Photostability of Quercetin under Exposure to UV irradiation

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SUMMARY. Recently, it was demonstrated that topical formulations containing quercetin were able to inhibit the UVB-induced cutaneous oxidative stress and inflammation. Nevertheless, the photostability of this flavonoid under exposure to UV irradiation have not been investigated and it is well-known that the photochemical stability of this substance is essential for its activity. Therefore, in the present work was investigated the photostability of a propylene glycol solution of quercetin under forced exposure to UVB irradiation. The content of quercetin in each solution was determined quantitatively by HPLC, its ultraviolet spectra (UV-vis) and its antioxidant activity by DPPH®. The HPLC and DPPH® methods were validated and the results demonstrated that these methodologies are adequate and reliable to quantify quercetin and to determine its antioxidant activity, respectively. The photostability study showed that quercetin is resistant of degradation caused by UVB radiation exposure.

RESUMEN. “Fotostabilidad de Quercetina bajo Exposición a Irradiación UV”. Recientemente fue demostrado que formulaciones tópicas que contienen quercetina inhibieron la tensión oxidante y la inflamación cutánea inducida por la radiación UVB. No obstante, no fue aún investigada la fotostabilidad de este flavonóide bajo la exposición a la radiación de UV y es conocido que la estabilidad fotoquímica de esta sustancia es esencial para su actividad. Por consiguiente, en este trabajo se investigó la fotostabilidad de una solución de quercetina en propilenglicol bajo exposición a radiación UVB. Fue analizado el contenido de quercetina en cada solución por CLAR y su absorción al ultravioleta y su actividad antioxidante por DPPH®. Se validaron los métodos CLAR y de DPPH® y los resultados demostraron que estas metodologías son adecuadas y confiables para cuantificación y determinación de la actividad antioxidante de la quercetina, respectivamente. El estudio de fotostabilidad mostró que la quercetina es resistente a la degradación causada por la exposición a la radiación UVB.

INTRODUCTION

Ultraviolet (UV) irradiation is one of the most ubiquitous environmental hazards that impact every living creature under the sun. Skin is the largest human organ, and is the only organ directly exposed to UV irradiation. The exposure to UV irradiation induces profound biological changes in human skin and is believed to be the major cause of skin cancer and premature skin aging 1,2.

The UV irradiation can affect enzymatic and nonenzymatic antioxidants that protect the cells against deleterious effects of reactive oxygen species (ROS). When ROS overcome antioxidant defence, the damage occurs in the cells. Therefore, the use of antioxidants can protect the cells from UV-mediated tumor induction and subsequent development of precancerous lesions into tumors 3.

Quercetin is the most abundant and commonly investigated member of plant polyphenolic compounds 4. Moreover, quercetin exhibits the highest antiradical property toward hydroxyl radical, peroxyl, and superoxide anion compared to others flavonoids 5. These properties of quercetin are due to the presence of 3 active chemical functional groups in its structure: (i) the 3',4'-dihydroxy cathecol configuration on the B ring, which gives most stable phenoxy radicals after the donation of hydrogen atom; (ii) the 2,3-double bond in conjunction with the 4-carbonyl group on the C ring, which allows a delocalization of an electron from phenoxy radicals on the B ring to the C ring; and (iii) the 3-hydroxy group in combination with the 2,3-double bond, which increases the resonance stabilization for electron delocalization across the molecule 6,9.

KEY WORDS: DPPH®, HPLC, Photostability, Quercetin, UV-vis spectrum, Validation.

PALABRAS CLAVE: DPPH®, CLAR, Espectro UV-vis, Foto estabilidad, Quercetina, Validación.

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Topical administration of antioxidants provides an efficient way to enrich the endogenous cutaneous protection system and thus may be a successful strategy for diminishing ultraviolet radiation-mediated oxidative damage in the skin. It was recently demonstrated that formulations containing quercetin inhibit the UVB-induced cutaneous oxidative stress and inflammation, nevertheless, the photostability of this flavonoid have not been investigated and it is well-known that the photochemical stability of this substance is essential for its activity because a light-induced degradation can result in a decrease efficacy and sometimes also involve significant adverse side effects after drug administration.

