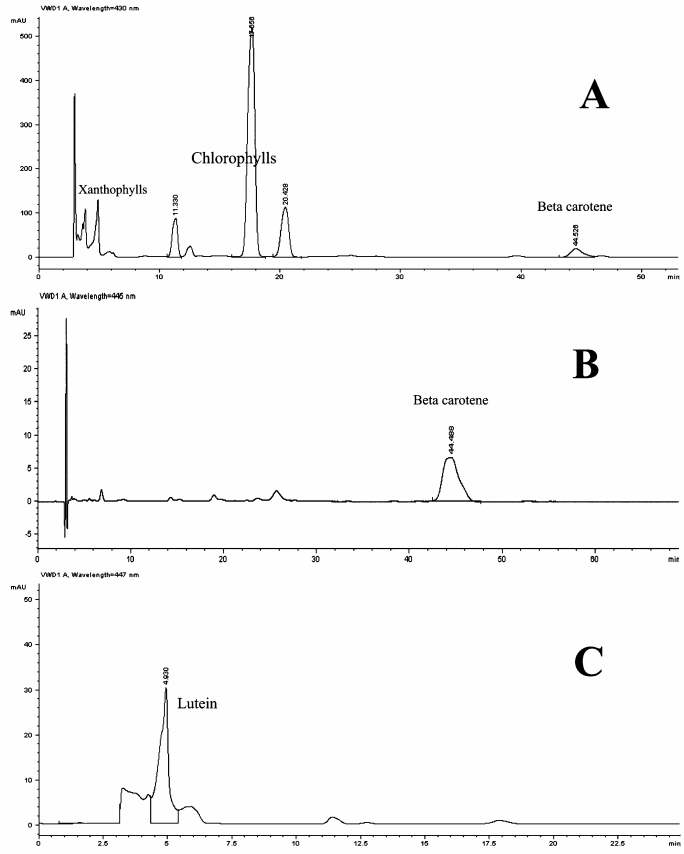

Fig. S4. HPLC chromatograms of spinach leaves extract (A), β-carotene fraction (B) and lutein fraction (C).

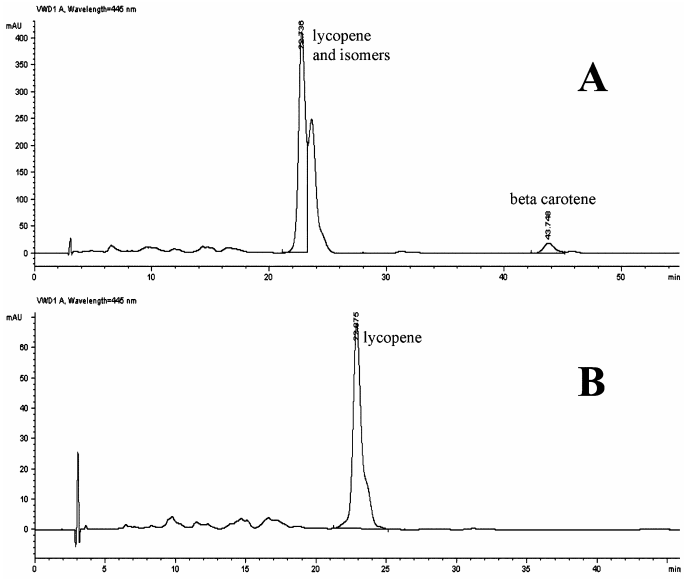

Fig. S5. HPLC chromatograms of tomato fruit extract (A) and lycopene fraction (B).

И З В О Д

ЕФЕКАТ УЛТРАЉУБИЧАСТОГ ЗРАЧЕЊА НА АНТИОКСИДАТИВНУ АКТИВНОСТ КАРОТЕНОИДА У ПРИСУСТВУ ЛЕЦИТИНА ПРАЋЕН ПРЕКО DPPH ТЕСТА

ДРАГАН ЦВЕТКОВИЋ и ДЕЈАН МАРКОВИЋ

Технолошки факултет, Универзитет у Нишу, Булевар ослобођења 124, Лесковац

Ефекти ултраљубичастог зрачења на антиоксидативну активност три изабрана каротеноида (β-каротена, ликопена и лутеина) у присуству липоидалне смеше лецитина, праћени су преко DPPH теста. DPPH тест се заснива на мерењу опадања абсорбанције DPPH радикала на 517 nm, проузрокованог антиоксидативним деловањем присутних каротеноида. Антиоксидативна активност испитиваних каротеноида примарно зависи од интензитета ултраљубичастог зрачења, односно од енергије упадних UV фотона.

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