Determination of quercetin in dietary supplements by isocratic RP-HPLC method

Dragica Doneva1*, Biljana Bauer2

1Institute of Public Health of the Republic of North Macedonia, 50 Division 6, 1000 Skopje, Republic of North Macedonia
2Institute of Pharmacognosy, Faculty of Pharmacy, Ss. Cyril and Methodius University, Mother Theresa 47, 1000 Skopje, Republic of North Macedonia

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Abstract

Quercetin is a natural flavonoid found abundantly in vegetables and fruits. It shows antioxidant, anti-inflammatory, antihypertoxic, antiallergic, antidiabetic and antiviral activity. Increasingly popular dietary supplements containing quercetin require critical examination of their quality. The aim of this study was to develop a simple and accurate HPLC method for quercetin determination in dietary supplements. Chromatographic separation was achieved by isocratic method, using a Purospher STAR® RP-18 reverse-phase column (150 x 4.6 mm i.d., particle size 5 μm), a mobile phase composed of acetonitrile and water (acidified to pH 3.0), in a ratio of 30:70 (V/V), run at a flow rate of 1.1 mL/min. The column temperature was kept at 30 °C. The DAD detector was set at 257 nm and 375 nm. The injection volume was 20 μL. Isopropanol was used as a solvent. The method was validated by determining system suitability, specificity, linearity, range, limit of detection and quantification, accuracy, precision, and robustness. It is characterized by simple preparation, good precision (RSD < 2%) and good analytical yield (100.09% and 100.29% at 257 nm and 375 nm, respectively). The limit of detection and quantification were 0.00071 μg/mL and 0.00215 μg/mL at 257 nm, and 0.00078 μg/mL and 0.00236 μg/mL at 375 nm, respectively. The system suitability test showed that method performance is similar at both wavelengths. This method can be recommended for routine analysis of dietary supplements containing quercetin in food quality control laboratories.

Key words: quercetin, HPLC, dietary supplements, flavonoid

Introduction

Quercetin is a natural flavonoid which belongs to the category called flavonols. It is ubiquitously present in various vegetables and fruits, like berries, lovage, capers, cilantro, dill, apples, and onions (Anand et al., 2016). It is one of the most common flavonoids found in herbal products and its biological activity is important in understanding the health benefits of these compounds (Ay et al., 2021; Lakanpal and Rai, 2007).

*donevadaci@gmail.com

The IUPAC chemical name of quercetin is 2-(3,4-dihydroxyphenyl)-3,5,7-trihydroxycromen-4-one, with a chemical formula C15H10O7 (Fig. 1). It is yellow in color and completely soluble in lipids and alcohol, insoluble in cold water, while sparingly soluble in hot water (Batiha et al., 2020).

Quercetin has shown many pharmacological activities, such as being anticancer, antiviral, and treating allergic, metabolic, and inflammatory disorders, eye and cardiovascular diseases, and arthritis (Dabeek and Marra, 2019; Harborn and Williams, 2000; Manach et al., 2005), and as an adjunct therapy for diseases related to thrombosis (Kovačević, 2000).
Quercetin is one of the most potent antioxidants among polyphenols (Prior, 2003; Russo et al., 2000), an anticancer and neuroprotective (Chen et al., 2017; Khan et al., 2019) and is characterized by very high safety, both in animals and in humans (Dajas, 2012). Through its flavonoid ingredients and together with ascorbic acid, introduced into both the human and animal organism, enters a series of oxidative processes (Kulevanova, 2004). The beneficial effect of quercetin is believed to be due to its ability to act as a free radical scavenger and its potency to inhibit the proliferation of transformed epithelial cells. It has effect on increasing the apoptosis of mutated cells, inhibiting cancer cell carcinoma, inhibiting DNA synthesis, reducing, and modifying cell signal transduction pathways (Erkoc et al., 2003) and is able to prevent cancer caused by oxidative stress (Bagel et al., 2012).

