PROTOLYTIC EQUILIBRIA AND PHOTODEGRADATION OF QUERCETIN IN AQUEOUS SOLUTION

Tatjana MOMIĆ[a1], Jasmina SAVIĆ[a2], Urh ČERNIGOJ[b1], Polonca TREBŠE[b2] and Vesna VASIĆ[a3,*]

[a] *Vinca Institute of Nuclear Sciences, Department of Physical Chemistry, P.O. Box 522, 11001 Belgrade, Republic of Serbia; e-mail: [1] momict@vin.bg.ac.yu, [2] jasnas@ vin.bg.ac.yu, [3] evasic@vin.bg.ac.yu*

[b] *Laboratory for Environmental Research, University of Nova Gorica, Nova Gorica, Slovenia; e-mail: [1] urh.cernigoj@p-ng.si, [2] polonca.trebse@p-ng.si*

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Studies of protolytic equilibria and investigations of stability of flavonoids at different acidities are necessary to better understand their antioxidant efficiencies and autoxidation characteristics. The protonation constant of carbonyl group and dissociation constants of OH groups of quercetin in aqueous solutions were determined spectrophotometrically. The distribution diagram of ionic species in aqueous solutions of various acidities was calculated. Study of the effects of UV irradiation on quercetin at pH 5.00, 7.50 and 10.00 indicated that UV irradiation accelerated quercetin autoxidation via the formation of the oxidation product. The stability of quercetin and oxidation product was investigated as a function of irradiation time by using spectrophotometric and HPLC techniques. The apparent pseudo-first-order rate constants for quercetin degradation and oxidation product formation were calculated and discussed.

Keywords: Autoxidation; Quercetin; Protolytic constants; Rate constants; UV irradiation.

The most important class of polyphenolic secondary metabolites in plants is bioflavonoids. The increasing interest in flavonoids is due to appreciation of their broad pharmacological potency. Quercetin (3,5,7,3′,4′-pentahydroxyflavone) (Scheme 1) is the most prevalent member of this group of compounds[1]. It is highly labile in biological systems, leading to rapid degradation, along with metabolism, to form multiple potential biologically active products[2]. Besides its important biological roles, it exerts anticancer, antiviral and anti-inflammatory properties, which are a consequence of its affinity to proteins[3–6]. Due to its phenolic structure, this compound is a strong antioxidant and free radical scavenger[7–11].

In general, the antioxidant efficiency of flavonoids has been related to their hydrogen donating abilities and the number of ionizable hydroxy

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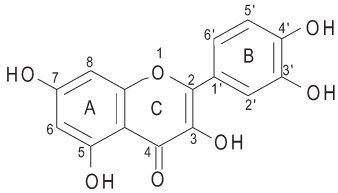
groups in the molecule[12–15]. Differences between reported values of pK_a, as well as the possibility to determine two or more pK_a values for quercetin, are caused by experimental conditions and applied methods[15–17]. Acidity and basicity, as well keto–enol prototropic tautomerism of quercetin molecule were also elucidated by quantum chemical calculations[18,19]. Moreover, flavonoids undergo autoxidation – the non-enzymatic reaction with atmospheric oxygen. This process was usually investigated in organic solutions in the presence of various oxidants, but in aqueous solutions it was strongly dependent on pH and the nature of buffer ions[20]. Autoxidation is usually accompanied by the production of the flavonoid radical anion, superoxide and hydrogen peroxide[21,22]. Also, autoxidation is related to the antioxidant properties of flavonoids, but is strongly dependent on OH group deprotonation, i.e. the ionic state of the molecule[23]. The absorption of UV-VIS radiation by some hydroxyflavones that are not stable to irradiation leads to their chemical changes followed by their eventual degradation[24,25]. However, it is assumed that the protective effect of quercetin against irradiation may be due to its antioxidant properties[26].



