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Original scientific paper

Effects of continuous UV-irradiation on the antioxidant activities of quercetin and rutin in solution in the presence of lecithin as the protective target

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Abstract: The stabilities and antioxidant action of two selected flavonoids, quercetin and rutin, dissolved in methanol and water, toward continuous UV--irradiation from three different sub-ranges (UV-A, UV-B and UV-C) were studied. The flavonoids underwent degradation (bleaching) following first-order kinetics. The bleaching rates were highly dependent on the energy input of the involved UV-photons. The antioxidant activities of the two flavonoids on UV-induced lecithin lipid peroxidation were studied by the thiobarbituric acid–malondialdehyde (TBA–MDA) test, and appeared to be also affected by the continuous UV irradiation. The energy input of the incident UV-photons again played a major governing role, but an impact of the flavonoids structures cannot be neglected.

Key words: flavonoids; UV-irradiation; antioxidant; kinetics.

INTRODUCTION

Sunlight reaching the Earth's surface contains an increasing fraction of its most energetic component, the UV-B fraction, due to the continuing damage of the ozone layer. Plants need sunlight to perform photosynthesis, but the enhanced level of UV-B radiation directly affects photosynthesis,[1] and the related processes, such as leaf morphology and the whole plant growth.[2–5] Both "light" and "dark" phase are affected; the light reaction itself[6,7] includes compositional changes in photosystems II[8] and I.[9] The changes mostly concern structural changes of the photosynthetic pigments,[10,11] especially chlorophyll,[6] but also of other components of the electron-transport chain.[12]

Naturally, plants have developed self-protective mechanisms against damaging UV-light. One of the major plant responses to enhanced UV-B radiation is an in-

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creased synthesis of pigments, capable of UV-B absorption, flavonoids above all. Flavonoids are mainly located in plant leaves but also in the other organs.[13,14] They are excellent UV-absorbers (because of their chemical structure) and hence are plant-protective screen-savers,[15] *i.e.*, they are very effective preventive antioxidants, since they reduce the production of the reactive radical oxygen species (ROS) generated by UV-radiation. However, in the case of free radicals chain reactions, they are also reported to be effective chain-breaking antioxidants.[15–21]

Flavonoids are benzo-γ-pyrone derivatives consisting of phenolic and pyran rings and are classified according to the substitutions in the basic skeleton. Their structures and functions differ in the presence/absence of hydroxyl, methoxy, glycosidic and other groups attached to the A and B rings of the basic skeleton (Fig. 1). In nature, flavonoids exist primarily as 3-*O*-glycosides and polymers.[16] The most important structural elements of flavonoids are: an *o*-dihydroxy group in the B ring (catechol structure) as a radical target; a double bond between positions 2 and 3 of the C-ring conjugated with keto group in position 4, because of its capacity to delocalize the uncoupled electron of a flavonoid radical; C-3,C-5 and C-7 hydroxyl groups (of the C and A rings, respectively) as scavengers of free radicals.[15–20]

The screen-saver function of flavonoids has practical applications, too. As excellent UV-absorbers, flavonoids are used as UV (UV-A and UV-B) protection agents in some cosmetics and pharmaceutical preparations, based on heterogeneous oil–water systems. While the general protective function of flavonoids in the formulations remains unquestionable, the whole picture covering the interactions of these formulations with UV-light, which includes not only flavonoids but also lipids, is less well known. Obviously, lipids undergo oxidation through free radical chain reactions, finishing with the formation of diene-type compounds at the very end, lipid peroxides; the whole process is known as lipid peroxidation.[22,23] Hence, the antioxidant function of flavonoids – toward lipids as the protection target – might be estimated through control of the lipid peroxidation process, partly through absorption of UV-radiation (as the peroxidation initiating agent), and partly through scavenging of the UV-created radical species, including lipid radicals and lipid-peroxy-radicals.[16–18,24]

The main objective of this study was to investigate the interaction of two flavonoids, rutin (flavonol glycoside) and quercetin (flavonol) with prolonged, continuous UV-radiation, in solution, in the absence and in the presence of soybean lecithin (a mixture of lipoidal compounds). The first goal was to obtain basic spectral and kinetic information concerning the stability of the flavonoids against UV-radiation in the absence of lecithin, which is also a measure of the ability of the flavonoids to prevent UV-initiated lecithin (lipid) peroxidation (when lipids are present). However, since prevention (through UV-absorption) is only partial and free radicals are certainly UV-generated in the system, the second goal, in

the presence of lecithin, was to examine the second type of flavonoid antioxidant ability, *i.e.*, to act as chain-breaking antioxidants. This type of approach was employed in previous studies, but with different antioxidants, selected carotenoids.[25,26] The same type of test, the thiobarbituric acid–malondialdehyde (TBA–MDA) test[26] was used to measure the amount of lipid peroxidation and to estimate its control by the flavonoids employed in this work.