Quercetin is effective against various viruses. It is potent against bursting in MT2 cells caused by human T-lymphotropic virus 1 (Coelho-Dos-Reis et al., 2011). Quercetin-3-O-β-D-glucuronide is effective against influenza A virus (Fan et al., 2011) and quercetin 7-rhamnose is effective against swine diarrhea epidemic virus (Song, 2011). It acts as a bacteriostatic agent by inhibiting bacterial growth (Wu et al., 2008) with excellent results in Escherichia coli, Salmonella enteric and Listeria monocytogenes (Božič et al., 2012) and helps reduce microbial growth (Andres et al., 2013).

Administration of the increasingly popular dietary supplements containing quercetin requires critical examination of quality, safety, and efficacy of these products. Although many high-quality dietary supplements exist on the market, quality concerns are in general justified. There are several studies that report that active ingredients dramatically differ from label claims and among products (Abdel-Tawab, 2018). Commonly identified problems include failure to verify that a finished batch meets product specifications for identity, purity, strength, and composition (Kapoor and Sharfstein, 2016). Quality control is one of the very important and essential steps in the manufacturing of dietary supplements as quality of products affect their safety and efficacy (Kagawad et al., 2021). Hence, the determination of the active ingredients in the finished products is required to check the compliance with the product specifications and to prove they contain the ingredients listed on the label in the declared potency and amount.

Quercetin in plants, biological materials, and pharmaceutical preparations is determined by spectrophotometric, chromatographic, and electromigration methods (Kurzawa, 2010). HPLC technique is used for determination of quercetin in different matrices, e.g., in the red wines (Fang et al., 2007; Sakkiadi et al., 2001; Stecher et al., 2001); phyto-pharmaceutical preparations (Mesbah et al., 2005); and in human urine after oral administration of a tablet of Ginkgo biloba extract (Wang et al., 2003). To date, there is no official monograph on quercetin in the European Pharmacopoeia (Ph. Eur., 10.0, 2019).

Therefore, the aim of this study was to develop simple, accurate, and reproducible high-performance liquid chromatography (HPLC) method for routine determination of quercetin in commercially available dietary supplements.

**Materials and methods**

**Materials and reagents**

We used commercially available capsules with a standardized content of 250 mg quercetin and 250 mg ascorbic acid, manufactured by PharmaVital GmbH, Germany, as samples. We chose this product because of its availability on the local market. In addition, the presence of vitamin C, beside quercetin, will allow us to confirm that quercetin can be accurately determined in the presence of vitamin C. Quercetin standard substance (a purity of the standard substance was 0.98) was purchased from Sigma-Aldrich (St. Louis, MO, USA). Ascorbic acid standard substance was purchased from Sigma-Aldrich (St. Louis, MO, USA). HPLC-grade acetonitrile and isopropanol were purchased from Merck (Darmstadt, Germany). Double-distilled water was used for preparation of mobile phase solutions. Phosphoric acid was supplied from Sigma-Aldrich (St. Louis, MO, USA). The volumetric glassware used in the study were class A. Prior to injection into the HPLC system, all solutions were filtered through a membrane nylon filter (0.45 μm pore size).

**Instrumentation**

In this study HPLC analyses were performed using an Agilent Technologies chromatographic system (Hewlett Packard, Avondale, USA) consisting of a Model 1200 Series SL pump with a Model Agilent series G-1315C DAD detector and a Model Agilent 1200 series G-1329B ALS auto sampler. Data analyses were done using Agilent Technologies HPLC 1100 software. The UV spectra of quercetin dissolved in isopropanol were obtained in the

Fig. 1. Chemical structure of quercetin.
Table 1. Results of system suitability test

