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Research Article

Benzophenone Suppression of Quercetin Antioxidant Activity towards Lipids under UV-B Irradiation Regime: Detection by HPLC Chromatography

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Quercetin, a well-known flavonoid antioxidant, has been employed to control benzophenone-sensitized peroxidation of the lipid mixture in methanol solution, induced by continuous UV-B irradiation. Surprisingly, the detected quercetin antioxidant activity was almost negligible. The presented data suggests that the reason is not in its own UV-B-induced degradation but rather in its interrelationship with benzophenone during UV-B stress. On the other side of this relationship, benzophenone anticipated sensitizing role towards lipids; that is, the initiation of lipid peroxidation has been affected as well. These results, obtained by HPLC chromatography, partly confirm but partly relativize to some extent recent results obtained with the same system by spectrophotometric method.

1. Introduction

It has already been widely recognized that UV-B radiation, the most damaging part of total natural sunlight spectrum (280–320 nm), induces events which affect some crucial biologically important processes of global importance such as DNA replication [1, 2] or photosynthesis [3, 4]. It has also been especially recognized as one of the major agents to initiate a lot of harmful free radicals-mediated processes, such as lipid peroxidation (LP). Lipid peroxidation is a free radical chain reaction (Type I), or it occurs through a nonradical pathway (Type II), by direct reaction with singlet oxygen created in the presence of a photosensitizer [5–9].

In a form of chain reaction, lipid peroxidation consists of an initiation step (leading to formation of lipid radicals—$L^{\bullet}$), a propagation step (where lipid radicals react with oxygen to form lipid peroxy radicals—$LOO^{\bullet}$), and a termination step (formation of dienes type lipid hydroperoxides—LOOH) [8, 10]. Lipid peroxidation initiators belong to reactive oxygen species (ROS) like hydroxy radicals ($OH^{\bullet}$) or peroxy radicals ($ROO^{\bullet}$). They can be created through different pathways, including variety of external stresses [8], implying very commonly an external radiation [10–12]; in case of UV-irradiation, LP may include a special type of LP initiators photosensitizers such as benzophenone (BZP), in very different media [13–15].

Photochemical reactions of benzophenone (BZP—Figure 1), including H-abstraction by its long-lived triplet state [13–15], have been relatively long-known in organic photochemistry. The related mechanisms are very complex and depend on particular solvent [16–18] or on particular BZP interactive compound in the given solvent [19, 20]. Upon the absorption of UV-light, benzophenone may reach its long-lived triplet state (^{3}BZP), very reactive toward its surroundings (e.g., phospholipids mixture and quercetin), including a direct reaction with lipids; this interaction results in the production of lipid radicals ($L^{\bullet}$), (1), which is the initiation step of lipid peroxidation (LP) chain reaction [21] as follows:

$$^3\text{BZP} + \text{LH} \longrightarrow \text{BZPH} + \text{L}^{\bullet} \quad (1)$$

(^{3}BZP—triplet state of benzophenone; BZPH—BZP-ketyl radical; LH—unsaturated lipid; $L^{\bullet}$—lipid radical).

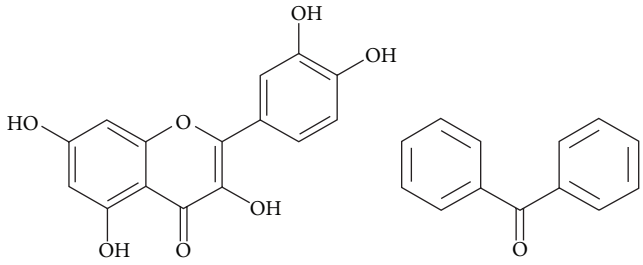

Figure 1: Structures of quercetin (left) and benzophenone (right).

On the other hand, lipid peroxidation is mostly controlled by antioxidants action *in vivo*. Many biomolecules (and classes of biomolecules) serve as antioxidants including flavonoids [22, 23] and quercetin (QC) among them (Figure 1). The updated studies connect high flavonols antioxidant activity with the presence of OH-group in C-ring 3-position, in combination with catechol B-ring structure [22, 24–27].