Thus, in the present work was investigated the photostability of quercetin with regard to its concentration, its absorbance spectrum (UV-vis) and antioxidant activity, under forced exposure to UVB radiation. A selective and reliable liquid chromatographic method (HPLC) was developed and validated for the quantitative evaluation of the drug photodegradation process. The use of a stable free radical (DPPH) in solution, to determine antioxidant activity of quercetin was also validated.

**MATERIAL AND METHODS**

**Chemicals**

Quercetin dihydrate 99% (C15H10O7·2H2O, Mw= 338.26) was purchased from Acros Organics (New Jersey, USA). Methanol (MeOH) and glacial acid acetic, both high-performance liquid chromatography (HPLC) grades were provided from J.T.Baker (USA) and Merck (Darmstadt, Germany), respectively. The water used to prepare the solutions or mobile phase was purified in a Milli-Q-plus System (Millipore, Bedforte, MA, USA). DPPH (2,2-diphenyl-1-picryl-hydrazyl) were purchased from Sigma (Germany).

**UV irradiation**

Quercetin solution was prepared in propylene glycol in the concentration of 5 mg/mL and exposed for 7 days consecutively under UVB irradiation.

The UVB source of irradiation consisted of a Philips TL40W/12 RS lamp (Medical Holand) emitting a continuous wave between 270 and 400 nm with a peak emission at 313 nm. The UVB output was measured using a model IL-1700 Research Radiometer (International Light, USA; calibrated by IL service staff) with a radiometer sensor for UV (SED005) and UVB (SED240), which detected that UVB was 73% of the total UV irradiation in the present experimental conditions. The UVB irradiation rate was 0.26 mW/cm².

A control solution, which was prepared in the same conditions, was not exposed to the irradiation. The possible photodegradation of quercetin was evaluated using three different methodologies. The content of quercetin in each solution was determined quantitatively by HPLC, its ultraviolet spectra (UV-vis) and its antioxidant activity by DPPH* free radical assay.

**HPLC method: apparatus and chromatographic conditions**

The HPLC analyses were performed using a Shimadzu (Kyoto, Japan) liquid chromatograph, equipped with an LC-10 AT VP solvent pump unit and an SPD-10A VP UV-Visible detector. Injections were performed manually through a 20 µL loop with a Rheodyne injector. Data were collected using a Chromatopac CRI8A integrator (Shimadzu, Kyoto, Japan). The separation was performed by a C18 Hypersyl BDS-CPS ciano (5 μm), 250 x 4.6 mm column. A mobile phase of 60% methanol and 40% acetic acid 2% (flow rate of 1 mL/min) was used, and quercetin was detected at 254 nm.

For the photostability analyses the non-irradiated and irradiated quercetin solutions (5 mg/mL in propylene glycol) were diluted to the final concentration of 50 μg/mL in a methanol: acetic acid 2% (60:40) solution.

**Hydrogen-donating ability by DPPH* assay**

The DPPH* assay has been widely used to evaluate the ability of several free radical scavenger molecules. The reactivity of phenolic derivatives with diphenylpicrylhydrazyl was determined from the change in absorbance at 517 nm, according to the method of Blois. For radical scavenging measurements, 1 mL of 100 mM acetate buffer, pH 5.5, 1 mL of ethanol, and 0.5 mL of 250 μM ethanolic solution of DPPH* were mixed, and 50 μL of each quercetin solution were added, then the change in absorbance was measured after 10 min using a CECIL CE 1021 Spectrophotometer (Cambridge, England). For the photostability analyses the non-irradiated and irradiated quercetin solution (5 mg/mL in propylene glycol) were diluted to the final concentration of 50 μg/mL in a methanolic solution. The positive control was prepared in the absence of quercetin solution, and it indicates the maximum odd electrons of DPPH*, which was considered 100% of free radicals in the solution to calculate the hydrogen-donating ability.
(%) of quercetin. The blank was prepared from the reaction mixture without DPPH* solution. All measurements were performed in triplicate.