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Results</th>
<th>Parameter Limit</th>
</tr>
</thead>
<tbody>
<tr>
<td>RSD of retention time</td>
<td>0.09%</td>
<td>&lt; 1%</td>
</tr>
<tr>
<td>RSD of peak area</td>
<td>0.07%</td>
<td>&lt; 1%</td>
</tr>
<tr>
<td>Capacity factor k’</td>
<td>4.43</td>
<td>&gt; 2</td>
</tr>
<tr>
<td>Tailing factor T</td>
<td>1.05</td>
<td>&lt; 2</td>
</tr>
<tr>
<td>Theoretical plate</td>
<td>4550</td>
<td>&gt; 2000</td>
</tr>
</tbody>
</table>

wavelength region from 200 to 400 nm using Model Lambda 12 (Perkin Elmer) UV-visible spectrophotometer. The wavelength of 257 nm and 375 nm was chosen for the quantification of quercetin.

Chromatographic Conditions

Chromatographic separation was achieved using a LiChroCART® Purospher® STAR RP-18 end-capped column (150 x 4.6 mm i.d., particle size 5 μm). The mobile phase was composed of mixture of acetonitrile and water acidified with o-phosphoric acid (pH 3.0) in ratio 30:70 (V/V), filtered through 0.45 μm nylon filter. The column temperature was maintained at 30 °C, while the eluent was run at 1.1 mL/min. The injection volume was 20 μL. Isopropanol was used as a solvent in preparation of standard and sample solution. The operating wavelength was selected by scanning a mix standard solution (quercetin and ascorbic acid in isopropanol) in the wavelength region from 200 to 400 nm using a Model Lambda 12 (Perkin Elmer) UV-visible spectrophotometer (Fig. 2).

Calibration curve

A quercetin stock solution was prepared by dissolving quercetin standard substance (25.12 mg) with isopropanol in 25 mL amber volumetric flask (c = 0.9851 mg/mL). The mixture was sonicated in an ultrasonic bath for 10 min and stored at room temperature (25 ±2 °C) during the study.

Working standard solutions were prepared daily, by dilution of quercetin stock solution with the same solvent (isopropanol) to obtain final concentrations ranging from 4.93 – 118.21 μg/mL (4.93; 9.85; 19.70; 39.40; 98.50 and 118.21 μg/mL). Isopropanol was used as a blank.

Sample preparation

The following procedure was used to determine the quercetin content in capsules. Twenty capsules were accurately weighed and used to calculate the average mass of one capsule. Then, a capsule content containing 250 mg quercetin (approximately an average capsule content) was accurately weighed, transferred to an amber 100 mL volumetric flask, about 70 mL of isopropanol were added in the flask and the solution was sonicated in an ultrasonic bath for 10 min. The volume was made up to the mark with the same solvent (target concentration of 0.02 mg/mL quercetin). All solvents and solutions were filtered through a nylon membrane filter with a pore size of 0.45 μm. The amount of quercetin per capsule was calculated using the standard calibration curves at two wavelengths (257 nm and 375 nm).

Results and discussion

Various mobile phases and chromatographic columns have been used to optimize the chromatographic conditions of a HPLC method for determination of quercetin in dietary supplements. The selection of the mobile phase and HPLC column was done on a basis of the system suitability parameters (capacity factor, tailing factor, theoretical plate etc.). Thus, the mobile phase consisting of acetonitrile and water acidified with o-phosphoric acid (pH 3.0), 30:70 (V/V), was found optimal for isocratic determination of quercetin in capsules (Table 1).

The wavelengths were selected by scanning the mix standard solution (quercetin and vitamin C in isopropanol) over 200 – 400 nm and the wavelengths of 257 nm and 375 nm were selected for determination of quercetin in dietary supplements also containing ascorbic acid. While quercetin exhibits maximum absorbance at 375 nm, ascorbic acid has showed very poor absorbance at this wavelength (Fig. 2). Therefore, we decided to include the additional wavelength of 257 nm (besides the wavelength of 375 nm) in order to monitor whether the proper chromatographic separation of the quercetin and ascorbic acid is achieved.
Method validation

We used the ICH guideline on the validation of analytical procedures to demonstrate that the developed method is suitable for its intended purpose (ICH, 2005). As the method will be used for determination of quercetin in capsules, the mandatory parameters for method validation are specificity, selectivity, linearity, limit of detection (LOD), limit of quantification (LOQ), accuracy, precision and robustness.