EXPERIMENTAL

Samples preparations

Quercetin and rutin standards (Merck) were dissolved in methanol (quercetin and rutin) and in water (rutin), at a concentration of 8×10^{-5} M.

UV–Vis spectroscopy

The UV–Vis spectra of rutin and quercetin in different solvents were recorded on a Varian Cary-100 spectrophotometer. All spectra, before and after irradiation with UV-light, were recorded in the range 200 to 500 nm.

UV-treatment

The irradiation of pigments in different solvents was performed in a cylindrical photochemical reactor "Rayonnet", with 14 symmetrically placed lamps, with emission maxima in three different UV sub-ranges: 254 (UV-C), 300 (UV-B) and 350 nm (UV-A). The samples were irradiated in quartz cuvettes (1 cm×1 cm×4.5 cm) placed on a rotating circular holder. Quercetin was irradiated in methanol, while rutin was irradiated in methanol and water. The concentration of the flavonoids in both solvents was 8×10^{-5} M.

TBA–MDA test

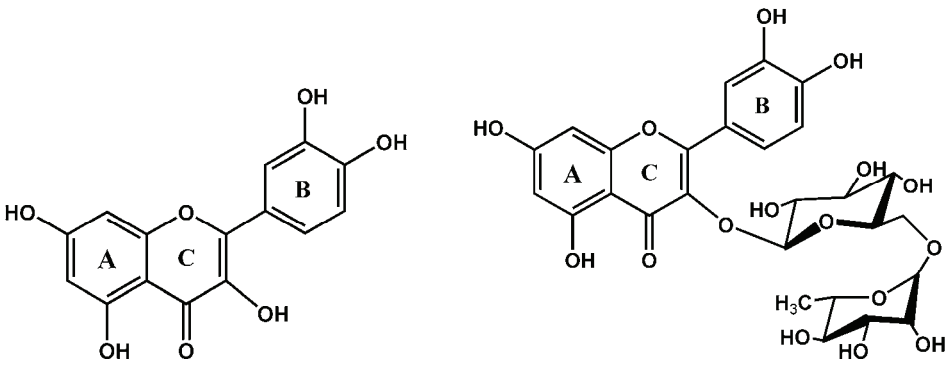
Lecithin peroxidation, as well as its inhibition in the presence of flavonoids, was measured by the TBA–MDA test.[27,28] This method is based on the reaction of malondialdehyde (MDA – a secondary product of lipid peroxidation) with thiobarbituric acid (TBA), whereby a red-coloured complex with a maximum absorption at 532 nm is obtained. The reaction mixture contained 2.2×10^{-3} M aqueous solution of lecithins and 7×10^{-5} M methanolic solution of a flavonoid, in a 10:1 (v/v) ratio. Lecithin peroxidation was initiated by continuous UV-irradiation during increasing times. Two cm^3 of aqueous trichloroacetic acid (5.5 %), and 2.5 cm^3 of Tris-buffer (pH 7.4), followed by 2 cm^3 of thiobarbituric acid (4.2×10^{-2} M in 5×10^{-2} M NaOH) were added in the reaction mixture immediately after irradiation. The mixture was incubated for 10 min at 37 °C in the dark and then centrifuged for 10 min at 4000 rpm. The Vis spectrum of the TBA–MDA complex was subsequently recorded from 400 to 800 nm. The absorbance of the complex in the supernatant was read at 532 nm and used to calculate the percentage inhibition of lecithin peroxidation using the following equation:

$$\text{Inhibition of lecithin peroxidation}\,(\%) = (A_C - A_S)\times\frac{100}{(A_C - A_B)} \quad (1)$$

where A_C – absorbance of the control (aqueous solution of pure lecithin) which was UV-irradiated and treated with TBA solution, A_S – absorbance of the sample (lecithin/flavonoids mixture) which was UV-irradiated and treated with TBA solution and A_B – absorbance of the blank (aqueous solution of pure lecithin which is not UV-irradiated but treated with TBA solution, thereby monitoring the MDA level in the lecithin before UV-irradiation).