SCHEME 1

The present paper deals with the detailed study of the protolytic equilibrium of quercetin and its stability in water. In addition, the stability of various ionic forms of quercetin before and after exposure to UV irradiation was investigated. The aim was to complete the literature data concerning the pK_a values of quercetin of a wide range of acidities, as well as to elucidate the autoxidation mechanism of this compound as a consequence of the irradiation of aqueous solutions.

EXPERIMENTAL

Chemicals

Quercetin dihydrate (Sigma–Aldrich) of the highest quality available (98%) was used without purification. 1×10^{-3} M stock solution of quercetin was freshly prepared in methanol immediately before the experiments. For equilibrium and stability experiments, quercetin solutions were prepared in Britton–Robinson buffer. The ionic strength was maintained constant (0.1 M) with NaCl in the pH range 2.10–12.20. Acidity of concentrated $HClO_4$ solutions was

characterized by Hammett (H_0)[27] and Cox–Yates (X)[28] acidity functions. Redistilled water was used in all experiments.

Methods

Spectroscopic and pH measurements. UV spectra (λ, nm) were recorded immediately after preparation of the solutions on a Perkin–Elmer Lambda 35 UV-VIS spectrophotometer equipped with thermostatted quartz cell. The temperature in the cell was kept at 25 ± 0.05 °C with a water-thermostatted bath. pH values of the solutions were measured with a Metrohm pH-meter Model 713 equipped with glass electrode.

HPLC analysis. HPLC equipment consisted of an HP 1100 Series chromatograph coupled with a DAD. Chromatographic separations were run on a C18 Pinnacle ODS column (Restek, 250 × 4.6 mm, 5 μm) using an 80:20 mixture of 2% v/v H_3PO_4 and acetonitrile (Baker) as the eluent for the first 2 min. The linear gradient was applied from 20 to 45% acetonitrile from 2 to 7 min. An isocratic 55:45 mixture was applied between 7 and 13 min. The eluent flow rate was 1.0 ml min^{-1} and the injection volume was 10 μl. The elutions were monitored with DAD at different wavelengths between 200 and 400 nm.

Photodegradation studies. Experiments were carried out in an annular (double skin) photoreactor. A low-pressure mercury fluorescent lamp (Philips CLEO 20 W with broad maximum at 355 nm or Sankyo denki G15T8E with broad maximum at 310 nm) was encircled by two Duran borosilicate glass tubes of different diameters. Aqueous solutions (70 ml) of quercetin at different pH values were pumped and irradiated between the two quartz glass tubes. During irradiation, aliquots of samples were taken from the cell and analyzed with an HPLC and UV-VIS spectrophotometer.

RESULTS

Spectral and Acid-Base Properties of Quercetin

The absorption spectra of 5 × 10^{-5} M quercetin in Britton–Robinson buffer were recorded as a function of acidity in the pH range 2.00–12.20 (Fig. 1). The spectra showed two characteristic absorption maxima in the range 254–313 nm and 367–417 nm. The change of acidity induced a slight shift of both maxima, which was usually followed by poor isosbestic points. These changes in absorption spectra, due to variable medium acidities, are the consequence of the charged ionic forms of quercetin according to the equilibrium:

$$\mathrm{RH}_n^{n-5} \overset{K_{a(6-n)}}{\rightleftharpoons} \mathrm{RH}_n^{n-6} + \mathrm{H}^+ \qquad (1)$$

where n = 1–5 and $K_{a(6-n)}$ are the dissociation constants of quercetin. The dissociation constants of two consecutive overlapping equilibria were deter-

mined from the change of absorbance (A) vs pH (S-shaped curves) at the wavelengths which showed the largest difference between A_{max} and A_{min} (data not shown). A non-linear least-squares regression analysis on Eq. (*2*) was performed in the appropriate pH range[29]:

$$A = \frac{A_{(5-k)}10^{-2\times pH} + A_{(6-k)}K_{a(6-k)}10^{-pH} + A_{(7-k)}K_{a(6-k)}K_{a(7-k)}}{10^{-2\times pH} + K_{a(6-k)}10^{-pH} + K_{a(6-k)}K_{a(7-k)}} \qquad (2)$$