Quercetin and its glycosides have been reportedly synthesized in plants, as a part of its total response toward UV-radiation to prevent an extended induced damage [28, 29]. Quercetin absorbs UV radiation with absorbance maxima in the UV-A (λ_{max} = 365 nm, ε = 28,400 mol^{-1} dm^3 cm^{-1}) and UV-C range (λ_{max} = 256 nm, ε = 28,300 mol^{-1} dm^3 cm^{-1}). However, flavonoids can also act as free-radical scavengers to prevent oxidative skin damage [30–34], and their topical application has met considerable interest [35–38]; generally, they are of significant interest for medicinal chemistry [39, 40].

So, quercetin may act as a protector against UV radiation through (i) either absorption (preventive, inhibition mode) or (ii) scavenging activities ("chain-breaking", antioxidant mode). In the former case, the absorbed UV energy may be dissipated as heat [41] or converted into quercetin decomposition products, *in vitro* as *in vivo* [27, 42, 43]; in the latter case-quercetin scavenges already-created free radicals (such as lipid peroxy radicals, LOO$^\bullet$), mostly by hydrogen atom transfer mechanism, shown in (2) [44, 45] as follows:

$$\text{Fl-OH} + \text{L}^\bullet \longrightarrow \text{Fl-O}^\bullet + \text{LH} \quad (2)$$

The oxidized Fl-O$^\bullet$ radical (which can be stabilized by one intramolecular H-bonding in the B-ring: –O$^\bullet \cdots$ H–O–) [45] may react with a second radical (L$^\bullet$) acquiring a quite stable quinone structure [23].

In our former paper, we have tested QC antioxidant ability toward lipids in the presence of sensitizing BZP (as LP initiator) in methanol solution under continuous UV-B irradiation by spectrophotometric method [46]. We have got somewhat surprising conclusions on QC activity in the presence of BZP (under UV-B stress), which led us to reexamine the same system (BZP + QC + lipids, in MeOH) by HPLC chromatography, to confirm or at least relativize these conclusions. HPLC separation should provide "cleaner" picture since providing satisfying separation of the involving species eliminates partial overlapping of the absorption spectra and potentially doubtful related interpretations.

2. Materials and Methods

Phospholipids (Phospholipon 90) were gifted by PHOSPHOLIPID GmbH, Köln, Germany. According to the accompanied declaration, the mixture content is Phospholipon 90 (PL90)—phosphatidylcholine 98%, lysophosphatidylcholine 2.1%; fatty acid composition: palmitic acid 12 ± 2%, stearic acid 3 ± 1%, oleic acid 10 ± 3%, linoleic acid 66 ± 5%, linolenic acid 5 ± 2%, peroxide value max. 1.3. The phospholipids mixtures were kept in dark to prevent at least the photooxidation process. Benzophenone (BZP) was obtained from Sigma Chemical Co. (St. Louis, USA). Standard of quercetin was purchased from Merck (Darmstadt, Germany).

2.1. The Samples. The (studied system) components' final concentrations in methanol solution were phospholipids, $1.3 \cdot 10^{-3}$ mol dm^{-3}; quercetin, $1 \cdot 10^{-4}$ mol dm^{-3}; BZP, $1.1 \cdot 10^{-4}$ mol dm^{-3}. The pH was 7.6, and the experiments were done at room temperature.

2.2. UV-Irradiation. Continuous irradiations of samples in methanol were performed in cylindrical photochemical reactor "Rayonnet," with 10 symmetrically placed lamps with emission maxima at 300 nm (UV-B). The samples were irradiated in quartz closed cuvettes (1 × 1 × 4.5 cm) placed on rotating circular holder. The total measured energy flux was 15.0 W m^{-2} for 300 nm at 10 cm distance from the lamps. The methanol solutions of BZP only and phospholipids (with or without BZP), as a kind of blank, were irradiated and analyzed simultaneously with the phospholipids/quercetin and phospholipids/quercetin/BZP mixtures.

2.3. HPLC. The irradiated samples were immediately analysed with Agilent 1100 Series system (Waldbron, Germany), on Zorbax Eclipse XDB-C18 column, 4.6 × 250 mm, 5 μm, with DAD detector. The samples volume was 20 μL. The isocratic regime with 100% methanol was applied, under flow of 1.0 mL/min. The column temperature was 25°C. The chromatograms were recorded at 210 nm (unoxidized lipids), 234 nm (peroxides-[47, 48]), 250 nm (BZP), 295 nm (possible quercetin decomposition products) and 371 nm (quercetin).