RESULTS

The absorption spectra of flavonoids consist of two distinctive bands in a broad range of 240–400 nm. Band I, covering the range 300–380 nm, is attributed to the B-ring (with A_{max} around 350–370 nm), while band II, covering the range of 240–280 nm (with A_{max} around 260–270 nm) is attributed to the A–C benzoyl system; a weak band with an absorption maximum around 300 nm was also detected; it is attributed to the C-ring only (Figs. 1, 2A, 3A and 4A).



(A) (B)

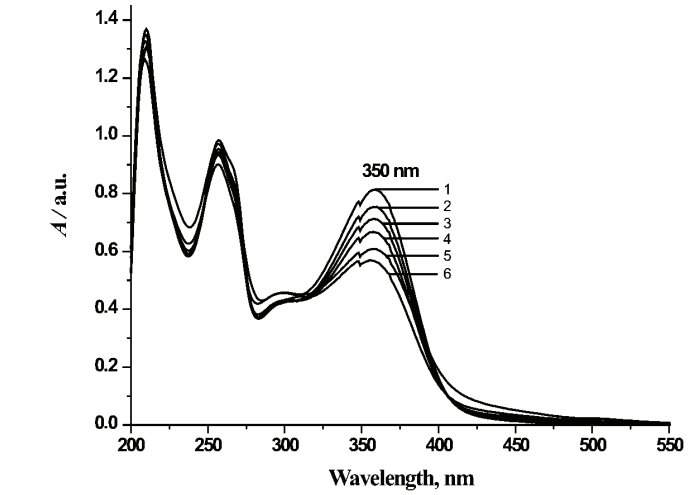
Fig. 1. Structure of A) quercetin and B) rutin.

The changes in the absorption spectra of rutin exposed to UV-C radiation in methanol are shown in Fig. 2A. The maximum absorbance of rutin (A_{max}= 350 nm) decreased proportionally with the length of the irradiation time (t_{irr}). A kinetic log plot, log A_{max}(350 nm) = $f(t_{irr})$, is shown in Fig. 2B. The plot shows a good linear fitting, with R values of about 0.99. The bleaching kinetics seems to obey a first-order law. Rutin showed a very similar response during irradiation with UV-B and UV-A light (not shown).

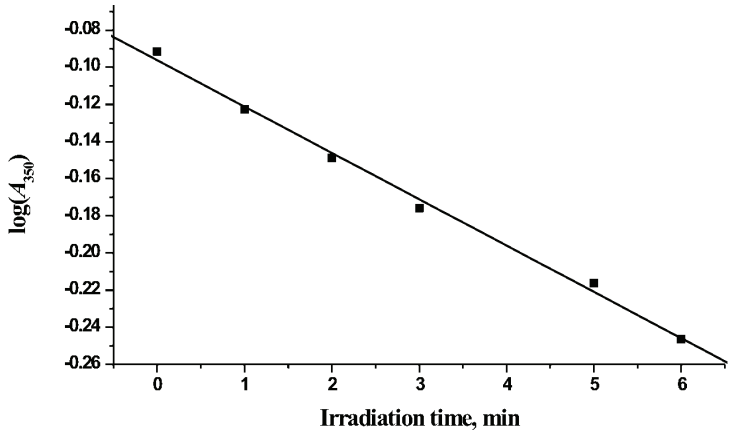
The changes in the absorption spectra of rutin exposed to UV-B radiation in water are shown in Fig. 3A. The maximum absorbance of rutin (A_{max} = 350 nm) decreased proportionally with the irradiation time (t_{irr}). A kinetic log plot, log A_{max}(350 nm) = $f(t_{irr})$, is shown in Fig. 3B. The plots show a good linearity, with R values of about 0.99. The bleaching kinetics seems to obey a first-order law again. Rutin expressed very similar responses during irradiation with UV-C and UV-A light in water (not shown).