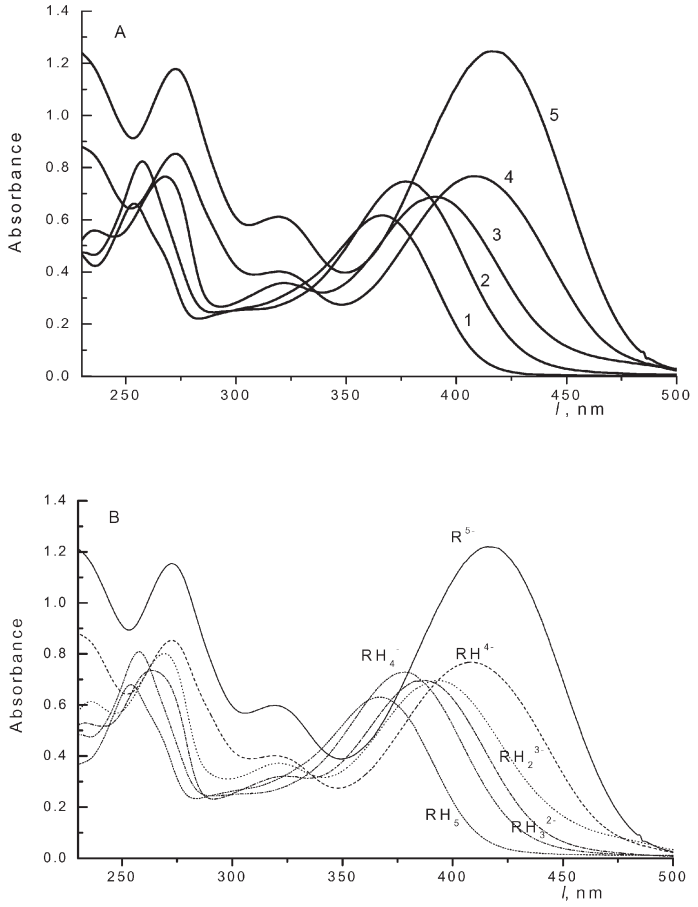


FIG. 1

Absorption spectra of 5×10^{-5} M quercetin at various pH: *1* 2.10, *2* 6.70, *3* 8.50, *4* 10.00, *5* 12.20 (A) and of ionic forms of quercetin (B)

where A is the absorbance of the quercetin solution consisting of at least three ionic forms such that A_{7-k}, A_{6-k} and A_{5-k} are the absorbances of the minimum, middle region plateau and maximum of A vs pH dependence, k = 2–5. These values can be ascribed to different ionic forms in overlapping equilibrium. The values obtained for the dissociation constants together with the pK_a values from literature for quercetin are presented in Table I. The distribution species diagram shown in Fig. 2 was calculated using the

TABLE I
Protolytic constants of quercetin in aqueous media

pK_{a1}	pK_{a2}	pK_{a3}	pK_{a4}	pK_{a5}	Ref.
		Dissociation of OH groups			
5.50 ± 0.25	7.15 ± 0.42	8.00 ± 0.46	9.57 ± 0.25	11.40 ± 0.24	this work
5.7[a]	7.1[a]	8.0[a]	9.9[a]	11.0[a]	16
		Protonation of carbonyl group			
HAFM[b]	p$K_{RH_6^+}$	–2.02 ± 0.03	n = 0.96 ± 0.02		this work
EAFM[c]	p$K_{RH_6^+}$	–2.65 ± 0.02	m^* = 0.93 ± 0.01		this work

[a] Determined spectrophotometrically. [b] Hammett acidity function method. [c] Cox–Yates "excess acidity" function method.