**VALIDATION OF THE METHODS**

**Linearity**

To validate these methodologies linearity was checked in the concentration range of 0.1 - 300 µg/mL to HPLC, in which no internal standard was used, and 0.1 - 4.0 µg/mL to DPPH*. The linearity was evaluated by linear regression analysis.

**Precision and Accuracy**

The precision of the methods was determined by repeatability (intra-day) and intermediate precision (inter-day). Repeatability was evaluated by assaying samples with the same concentration and during the same day. The intermediate precision was studied by comparing the assays on different days (3 days). Accuracy was determined in the same conditions as precision.

Precision was expressed as relative standard deviation (R.S.D.) and accuracy as the agreement between the measured and the true values. To check the precision and accuracy of the HPLC method, intra (n=7) and inter-day (n=15) respectively, concentrations levels of 5; 50 and 75 µg/mL were used. To check these parameters in DPPH* method, intra (n=10) and inter-day (n=15) respectively, concentrations levels of 0.5; 1 and 1.5 µg/mL were used.

**Detection and quantification limits (LOD and LOQ)**

The parameters LOD and LOQ were determined on the basis of response and slope of the regression equation for both methods.

**Ultraviolet spectrum**

The irradiated and non-irradiated quercetin solutions were diluted in a methanol solution in the final concentration of 20 µg/mL and its UV-vis spectrum were carried out on a Hitachi spectrophotometer (model U-2001). The spectra represent the average of 100 scans/min resolution in the spectral range 500-200 nm.

**RESULTS AND DISCUSSION**

**Validation of the HPLC method**

The HPLC method has been widely used to separate and quantify flavonoids presents in plants and foods.10.15. The chromatographic method developed for the quantitative determination of quercetin was validated according to ICH and ANVISA guidelines,10 to obtain reproducible analyses with a high degree of accuracy and precision in the range of concentrations investigated. The parameters considered were linearity, precision, accuracy and detection and quantification limits.12,17.

Under the experimental conditions, the retention time of quercetin was approximately 5 minutes. The height of the peak was used to calculate the concentration range of quercetin. Linearity was achieved for concentrations between 0.1 and 200 µg/mL, with correlation coefficient (r) of 0.999; slope = 3716.8; intercept = -2475.6. The accuracy and precision of the assay were established across the specified range of the analytical procedure from intra and inter-day replicate analyses. Table 1 shows the results achieved with three concentrations in the evaluation of the precision and accuracy of the method. For the solutions in the range of 50 and 75 µg/mL neither RSDs nor relative errors exceeded a value of 2.52%, however, for the lowest concentration evaluated (5 µg/mL) theses values were higher, being 10.39% for the inter-day error. These results can be explained by the fact that RSD and error values tend to be higher as much as lower the solution concentration is. The average of RSD and error values from all the concentrations evaluated was no more than 4.68%, which indicates that the values obtained are in agreement with accepted validation produced and that the chromatographic methodology used is reliable to quantify the flavonoid quercetin in the range evaluated.12. Detection and quantification limits were 0.011 and 0.03 µg/mL respectively and the variation coefficient of the last one was 8.33% (n=5). Using this HPLC procedure, unidentified peaks were not detected.

<table>
<thead>
<tr>
<th>Quercetin (µg/mL)</th>
<th>Intra-day</th>
<th>Inter-day</th>
<th>Intra-day</th>
<th>Inter-day</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>R.S.D. (%)</td>
<td>R.S.D. (%)</td>
<td>Error (%)</td>
<td>Error (%)</td>
</tr>
<tr>
<td>5</td>
<td>0.07</td>
<td>4.93</td>
<td>6.74</td>
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<tr>
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<td>2.79</td>
<td>4.68</td>
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</table>

Table 1. Intra and inter-day precision and accuracy of the proposed HPLC method. R.S.D (%): relative standard deviation.
Validation of the DPPH* method

DPPH* is a stable free radical, which can accept an electron or hydrogen radical converting into a stable, diamagnetic molecule. Because of this odd electron, DPPH* has a strong absorption band at 517nm. As this electron becomes paired off, the absorption decreases stoichiometrically with respect to the number of electrons taken up. Such a change in absorbance produced in this reaction has been widely used to test the ability of several molecules to act as free radical scavengers 13.