The changes in the absorption spectra of quercetin exposed to UV-B radiation in methanol are shown in Fig. 4A. The maxima of the two absorption bands of quercetin (A_{max} = 370 nm and 250 nm) decrease proportionally with the irradiation time. The corresponding kinetic log plot, log A_{max}(370 nm) = $f(t_{irr})$, is shown in Fig. 4B. The plot shows a good linear fit, with R values of about 0.98. The bleaching kinetics seems to obey a first-order law. On the other hand and

contrary to the behaviour of rutin irradiated in a methanolic solution, a slow but continuous rise in the absorbance of quercetin at 290 nm was detected with increasing irradiation time; the log A_{max}(290 nm) = $f(t_{irr})$ dependence also corresponded to 1st order kinetics. Quercetin exhibited very similar behaviour during irradiation with UV-C and UV-A light in methanol (not shown).



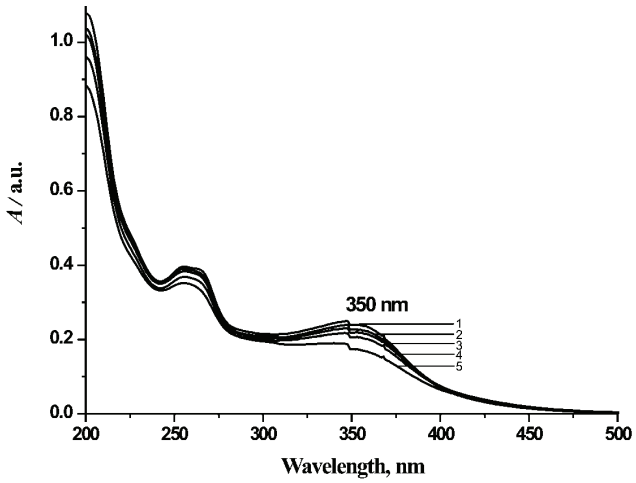
(A)



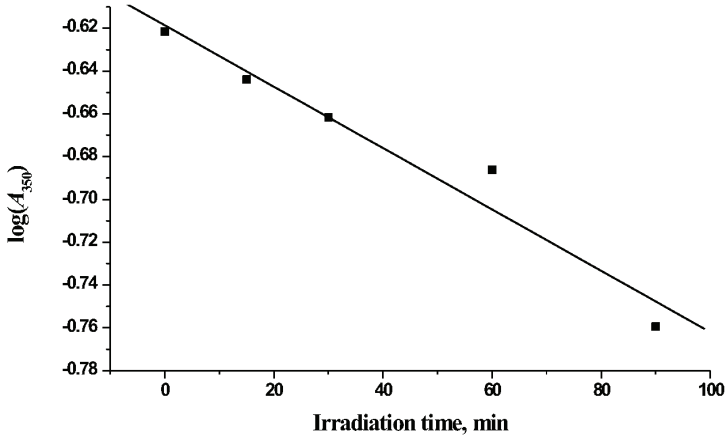
(B)

Fig. 2. Rutin in methanol irradiated with UV-C light. A) The changes in the absorption spectra of rutin exposed to UV-C radiation in methanol for 1 – 0; 2 – 1; 3 – 2; 4 – 3; 5 – 5; 6 – 6 min. B) The corresponding kinetic log plot obtained by following the A_{max} values at 350 nm (band I), as a function of the irradiation period (t_{irr}), log A_{max}(350 nm) = $f(t_{irr})$. The concentration of rutin was 8.5×10^{-5} M.

The time dynamics of the production of lipid peroxides in the lecithin lipoidal mixture because of UV-irradiation in the absence (control) and in presence of quercetin and rutin (sample) are shown in Fig. 5. The amount of peroxidation was estimated *via* the TBA–MDA test, by measuring the absorbance of the formed TBA–MDA complex at 532 nm.



(A)



(B)

Fig. 3. Rutin in water irradiated with UV-B light. A) The changes in the absorption spectra of rutin exposed to UV-B radiation in water for 1 – 0; 2 – 15; 3 – 30; 4 – 60; 5 – 90 min. B) The corresponding kinetic log plot obtained by following the A_{max} values at 350 nm (band I), as a function of the irradiation period (t_{irr}), log A_{max}(350 nm) = $f(t_{irr})$. The concentration of rutin was 8.5×10^{-5} M.

The kinetics of change in the percent inhibition of UV-induced lipid peroxidation, by quercetin and rutin in aqueous solutions containing the two flavonoids

(7×10^{-5} M) and soybean lecithin lipoidal mixture (2×10^{-3} M) are shown in Fig. 6. The inhibition was calculated from Eq. (1).