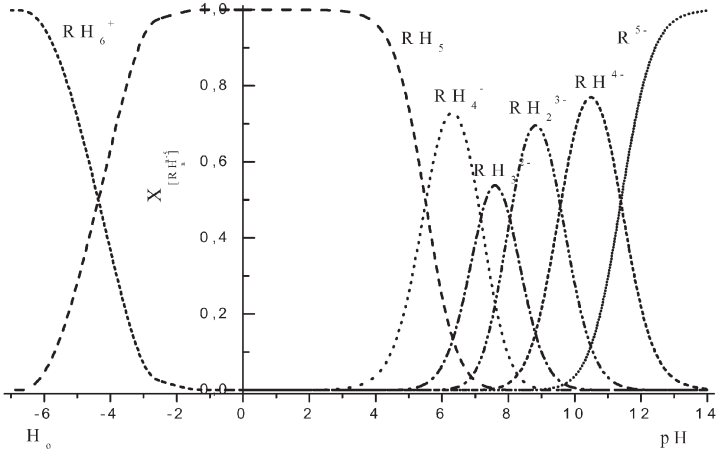


FIG. 2
Distribution of ionic forms of 5 × 10^{-5} M quercetin as a function of acidity

experimentally obtained dissociation constants of 5×10^{-5} M quercetin. However, it was interesting to present the absorption spectra of the various deprotonated forms of quercetin. The absorption spectra shown in Fig. 1A are not characteristic of one species. The poor isosbestic points due to the overlapping equilibrium indicate that they are the contributions of different ionic forms. Two of them, RH_5 and R^{5-}, were directly accessible from the experimental data at pH < 4 and pH > 12. The absorption spectra of the other forms were obtained by using the chemometric method[31]. Figure 1B shows the calculated absorption spectra of ionic forms of quercetin.

The formation of quercetin-derived pyrylium ion (positive ionic form) due to the protonation of the carbonyl group was confirmed by the change of UV-VIS spectra of 1×10^{-5} M quercetin in acidity range from 10.00 to 2.10 M $HClO_4$ (Fig. 3). The formation of the corresponding pyrylium cation modified drastically the UV-VIS spectrum by shifting both the characteristic absorption bands bathochromically[32]. Consequently, the new characteristic absorption band at about 430 nm appeared. Four well defined isosbestic points were observed (Fig. 3), indicating that there was no significant medium effect or build-up intermediates. Moreover, the reversibility of the protonation reaction was confirmed by comparison of the absorption

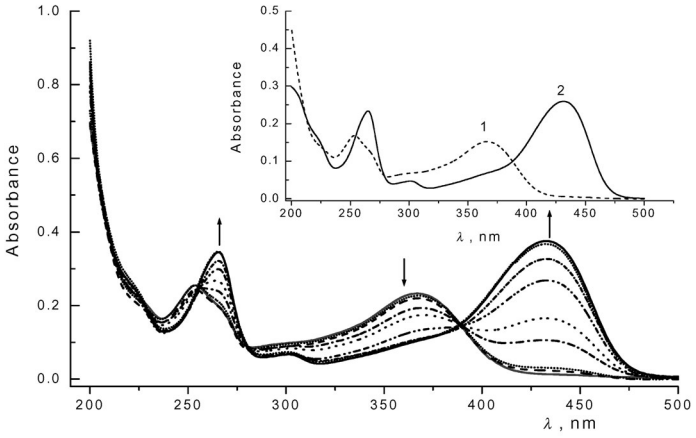


FIG. 3

Absorption spectra of 1×10^{-5} M quercetin in $HClO_4$ (mol l^{-1}): 2.10, 3.10, 4.30, 4.60, 5.10, 6.20, 7.20, 8.20, 10.00 (in the direction of arrows). Inset: Absorption spectra of 1×10^{-5} M quercetin in 2.10 M $HClO_4$ (*1*) and 10.00 M $HClO_4$ (*2*) obtained upon dilution of 5×10^{-5} M quercetin in 10.00 M $HClO_4$