Most of the experiments (UV-irrad., followed by HPLC analysis) were repeated at least once more; some of them were triplicated.

3. Results and Discussion

Structures of benzophenone and quercetin are shown in Figure 1.

The changes in the samples recorded chromatograms as a result of increased UV-B irradiation periods are shown in Figures 2(a)–4(a): Figure 2(a) shows degradation of quercetin (recorded at 371 nm), and Figure 3(a) shows degradation of BZP (recorded at 250 nm), while Figure 4(a) documents a proliferation of LP process, that is, a rise in peroxides production (recorded at 234 nm).

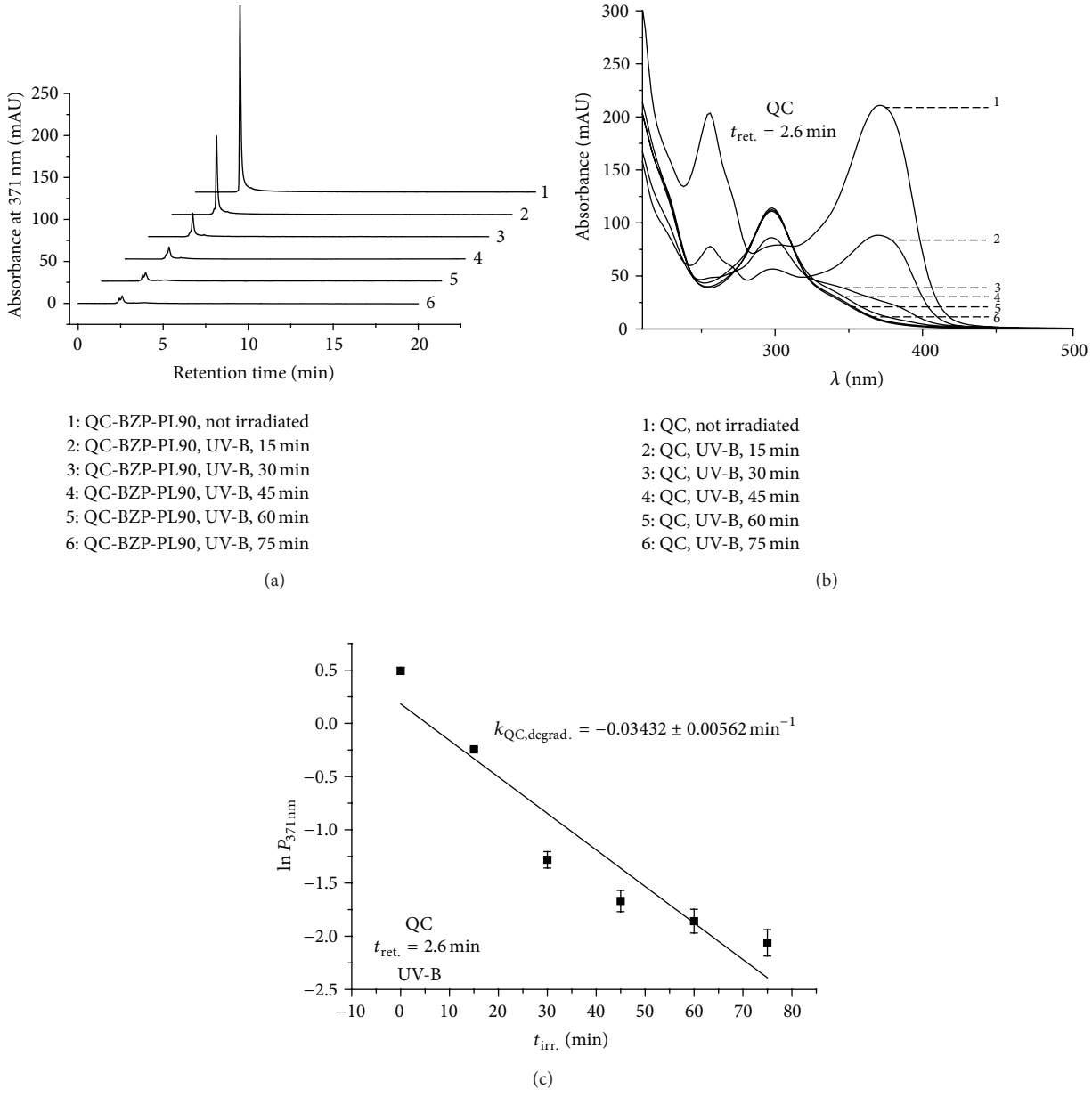

FIGURE 2: Degradation (bleaching) of quercetin from the UV-B-irradiated sample, that is, quercetin/BZP/phospholipids mixture in methanol. (a) The recorded samples chromatograms recorded at 371 nm (quercetin retention time, $t_{ret.}$, of 2.6 min). (b) Changes in quercetin absorption spectra taken from the upper (a) chromatogram (quercetin peak at $t_{ret.} = 2.6$ min), as a result of the increasing UV-B irradiation periods ($t_{irr.}$). (c) Kinetics of UV-B irradiation-induced bleaching, followed through a decrease of quercetin peak ($t_{ret.} = 2.6$ min) integrated areas as a result of the increased UV-B irradiation periods, $\ln P_{371\,nm} = f(t_{irr.})$. The corresponding bleaching rate constant and the related error bars are shown in the plot.

The corresponding changes in the absorption spectra of quercetin, BZP, and the two peroxides (taken for the corresponding peaks of the recorded HPLC chromatograms) are shown in Figures 2(b), 3(b), 4(b), and 4(c), respectively. The difference in the absorption spectra of the two detected peroxides—the absence and the presence of the "right shoulder" with A_{max} around 280 nm (Figures 4(b) and 4(c), resp.)—suggests they have somewhat different structures; still the basic peroxide diene chromophore, responsible for A_{max} at 234 nm, is obviously present in both cases.

The related kinetic plots referring to the degradation of quercetin, BZP, and production of the two peroxides are given in Figures 2(c), 3(c), 4(d), and 4(e), respectively.

The slopes calculated from kinetic ln-plots shown in Figures 2(c), 3(c), 4(d), and 4(e) representing the rates of quercetin degradation, BZP degradation, and the two peroxides production in the UV-B irradiated samples are shown in Tables 1(a) and 1(b), respectively.

In our recent publication [49], we have compared stability of two flavonoid components, quercetin and rutin, in solution