In the DPPH* method under the experimental conditions, a linear relationship was found between 0.1-2 µg/mL (r=0.996; slope = 39.47; intercept = 8.753), having the highest activity (approximately 90%) in the concentrations of 5 and 10 µg/mL (Figures 1A and 1B). The concentration of antioxidant needed to decrease the initial DPPH* concentration by 50% (IC₅₀) is a parameter widely used to measure the antioxidant activity. The lower the IC₅₀, the higher the antioxidant power 18. The IC₅₀ achieved was 0.834 µg/mL which is very similar from what Parejo et al. 18 found and which demonstrates the high antioxidant activity of quercetin. Tsimo-
giannis and Oreopoulou 19 showed that the loss of structural characteristics such as 2, 3-double bond and 3-OH group decreased the antioxidant power of flavonoids, what indicates that these structural characteristics are essential for the high antioxidant activity found in quercetin.

Table 2 shows the results achieved with three concentrations in the evaluation of the precision and accuracy of the method; neither RSDs nor errors of the concentrations analyzed exceeded a value of 9.4%, in agreement with literature recommendations 12. Detection and quantification limits were 0.32 and 0.6 µg/mL respectively.

The results explained above show that both methodologies HPLC to quantify the quercetin and DPPH* to determine its antioxidant activity are adequate and reliable for the analysis of this drug after exposure to UV irradiation.

Photostability tests

Chronic exposition of unprotected human skin to UVB radiation is known to induce an array of adverse reactions, including premature skin aging, erythema, inflammation and photocarcinogenesis, and this through the formation of reactive oxygen species (ROS) 20.

Fortunately, the skin posses a wide range of interlinked antioxidant defence mechanisms to protect itself from damage by UV-induced ROS. However, the capacity of these systems is not unlimited, and they can be overwhelmed by the

![Figure 1. H-donor ability of quercetin in methanol solution using stable radical DPPH® in 250 µM ethanolic solution. (A) Concentrations of quercetin utilized were 0.1; 0.5; 1; 1.5; 2; 5 and 10 µg/mL in medium reaction. (B) Linear relation between the concentration of quercetin and H-donor ability to DPPH® radical. Results are represented by means ± SEM.](image)

<table>
<thead>
<tr>
<th>Quercetin (µg/mL)</th>
<th>Intra-day R.S.D. (%)</th>
<th>Inter-day R.S.D. (%)</th>
<th>Intra-day Error (%)</th>
<th>Inter-day Error (%)</th>
</tr>
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<td>0.5</td>
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<td>0.24</td>
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<tr>
<td>Average</td>
<td>1.14</td>
<td>6.43</td>
<td>5.16</td>
<td>5.34</td>
</tr>
</tbody>
</table>

Table 2. Intra and inter-day precision and accuracy of the DPPH® method. R.S.D (%): relative standard deviation.
fact that nearly all of the defence systems have been shown to be decreased after UV exposure. This means that after chronic exposure to UV, ROS can reach damage levels 21.

A strategy to provide photoprotection would be the use of substances that have antioxidant activity such as the flavonoid quercetin. Recently study demonstrated the possible applicability of quercetin in topical formulations to prevent UVB-induced oxidative/inflammatory skin damages 11. Nevertheless, to prevent or decrease the oxidative damage in the skin induced by ultraviolet radiation is essential the photochemical stability of this flavonoid. For this reason, the photostability of quercetin, under forced exposure to UVB irradiation was investigated.

The high-performance liquid chromatography (HPLC) seems to be the most suitable analytical technique to achieve both qualitative and quantitative information on the photochemical reaction 12. Figures 2A and 2B show the chromatograms of non-irradiated (A) and irradiated (B) quercetin’s solutions. Both of chromatograms present the same elution profile, being the retention time and the height of the peak very similar. These results demonstrate that there was no loss in the quantity of quercetin neither the presence of other compounds, which were produced as a result of photochemical reaction.