spectra of two samples which were made by dilution of 5×10^{-5} M quercetin solution 10.00 M $HClO_4$. The concentration of quercetin upon dilution was 1×10^{-5} mol l^{-1} in both 2.10 M $HClO_4$ (Fig. 3, inset, *1*) and 10.00 M $HClO_4$ (Fig. 3, inset, *2*). The absorption spectrum of neutral ionic form in 2.10 M $HClO_4$ was obtained. These results gave strong evidence that the spectral changes resulted from the effect of deprotonation of pyrylium cation, so it was safe to deal with a single equilibrium:

$$RH_5 + H^+ \overset{K_{RH_6^+}}{\rightleftharpoons} RH_6^+ \quad \text{where} \quad K_{RH_6^+} = \frac{[RH_5][H^+]}{[RH_6^+]} . \qquad (3)$$

The value of dissociation constant of the protonated form of carbonyl group, $pK_{RH_6^+}$ was obtained using the Hammett (H_0)-HAFM [27] and Cox–Yates (X)-EAFM [28] acidity functions methods, according to Eqs (*4*) and (*5*), respectively:

$$\log I = pK_{RH_6^+} - nH_0 \qquad (4)$$

$$\log I - \log C_{H^+} = m^* X + pK_{RH_6^+} \qquad (5)$$

where $I = RH_6^+/RH_5$ represents the ionization ratio, n is the number of protons which are involved in reaction, C_{H^+} stands for the hydrogen ion concentration. The parameter m^*, or solvation coefficient, accounts for the susceptibility of the protonated base to be stabilized by solvation, especially by H-bonding, and reflects the behavior of the set of bases. In order to determine the ionization ratio I, absorbance was measured at 434 nm as a dependence of H_0 and X functions. The values of constant $pK_{RH_6^+}$ obtained by using two methods are given in Table I.

Photochemical Degradation of Quercetin

It is well known that quercetin undergoes autoxidation in organic solutions and aqueous media above pH 8.00. The autoxidation is strongly dependent on pH and the buffer solution, as well as the presence of oxidants[20]. As can be seen from Fig. 2, at pH < 5 quercetin is in the form of a neutral molecule or positive ion ($H_0 < -2$). Above pH 4.00, at least two ionic species are present in aqueous solution due to the dissociation of the OH groups. According to literature, the dissociation promotes the autoxidation of quercetin[20,32]. The influence of photochemical irradiation on 5×10^{-5} M

quercetin in aqueous solution was investigated at pH 5.00, 7.50 and 10.00 at 25 °C. The irradiation time varied from 1 to 1600 min and all samples were analyzed by HPLC. Additionally, absorption spectra of the irradiated samples were followed in the same time interval and were compared with the absorption spectra of the control sample, which was not irradiated. The results obtained at pH 7.50 and 10.00 are presented in Figs 4 and 5.

The absorption spectra of the irradiated and non-irradiated samples at pH 10.00 and the irradiated samples at pH 5.00 and 7.50 showed the decay of the quercetin absorption bands (260–273 and 385–409 nm depending on pH) and simultaneous rise of the absorption band at about 327–321 nm, typical of oxidation product formation[33]. The results obtained at pH 7.50 and 10.00 are presented in Figs 4C, 4D and 5C, 5D. Moreover, two well-defined isosbestic points at 284 and 359 nm were observed. These spectral changes are entirely analogous to those occurring upon autoxidation of quercetin in water as well as oxidation with some oxidants in organic solvents[33]. From the literature data and the obtained spectrophotometric and HPLC results in this work, we assume that the autoxidation product detectable at 321–327 nm results from H_2O addition on the *p*-quinomethane