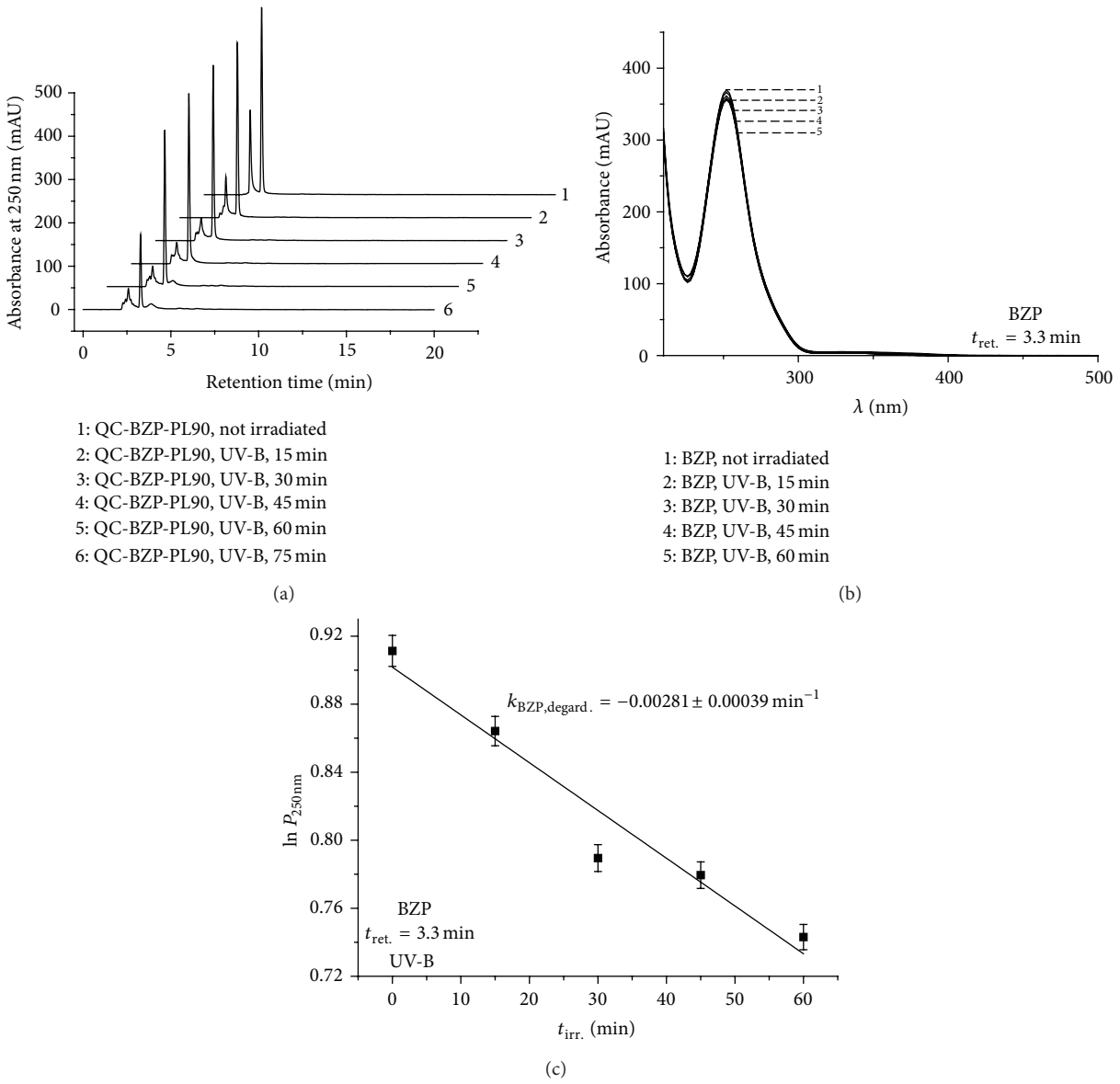

Figure 3: Degradation (bleaching) of benzophenone from the UV-B-irradiated sample, that is, quercetin/BZP/phospholipids mixture in methanol. (a) The recorded samples chromatograms recorded at 250 nm (BZP retention time, $t_{ret.}$, of 3.3 min). (b) Changes in BZP absorption spectra taken from the upper (a) chromatogram (BZP peak at $t_{ret.}$ = 3.3 min), as a result of the increasing UV-B irradiation periods ($t_{irr.}$). (c) Kinetics of UV-B irradiation-induced bleaching, followed through a decrease of BZP peak ($t_{ret.}$ = 3.3 min) integrated areas as a result of the increased UV-B irradiation periods, $\ln P_{250\,nm} = f(t_{irr.})$. The corresponding bleaching rate constant and the related error bars are shown in the plot.

toward UV-irradiation (from the three subranges UV-A, UV-B, UV-C), as well as their antioxidant activities in the presence of lipoidal mixture (i.e., lecithin) as the "protection target"; despite the lower QC stability against UV-irradiation, its antioxidant ability to protect lipid mixture from peroxidation was found to be higher compared to rutin. In addition, we have recently observed that UV continuous irradiation of quercetin and rutin in solution led to creation of products which absorb in spectral regions between 250 and 350 nm [50]. And, in the most recent report [46], we have added benzophenone in QC/phospholipids mixture. The purpose of BZP involvement in the system was to produce more radicals, for example, lipid radicals since it is not only very efficient but very selective sensitizer [5, 13, 14]. Therefore, the new system (BZP + QC + lipids, in MeOH) contributed to some extent to better understanding of both quercetin protective actions during prolonged continuous UV-irradiation: the preventive one (studied through UV-induced degradation) as well as the antioxidant one (studied by tracing expansion of UV-induced LP process, expressed through creation of LP peroxides dienes structures in the presence and in the absence of benzophenone). While quercetin suppression effect on LP