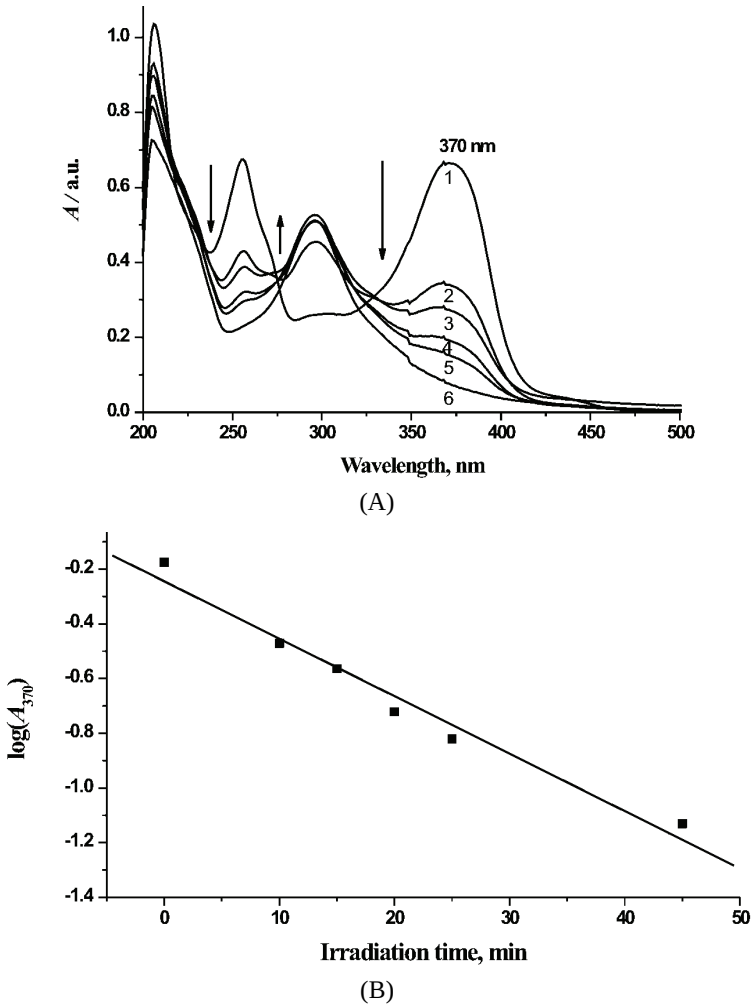
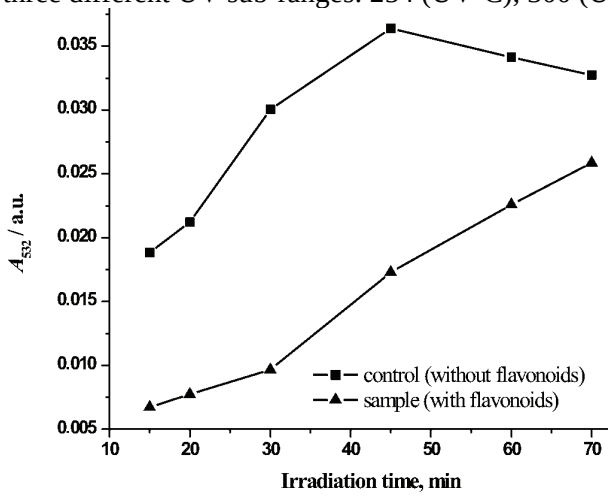


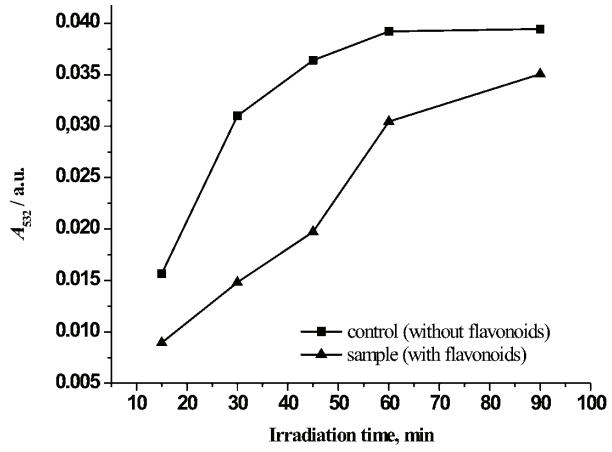
Fig. 4. Quercetin in methanol irradiated with UV-B light. A) The changes in the absorption spectra of quercetin exposed to UV-B radiation in methanol for 1 – 0; 2 – 10; 3 – 15; 4 – 20; 5 – 25; 6 – 45 min. B) The corresponding kinetic log plot obtained by following the A_{max} values at 370 nm (band I), as a function of the irradiation period (t_{irr}), $\log A_{max}(370\text{ nm}) = f(t_{irr})$. The concentration of quercetin was 8.5×10^{-5} M.