Ultraviolet-visible absorption spectroscopy is perhaps the single most useful technique available for flavonoid structure analysis. The flavonoid spectrum is usually determined for a methanol or, less satisfactorily, ethanol solution of the flavonoid and the spectrum typically consists of two absorption maxima in the ranges 240-285 nm (band II) and 300-550 nm (band I). The precise position and relative intensities of these maxima give valuable information on the nature of the flavonoid and its oxygenation pattern. In flavonoids, such as quercetin, the presence of a 3', 4'-diOH system is generally evidenced by a second peak (sometimes a shoulder) in a band II (250-280 nm) and their spectrum also consist of another peak in the range 350-385 nm (band I) 22.

The ultraviolet spectra (UV-vis) of the non-irradiated (A) and irradiated (B) quercetin in methanolic solutions are displayed in Figure 3. Both spectra showed the same absorption peaks, which demonstrate that no change in absorption spectrum of quercetin was detected after the forced exposure to UVB irradiation, corroborating with the previous analysis in HPLC. Furthermore, the quercetin spectra obtained in the present work showed a very similar profile with the spectrum obtained by Cornard et al. 25 and Mendoza-Wilson & Glossman-Mitnik 26 and what was described by Markham 22 as a typical flavonoid spectrum.

Results obtained by Pinelo et al. 25 clearly showed that quercetin oxidative degradation promotes peculiar changes in the antioxidant capacity. For this reason, quercetin photostability analyses under UV irradiation exposure have to investigate its antioxidant activity. This determination was carried by the H-donor ability of this compound using the stable radical DPPH•.

Figure 4 demonstrates that a very similar profile of dose-response curve was obtained for non-irradiated and irradiated solutions, being both of them concentration-dependent. The concentration of antioxidant needed to decrease

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**Figure 2.** Chromatograms of quercetin in a methanolic solution (50 µg/mL). (A) Non-irradiated and (B) irradiated solutions. Chromatographic conditions: C18 Hypersyl BDS-CPS ciano (5 µm), 250 x 4.6 mm column - attached to a pre column, mobile phase: methanol and acetic acid 2% (60:40), 1 mL/min. Detection at 254 nm.

**Figure 3.** Ultraviolet spectrum (UV-vis) of quercetin in methanol solution (20 µg/mL). (A) Non-irradiated and (B) irradiated solutions.
the initial DPPH\(^*\) concentration by 50% (IC\(_{50}\)) was 0.969 \(\mu\)g/mL for non-irradiated solution and 0.985 \(\mu\)g/mL for irradiated solution and in 2 \(\mu\)g/mL the highest H-donor capability was achieved (approximately 80% of inhibition) since in higher concentrations a plateau effect was observed.

The results explained above demonstrate that the forced exposure under UV irradiation did not cause a loss in antioxidant activity of quercetin in propylene glycol solution.

Even so Smith et al. \(^{26}\) achieved that quercetin was the most susceptible flavonoid to photobleaching after 15 hours of exposure to UV irradiation, the experiments elucidated above demonstrated that any degradation, structural alteration or loss of activity of this flavonoid occurred after more rigorous UV irradiation exposure.

In the study in which it was observed the beneficial effectiveness of topical formulations containing quercetin against UVB-induced oxidative stress in hairless mice \(^{11}\), the maximum UVB dose used were approximately 40 times lower than the one applied in the present study. This demonstrate the viability of using this flavonoid, in further studies, to decrease the harmful effects caused by UV irradiation in skin, since the results obtained showed that quercetin is resistant of degradation caused by UVB irradiation exposure.

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**Figure 4.** H-donor ability quercetin non-irradiated and irradiated using stable radical DPPH\(^*\) in 250 \(\mu\)M ethanolic solution. Concentrations of quercetin utilized were 0.1, 0.5, 0.75, 1.0, 1.5, 2.0, 5.0 and 10 \(\mu\)g/mL in medium reaction. Results are represented by means ± SEM.

**REFERENCES**