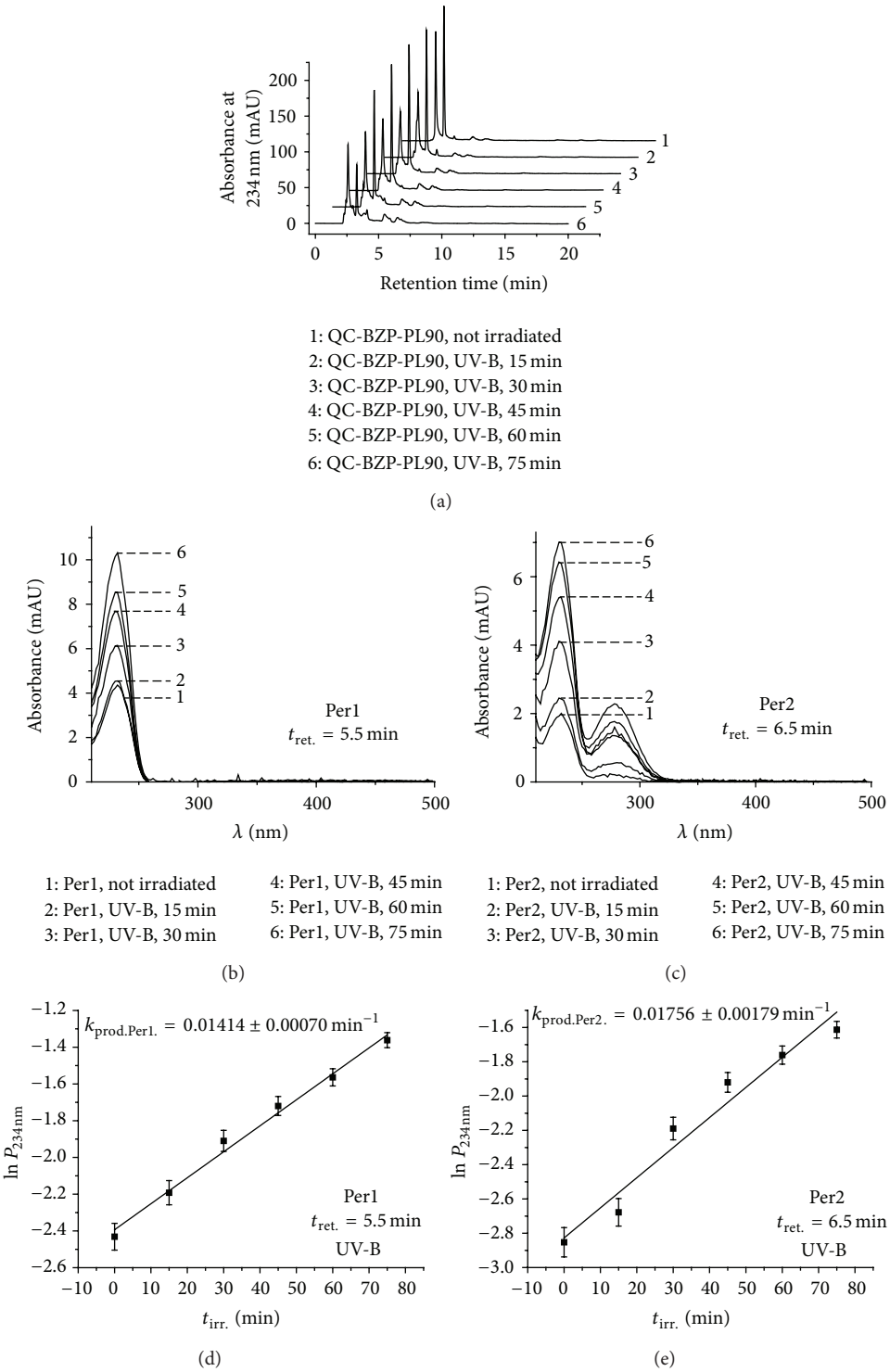

Figure 4: Formation of lipid peroxides in the UV-B-irradiated sample, that is, quercetin/BZP/phospholipids mixture in methanol. (a) The recorded samples chromatograms recorded at 234 nm (peroxides retention times, $t_{ret.}$, of 5.5 and 6.5 min). ((b) and (c)) changes in the two peroxides absorption spectra taken from the upper (a) chromatogram (Per1 $t_{ret.}$ = 5.5 min, Per2 $t_{ret.}$ = 6.5 min), as a result of the increasing UV-B irradiation periods ($t_{irr.}$). ((d) and (e)) Kinetics of rise of the two peroxides peaks (Per1 $t_{ret.}$ = 5.5 min, Per2 $t_{ret.}$ = 6.5 min) integrated areas as a result of the increased UV-B irradiation periods, $\ln P_{234\,nm} = f(t_{irr.})$. The corresponding rate constants and the related error bars are shown in the plots.