Specificity and selectivity

Specificity is applied to provide “an exact result which allows an accurate statement on the content or potency of the analyte in a sample in the presence of components that can be expected to be present (impurities, degradants, matrix, etc.)” (ICH, 2005). The resolution between quercetin peak and vitamin C peak was 7.4 at 257 nm which proved that critical separation between these two components which elute closest to each other was achieved (at 375 nm ascorbic acid has showed poor absorption) (Fig. 3).

We used typical chromatograms of standard solution, sample solution and blank solution (isopropanol) to demonstrate specificity. Specificity was demonstrated as there was no overlap of the main peak with the peaks from the blank solution, or eventually present impurities and degradants (Fig. 4 and Fig. 5).
Determination of quercetin in dietary supplements by isocratic RP-HPLC method

Furthermore, the UV spectrum of quercetin peak, eluted at retention time of 5.98 min from the standard solution was similar with the quercetin peak from the sample solution (containing quercetin and ascorbic acid), which proved there was no interference from the ascorbic acid and other ingredients (Fig. 6).

Considering the obtained results of the system suitability test, the method performance is similar at both wavelengths (257 nm and 375 nm) (Table 1). However, ascorbic acid is visible only at the wavelength of 257 nm because this component has shown poor absorbance at 375 nm.

**Linearity and range**

To show that the results of the analytical method are directly proportional to the concentration of the quercetin, we used 6 different concentrations of standard quercetin solutions (0.0049 – 0.11821 mg/mL). By mathematical
estimation of the degree of dependence, we obtain similar equations of the regression line at both wavelengths, as follows:

\[
y = 64179 \times x - 71.533 \quad (257 \text{ nm}) \\
y = 65032 \times x - 79.493 \quad (375 \text{ nm})
\]

Together with the correlation coefficients:
\[
R^2 = 1.0000 \quad (257 \text{ nm}) \\
R^2 = 0.9999 \quad (375 \text{ nm}).
\]

**Limit of detection and limit of quantification**

The limit of detection in a single analytical procedure is defined as the amount of analyte in a sample that can be detected but does not have to be measured as an exact value. The quantitative limit of an individual analytical procedure is the amount of analyte in a sample that can be determined quantitatively with appropriate precision and accuracy. LOD and LOQ are found using the ratios of 3.3 \( \sigma / S \) and 10 \( \sigma / S \) respectively, where \( \sigma \) represents the standard estimation error and \( S \) is the slope (ICH, 2005).

The established LOD and LOQ for quercetin were 0.00071 \( \mu g/mL \) and 0.00215 \( \mu g/mL \) at 257 nm, and 0.00078 \( \mu g/mL \) and 0.00236 \( \mu g/mL \) at 375 nm, respectively (Table 2).

![UV spectra of quercetin peak](image)

**Fig. 6.** UV spectra of quercetin peak, eluted at retention time of 5.98 min from the standard solution (a) and sample solution of capsules containing 250 mg quercetin and 250 mg ascorbic acid (b).