The calculated slopes (k_1) ($y_1 = k_1x + n$; y_1 – log (absorbance of rutin and quercetin in different solvents at 350 and 370 nm, respectively); x – UV-irradiation time) are presented in Table I. Such a presentation provides comparison of the corresponding slopes (obtained from the plots shown at Figs. 2B, 3B and 4B),

i.e., rates of the degradation (bleaching) of flavonoids during UV-irradiation of three different UV sub-ranges: 254 (UV-C), 300 (UV-B) and 350 nm (UV-A).



(A)



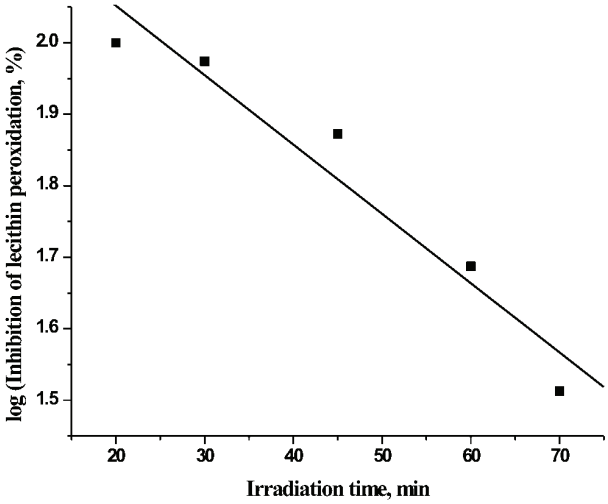
(B)

Fig. 5. Time dynamics of lipid peroxides production because of UV-A irradiation in the absence (control) and in the presence of A) quercetin and B) rutin (sample) in a lecithin lipoidal mixture, which was kept at the same concentration in both cases. The amount of peroxidation was estimated by TBA––MDA test, by measuring absorbance of the corresponding complex at 532 nm.

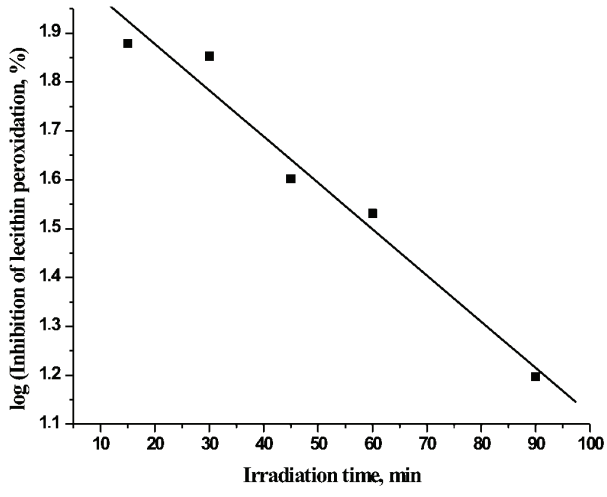
The calculated slopes represent the changes in the antioxidant activities of quercetin and rutin, followed by the TBA–MDA test (explained in the Experimental), toward the lecithin lipoidal mixture as the protected target, as a result of continuous UV-irradiation from three UV sub-ranges, expressed as the changes in the inhibition of UV-induced lecithin (lipid) peroxidation (by the two flavonoids), and are given in Table II. The slopes (k_2) were calculated from the plot: log (% inhibition of lipid peroxidation) = $f(t_{irr})$; the inhibition was calculated using the formula given in the Experimental.

DISCUSSION

As it was stated in the Introduction, antioxidant action of flavonoids in the presence of a "protective target" (lecithin lipids in this study) may be potentially expressed in *i*) a preventive and/or *ii*) chain-breaking manner; the former action does not exclude the latter one, moreover both actions are undoubtedly related. *i.e.*, the more expressed are the preventative effects of flavonoids (through UV--absorption), the less are their chain-breaking actions necessary.