Table 1: (a) Rate constants for degradation of quercetin (QC) and benzophenone (BZP) (min^{-1}) obtained from the slopes of the 1st order linear plots, ln $P_{371/250\,nm} = f(t_{irr.})$, representing proportional decrease of QC and BZP concentrations as a result of the increasing UV-B irradiation periods. $P_{371/250\,nm}$ presents integrated areas of QC and BZP peaks, from the HPLC chromatograms (shown in Figures 2(a) and 3(a)) recorded at 371 and 250 nm, respectively. (b) Rate constants for the two peroxides formation, obtained from the slopes of the 1st order linear plot, ln $P_{234\,nm} = f(t_{irr.})$, representing proportional increase of the two peroxides concentrations as a result of the increasing UV-B irradiation periods. $P_{234\,nm}$ presents integrated areas of the peroxides peaks (Per1 $t_{ret.}$ = 5.5 min, Per2 $t_{ret.}$ = 6.5 min), from the HPLC chromatograms (shown in Figure 4(a)) recorded at 234 nm.

(a) $k_{degrad.}$, min^{-1}

	QC + PL90 + BZP	QC + PL90	BZP + PL90
QC $t_{ret.}$ = 2.6 min	−0.03432	−0.00332	/
BZP $t_{ret.}$ = 3.3 min	−0.00281	/	−0.01288

(b) Peroxides production, $k_{prod.\,Per}$, min^{-1}

	QC + PL90 + BZP	QC + PL90	BZP + PL90
Per1 $t_{ret.}$ = 5.5 min	0.01414	−0.00009	0.01734
Per2 $t_{ret.}$ = 6.5 min	0.01756	/	0.01473

process has been proven, it was found that the suppression effect is less effective when BZP was present [46]; evidently, quercetin degradation was more favored in the presence of BZP. However, the deeper conclusions could not be offered partly because of the obvious limitations of the applied spectrophotometric method based on a clear overlapping of the involved species (BZP, quercetin, peroxides) absorption spectra (λ_{max} = 250 nm for BZP, 234 nm for peroxides, 260 and 370 nm for quercetin). This report is one step ahead since the method has been changed; now, the same system has been analysed by HPLC chromatography providing a kinetic analysis of the clearly separated involved species. As in the cited two reports [46, 49], due to the complexity of the studied system, the mixture of BZP and lipids has been irradiated as a blank in order to evaluate, in the next step, LP control by quercetin during UV-B irradiation (290–320 nm).

The chromatograms recorded at the involved species absorption maxima (Figures 2(a), 3(a), and 4(a)) deal with the separated compounds; the kinetic plots (Figures 2(c), 3(c), 4(d), and 4(e)) have been obtained from the corresponding peaks integrated areas, and the calculated rate constants for quercetin and BZP degradation as well as for the two peroxides (Per1 & Per2) formation represent much "pure behavior," compared to at least partly "mixed behavior" reported in the former paper [46].

Prolonged continuous irradiation of quercetin in methanol (in the presence of lipid mixture and BZP) causes a gradual decrease of the corresponding integrated peak ($t_{ret.}$ = 2.6 min) areas during increasing irradiation periods (Figure 2(a)); the corresponding kinetic plot (Figure 2(c)) and the changes in the corresponding absorption spectra shown in Figure 2(b) confirm this degradation pattern. This degradation pattern (of QC in MeOH) has been already noted in our two previous reports, where QC decomposition into ring C-opening products has been recorded by HPLC techniques [50] or by spectrophotometric method (in the last case in the presence of BZP, [46]), confirming the previous work (with BZP and QC in MeOH) and the proposed mechanism of Fahlman and Krol [51] (it should be added at this place that the oxidation pattern of quercetin is highly dependent on the applied initiating mechanism, but the related details are out of scope of this paper [52, 53]). However the real issue in QC degradation kinetics is the role of BZP. If one compares the QC degradation rate constants from Table 1(a) ($k_{QC,degrad.}$) in the presence and in the absence of BZP (the last one obtained under absolutely the same chromatographic conditions, not shown), 0.03432 versus 0.00332 min^{-1}, it is evident that BZP presence dramatically speeds up QC degradation. The same fact was seen in the previous report [46], when the same (BZP + QC + lipids) mixture was irradiated under the same UV irradiation regime, though the (degradation constants, in the presence and in the absence of BZP) relationship is not that huge as in this study (roughly, 3 versus 10). Still, having in mind objective limits of spectrophotometric method the HPLC data from Table 1(a) looks not only confirming but also more accurate. It is also important to note that the reported quercetin degradation rate constant in the presence of lipids but in the absence of BZP (0.00332 min^{-1}) is in a logical agreement with the one obtained for QC degradation under the same (UV-B) conditions and in the same solvent, methanol, in the absence of all other species (0.00183 min^{-1}) [50]; the latter one is obviously smaller, confirming quercetin antioxidant activity toward lipids before introduction of BZP.