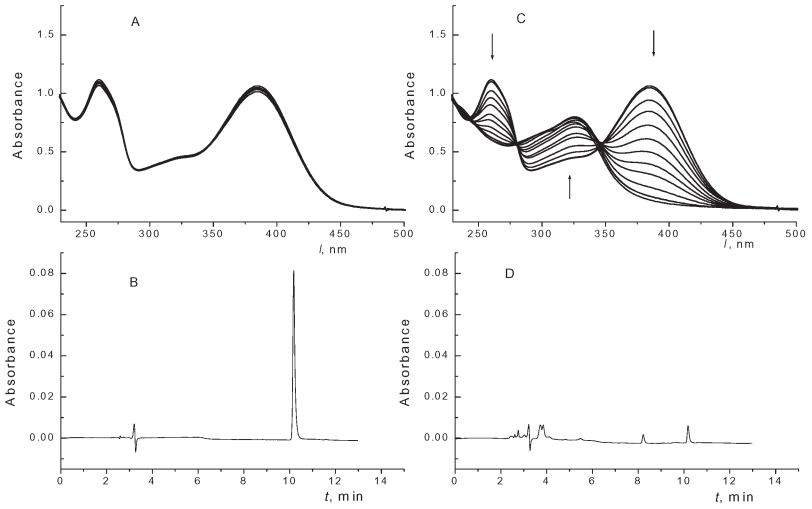


FIG. 4
Absorption spectra and HPLC chromatograms of 5×10^{-5} M quercetin at pH 7.50 in non-irradiated (A, B) and irradiated (C, D) samples. Spectra were recorded over a period of 300 min, arrows indicate the direction of the changes. Chromatograms were recorded after 300 min

formed by H-atom abstraction at 3-OH and 4′-OH of quercetin and subsequent rearrangement of the central ring[33]. The complete sequences of quercetin autoxidation induced by oxidants and catalyzed by metal ions are described earlier[32–34].

According to the distribution diagram of ionic forms (Fig. 2), quercetin is in neutral and monoion forms at pH 5.00 and mainly in the form of mono- and dianion at pH 7.50. It may be tempting to consider the first step of quercetin autoxidation as a direct electron transfer from quercetin anions to dioxygen because this process is thermodynamically unfavorable and very slow[35]. However, irradiation initiated and enhanced the electron transfer even in the case of neutral quercetin in water solution. Furthermore, oxidation product formation was not observed in the non-irradiated samples at pH 5.00 and 7.50 (Figs 4A, 4B). Analysis of the HPLC chromatograms indicated that the irradiation led to different absorbing intermediates found at lower retention times in the liquid chromatogram, at all pH values (Figs 4D, 5D). These more polar intermediates were not identified, but it was observed that the products differ when comparing the experiments at various pH.

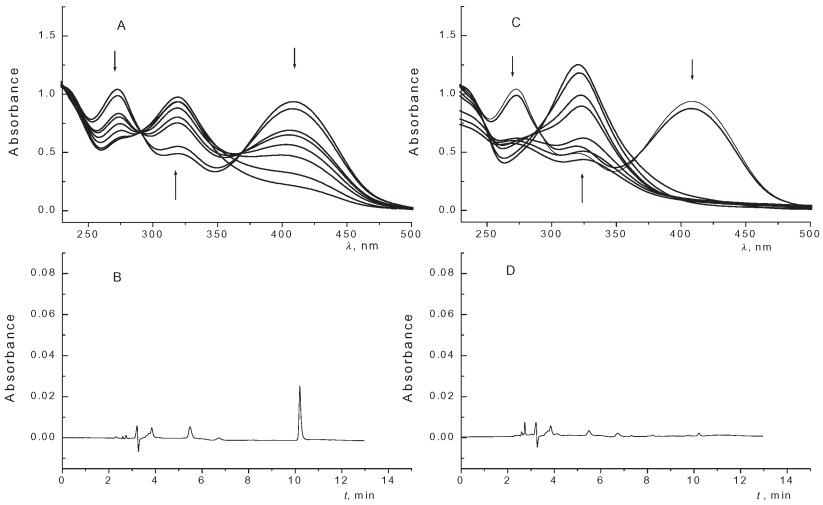


FIG. 5
Absorption spectra and HPLC chromatograms of 5×10^{-5} M quercetin at pH 10.00 in non-irradiated (A, B) and irradiated (C, D) samples. Spectra were recorded over a period of 220 min, arrows indicate the direction of the changes. Chromatograms were recorded after 120 and 40 min, respectively