(A)



(B)

Fig. 6. Kinetics of change in the percentage (%) inhibition of UV--A-induced lipid peroxidation by quercetin (A) and rutin (B), in aqueous solutions containing the two flavonoids (7×10^{-5} M) and a soybean lecithin lipoidal mixture (2×10^{-3} M). The inhibition was calculated as described in the Experimental.

Flavonoids stability in the absence of lecithin: a measure of preventive antioxidant action

Both flavonoids expressed clear sensitivity to the action of UV radiation from all three UV-ranges. They underwent substantial bleaching as measured through the proportional decrease in the maximal absorbance of Band I as a function of radiation duration (t_{irr}), *i.e.*, log A_{max}(350 or 370 nm) = $f(t_{irr})$ (Figs. 2–4). These dependences are expressed as linear plots (Figs. 2–4), indicating first order kinetics of the degradation of the flavonoids. This conclusion does not question the possible presence of other species, mostly radical ROS, in the irradiated system (as a result of UV-irradiation), as well as their potential role in the mechanism of flavonoid degradation.[17,24]

TABLE I. Kinetics of rutin and quercetin degradation (bleaching) in methanol and water, during UV-irradiation in three different UV sub-ranges: 254 (UV-C), 300 (UV-B) and 350 nm (UV-A). The absorbances of rutin and quercetin were taken from the corresponding absorption spectra (Figs. 2–4) at 350 and 370 nm, respectively

Solvent	k_1 / min^{-1} (R)		
	Wavelength, nm		
	254 (UV-C)	300 (UV-B)	350 (UV-A)
	Rutin		
Methanol	–0.0250 (–0.998)	–0.00173 (–0.917)	–0.00016 (–0.923)
Water	–0.0514 (–0.990)	–0.00143 (–0.977)	0.00170 (0.996)
	Quercetin		
Methanol	–0.111 (–0.985)	–0.0210 (–0.986)	–0.00312 (–0.995)

TABLE II. Kinetics of the change in the percentage (%) of inhibition of (UV)-induced lipid peroxidation, by quercetin and rutin, in water solution containing two flavonoids (7×10^{-5} M) and a soybean lecithin lipoidal mixture (2×10^{-3} M), during UV-irradiation in three different UV sub-ranges: 254 (UV-C), 300 (UV-B) and 350 nm (UV-A). The change was followed by the TBA–MDA test

Wavelength, nm	k_2 / min^{-1} (R)	
	Quercetin	Rutin
254 (UV-C)	–0.170 (–0.944)	–0.28680 (–0.999)
300 (UV-B)	–0.0414 (–0.917)	–0.06880 (–0.970)
350 (UV-A)	–0.00970 (–0.969)	–0.00946 (–0.984)

Evidently, flavonoids bleaching are highly dependent on the energy input of the UV radiation; in both cases, a switch from the UV-A to the UV-B lamp and from the UV-B to the UV-C lamp led to a corresponding increase in the related bleaching rate constants by an order of magnitude (Table I). This behaviour was observed in the other studies related to continuous UV-irradiation.[29] Even the mutual relationship of the bleaching rate constants for the two flavonoids ($k_{bleach.,querc.}/k_{bleach.,rutin}$) is UV-energy dependent: from 4.4 (UV-A) over 12.1

(UV-B) to 19.4 (UV-C) in favour of quercetin, which appears to be less UV-resistant, compared to rutin in the same solvent (methanol). It is evident that the substituent at position 3 in the C-ring affects this difference as it was proposed the OH-group at this position makes quercetin more vulnerable to UV-action, compared to the glycoside residue in the case of rutin.[30]

In addition, a clear increase in the absorbance of quercetin was detected in the range between 280 and 320 nm, with a maximum around 290 nm, implying the formation of a UV-irradiation product. However, this was not of relevance for this work.

Flavonoids suppression of lecithin peroxidation

An estimation of flavonoids suppression of lipid peroxidation and its change because of UV-irradiation was realised in an indirect manner. Direct measurements of possible flavonoids scavenging of UV-created lipid ($L^{\bullet}$) and lipoperoxy ($LOO^{\bullet}$) radical species in investigated aqueous system (chain-breaking antioxidant action) was not possible because the required techniques were not available. Instead, the TBA–MDA test (which indirectly measures the amount of lipid peroxidation)[31,32] was employed in the presence and in the absence of the two flavonoids and the difference was attributed to the antioxidant activities of the flavonoids, not only through the chain-breaking action but also through direct UV-absorption.