However, it appears that not only BZP largely affects quercetin degradation (and so its antioxidant ability) but also its own main anticipated activity towards lipids [5, 13, 14]; BZP-sensitizing (H-abstraction) activity becomes almost 5 times slower in the presence of quercetin, rate constants for BZP degradation ($k_{BZP,degrad.}$) of 0.01288 and 0.00281 min^{-1},

respectively (Table 1a, calculated from the kinetic plot shown in Figure 3(c)).

That clearly suggests that the cause has to be somehow related to BZP (i.e., ^{3}BZP)-QC mutual interaction; no other interaction can match an explosive ^{3}BZP attack to double bonds in the used lipoidal mixture and the abstraction of allylic and double-allylic H-atoms [5, 13, 14], especially having in mind high percentage of present linoleic acid (more than 50%), with its two double bonds in each of the two hydrophobic branches.

As reported-quercetin has two absorption maximums, in UV-A (λ_{max} = 372 nm) and UV-C (λ_{max} = 260 nm) spectral ranges [49] which partly overlap with the applied UV-B irradiation range used in this work. In recently published paper, Fahlman et al. [51] studied UV-A and UV-B irradiation of quercetin in BZP-containing methanol solution, yielding an irreversible degradation, followed by formation of several C-ring-opened photoproducts; they noticed BZP impact on both, QC degradation as well as on photoproducts formation [51]; this confirms that BZP-QC connection is found in this work and in the previous report [46].

The other side of the anticipated BZP-QC interactions is a question of QC antioxidant activity against lipids: how much has it been affected by this interaction? To be able to evaluate the possible change, a blank experiment has been done with QC and the lipids (without BZP); from the corresponding slope (Figure Add-1 in Supplementary Materials available online at http://dx.doi.org/10.1155/2013/761675) for the peroxides production, a rate constant has been calculated ($k_{prod.\,Per}$ = −0.00009 min^{-1}, Table 1). The linear decrease in the peroxides production represents an obvious QC-governing effect. However, when BZP is brought in the system, the situation dramatically changes. First of all, the sign of peroxides production has been changed, that is, reversed, from minus (−) in BZP absence to plus (+) in its presence (Figures 4(d) and 4(e)). In addition, the peroxides production rate constants, for the two detected and separated peroxides species, $k_{prod.\,Per\,1}$ and $k_{prod.\,Per\,2}$, are almost 200 times bigger. And, the most important, it is hard to see almost any QC antioxidant activity on BZP-induced lipid peroxidation (more expressed than in the former, [46], "spectrophotometric" report): the two peroxides production rate constants for the two peroxides peaks, Per1 $t_{ret.}$ = 5.5 min and Per2 $t_{ret.}$ = 6.5 min, obtained from kinetic plots shown in Figures 4(d) and 4(e) in the presence and in the absence of quercetin are almost the same (0.01414 versus 0.01734 min^{-1} and 0.01756 versus 0.01473 min^{-1}, Table 1).

4. Conclusion

To conclude, it appears that while a QC general control effect on LP process is not ultimately denied, the suppression is definitely not only less effective but practically absent when BZP is present. HPLC results obtained in this paper not only support spectrophotometric data obtained for the same system in the last report [46] (concerning this fact) but also strengthen it. The very newly found fact, that is, the novelty of this study (compared to [46]), is that suppression has the other side, a slower BZP sensitizing activity (against lipids as the target). This emphasizes a necessity of further efforts to determine more precisely BZP-QC relationship in the presence of lipids, under conditions of external continuous UV-B irradiation stress.

Acknowledgments

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