According to spectrophotometric and HPLC experimental data, quercetin degradation (Eq. (*6*)) and oxidation product formation (Eq. (*7*)) as the function of irradiation time followed the relations:

$$A_{\mathrm{Q},t} = A_0 \mathrm{e}^{-kt} \tag{6}$$

$$A_{\mathrm{oxid},t} = A_\infty (1 - \mathrm{e}^{-kt}) \tag{7}$$

where $A_{\mathrm{Q},t}$ and $A_{\mathrm{oxid},t}$ are the absorbances proportional to the quercetin and its oxidation product concentrations, respectively, after an irradiation period of time t and k is the overall pseudo-first-order rate constant. A_0 and A_∞ are the absorbances proportional to the initial concentration of quercetin and the concentration of the oxidation product on the plateau of the kinetic curve, respectively.

The kinetic curves that describe the quercetin degradation and the formation and degradation of the oxidation product are shown in Figs 6 and 7. The overall apparent first-order rate constants for the oxidation product formation and quercetin transformation to its degradation products were determined from the kinetic curves, and are presented in Table II.

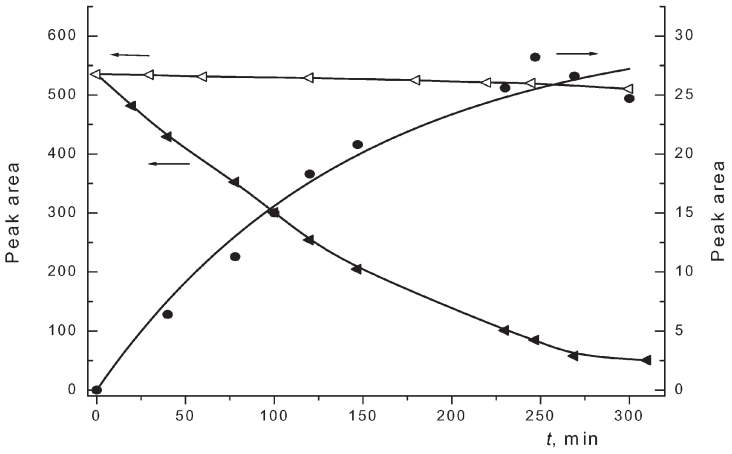


FIG. 6
Kinetics of oxidation product formation (circles) and quercetin degradation (triangles) based on HPLC data for 5×10^{-5} M non-irradiated (open symbols) and irradiated (solid symbols) samples at pH 7.50

It is clear from the obtained results that irradiation initiates the oxidation product formation at pH 5.00 and 7.50 and accelerates its rate at pH 10.00. Moreover, after 300 min irradiation the oxidation product concentration reaches a plateau at pH 7.50 since at pH 5.00 this process was slower, i.e. the plateau was reached after 800 min. At pH 10.00 the maximum of its

TABLE II
Rate constants of quercetin degradation (in min^{-1})

pH	Sample	$k \times 10^3$		
		qurcetin degradation[a]	water adduct formation[a]	water adduct degradation[a]
5.00	Non-irradiated	–	–	–
	Irradiated	4.22 ± 0.21	0.91 ± 0.17	–
7.50	Non-irradiated	–	–	–
	Irradiated	4.46 ± 0.15	5.60 ± 0.30	–
10.00	Non-irradiated	8.73 ± 0.34	25.12 ± 0.93	–
	Irradiated	110.00 ± 35.25	96.91 ± 0.12	10.13 ± 0.56

[a] (–) indicates that absorbance changes were not observed in the period from 0 to 1600 min.