The patterns of the peroxidation of the studied lecithin in the absence (control) and in the presence of the flavonoids (sample) are shown in Fig. 5; both plots were obtained with UV-A irradiation to be more comparable. As can be seen, the control pattern expresses a typical curve shape, beginning with a relatively sharp rise, reflecting the fast proliferation of the lipid peroxidation chain in the initial period of UV-irradiation, and finishing at some plateau level. In the latter case, for longer periods of UV-irradiation, equilibrium between the UV-induced production of peroxides and their simultaneous degradation exists. It is well known that (lipid) peroxides themselves are very unstable species, prone to self-degradation.[33] The sample patterns in the presence of both quercetin and rutin are obviously different (Fig. 5). Even in the initial phase, the flavonoids significantly reduce the amount of peroxidation, and continue to do so in an almost in linear manner until the end of the UV-irradiation process, when the plateau level (reflecting the production/degradation equilibrium of the peroxides) had already been established. Similar responses were obtained with UV-B and UV-C irradiation.

Thus, both quercetin and rutin retained their antioxidant behaviour under continuous UV-irradiation regime. However, it is questionable whether the "quantity" of their antioxidant activities remained at the same level, or changed under the employed UV-irradiation regime. Ioku and coworkers[34] found that

quercetin expressed a higher peroxyl radical-scavenging activity against methyl linoleate in solution (as the protective target) compared to its glucosides, similar to rutin, when peroxidation was initiated by a thermal 2,2'-azobis(2,4-dimethyl-valeronitrile (AMVN) initiator. Figure 6 explains what happens when UV-initiation was employed. The plots show a linear decreasing change in the flavonoid inhibition of lecithin peroxidation with increasing duration of UV-irradiations; in other words, the flavonoids antioxidant capacities are reduced under a continuous UV-irradiation regime. As can be seen from Table II, the quantity of change is directly proportional to the energy of incident photons. In the case of quercetin, the (antioxidation activity) attenuation factor (on switching from UV-A to UV-B, and from UV-B to UV-C) is about 4; in the case of rutin, the attenuation (diminution) factor for the UV-A/UV-B switch is about 7, and for UV-B/UV-C switch about 4, the same as for quercetin. Bearing in mind the previously cited greater stability of rutin, compared to quercetin, under a similar UV-irradiation regime when lecithin was absent, this could seem somewhat surprising. It evidently suggests that the higher resistance of rutin towards UV-irradiation (compared to quercetin) plays only a partial role in the protection of its antioxidant activity under the same UV-irradiation regime; the other, probably more significant factor might be related to the free radical species created in the system because of prolonged UV-irradiation, and the involved mechanisms.

ИЗВОД

ЕФЕКТИ КОНТИНУАЛНОГ УЛТРАЉУБИЧАСТОГ ОЗРАЧИВАЊА НА АНТОКСИДАТИВНУ АКТИВНОСТ КВЕРЦЕТИНА И РУТИНА У РАСТВОРУ У ПРИСУСТВУ ЛЕЦИТИНА КАО ЗАШТИТНЕ МЕТЕ

ДРАГАН ЦВЕТКОВИЋ[1], ДЕЈАН МАРКОВИЋ[1], ДРАГАНА ЦВЕТКОВИЋ[2] и БЛАГА РАДОВАНОВИЋ[3]

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У овом раду је проучавана стабилност и антиоксидативна активност два изабрана флавоноида, кверцетина и рутина, растворена у метанолу и води, према дејству контунуалног ултраљубичастог (UV) озрачивања из три различита под-опсега, UV-A, UV-B и UV-C. Флавоноиди подлежу разградњи пратећи кинетику првог реда. Константе брзине деградације (bleaching-a) су веома зависне од енергије UV-фотона. Антиоксидативна активност два флавоноида у односу на UV-индуковану липидну пероксидацију лецитина проучавана је TBA–MDA тестом, и нађено је да на њу такође утиче режим UV континуалног озрачивања. Енергија UV-фотона и овде игра главну улогу, али се ни утицај флавоноидних структура не може занемарити.

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