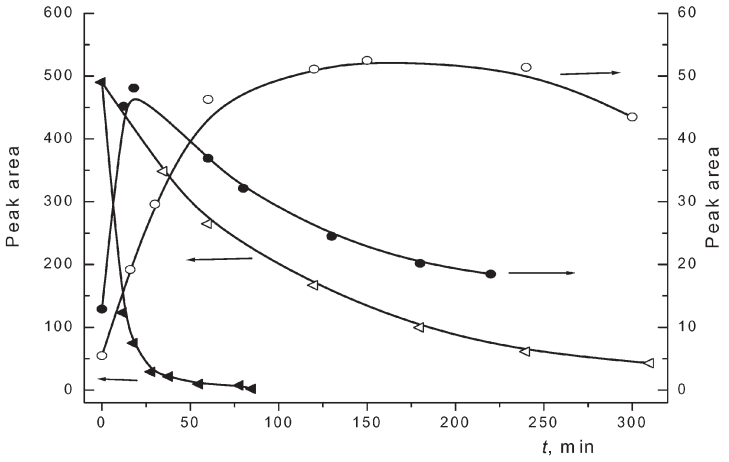


FIG. 7
Kinetics of oxidation product formation (circles) and quercetin degradation (triangles) based on HPLC data for 5×10^{-5} M non-irradiated (open symbols) and irradiated (solid symbols) samples at pH 10.00

concentration was achieved after 20 min of irradiation, which led to its complete degradation during the next 300 min. The concentration of quercetin in the non-irradiated samples at pH 5.00 and 7.50 did not change in the course of 1600 min, while after irradiation quercetin was almost completely degraded.

DISCUSSION

The pK_a values determined from our experimental data are in good agreement with other spectrophotometrically determined pK_a values reported earlier[16]. In particular, all polyphenolic compounds are in the neutral or cationic form at pH values below 5[36]. From a comparison of the pK_a values of similar polyhydroxyflavonols, it can be concluded that the preferential order for OH deprotonation is: 5-OH < 3-OH < 3′-OH < 7-OH < 4′-OH[37,38]. This assumption is rather reasonable and is based on electron delocalization in the corresponding phenolate or enolate ions and intramolecular H-binding. However, the literature surveys based on ^{13}C NMR study suggests that the order 5-OH < 3′-OH < 3-OH < 7-OH < 4′-OH can also be taken into account[39]. Altogether these data illustrate that only the C3 and C5 hydroxy groups have pK_a values that are always above the physiological pH range, indicating that only hydroxy groups at the other positions may be deprotonated at physiological pH[38,40].

Protonation of carbonyl group was observed in highly concentrated perchloric acid. According to the literature data, in the case of the mesomeric structure, a strongly polar structure with a positive charge on the oxygen from the ring and a negative charge on the oxygen of the carbonyl group presents a significant contribution, which may result in easy protonation of the carbonyl group[41,42].

The enhanced lability of quercetin in irradiated samples indicates that a photon absorbed by quercetin induces electronic excitation of the molecule that enhances the degradation process[25,43]. This is probably because of the more facile transfer of the excited electron compared with the electron transfer in the ground state. In our experiments there was no evidence of the free quinone or its charge transfer complex with quercetin in the absorption spectra, probably because of a fast addition of water to the quinone under our experimental conditions[32,44]. However, the apparent first-order kinetics of autoxidation can be accounted for by a rate-determining one-electron oxidation of the quercetin anion followed by two fast steps of radical disproportionation and water addition to the resulting quinones[20].

It is worth noting that the rate constants of quercetin degradation are very close to the rate constants of formation of oxidation products in all cases. The rate of quercetin degradation in the irradiated samples was faster compared with non-irradiated samples. At pH 10 it was about 13-fold higher compared with the rate of the non-irradiated samples at pH 10. However, the oxidation product half-life is much longer than the half-life of quercetin in the irradiated samples at all pH values. This finding is very important from the biological and biochemical points of view for the design of biochemical experiments in vitro.

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