**Table 2.** Characteristics of the linear regression analysis

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Quercetin API*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Linearity range (( \mu g/mL ))</td>
<td>257 nm</td>
</tr>
<tr>
<td></td>
<td>4.93 – 118.21</td>
</tr>
<tr>
<td>Slope</td>
<td>64179</td>
</tr>
<tr>
<td>Intercept</td>
<td>-71.53</td>
</tr>
<tr>
<td>Determination coefficient (r^2)</td>
<td>1.0000</td>
</tr>
<tr>
<td>SE of the intercept</td>
<td>13.78</td>
</tr>
<tr>
<td>SE of the slope</td>
<td>210.50</td>
</tr>
<tr>
<td>P-value of the slope</td>
<td>0.007</td>
</tr>
<tr>
<td>LOD (Limit of detection)</td>
<td>0.00071</td>
</tr>
<tr>
<td>LOQ (Limit of quantification)</td>
<td>0.00215</td>
</tr>
</tbody>
</table>

*Mean value of 3 determinations

SE – Standard error

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Table 3. Accuracy of the method

<table>
<thead>
<tr>
<th>Amount added (µg/mL)</th>
<th>Amount found (µg/mL) *</th>
<th>Recovery (%)</th>
<th>RSD (%)</th>
<th>Bias (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>257 nm</td>
<td>375 nm</td>
<td>257 nm</td>
<td>375 nm</td>
</tr>
<tr>
<td>16.12</td>
<td>16.39</td>
<td>16.37</td>
<td>101.69</td>
<td>101.56</td>
</tr>
<tr>
<td>18.48</td>
<td>18.42</td>
<td>18.38</td>
<td>99.65</td>
<td>99.36</td>
</tr>
<tr>
<td>23.21</td>
<td>23.09</td>
<td>23.39</td>
<td>99.49</td>
<td>100.67</td>
</tr>
<tr>
<td>25.57</td>
<td>25.90</td>
<td>25.82</td>
<td>101.26</td>
<td>100.95</td>
</tr>
<tr>
<td>32.67</td>
<td>31.89</td>
<td>31.97</td>
<td>98.35</td>
<td>98.95</td>
</tr>
<tr>
<td>X</td>
<td>100.09</td>
<td>100.29</td>
<td>0.06</td>
<td>0.16</td>
</tr>
</tbody>
</table>

* n = 3 determinations

**Accuracy**

Accuracy as a procedure for measuring uncorrected bias (system error) in analysis was performed using a standard addition method (Nethercote and Ermer, 2014). To study the accuracy of the proposed analytical method, we conducted recovery tests using the standard addition method. In order to discover whether excipients interfered with the analysis, known amounts of standard were added to the samples and the resulting mixtures were analyzed by the proposed method. The analytical yield (recovery) was calculated at both wavelengths (Table 3).

The results obtained in the validation study (recovery within the range of 98.0 - 102.0%) show that the method accuracy is in accordance with the criteria set by Bliesner (2006).

**Precision**

The precision of the analytical procedure was studied by analyzing six samples of the same sample batch at the same conditions (at the working concentration of quercetin (0.02 mg/mL) on the same day. The analysis of the samples was performed by the same analyst, using the same equipment. Intermediate precision of the analytical procedure was examined by sample analysis over three consecutive days. The precision of the analytical procedure was evaluated and expressed as a relatively standard deviation of the series of measurements (Table 4).

The results obtained in the validation study (RSD ≤ 2.0%) show that the method precision is in accordance with the criteria set by Bliesner (2006).

Table 4. Precision of the method

<table>
<thead>
<tr>
<th>Precision Parameter</th>
<th>% of declared content (X)</th>
<th>RSD (%)</th>
<th>Bias (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>257 nm</td>
<td>375 nm</td>
<td>257 nm</td>
</tr>
<tr>
<td>Intra-assay Precision *</td>
<td>100.86</td>
<td>100.22</td>
<td>0.32</td>
</tr>
<tr>
<td>Intermediate Precision</td>
<td>Day 1</td>
<td>100.02</td>
<td>99.96</td>
</tr>
<tr>
<td>Day 2</td>
<td>99.88</td>
<td>99.72</td>
<td>0.14</td>
</tr>
<tr>
<td>Day 3</td>
<td>99.55</td>
<td>99.66</td>
<td>0.27</td>
</tr>
<tr>
<td>X</td>
<td>99.82</td>
<td>99.76</td>
<td>0.17</td>
</tr>
</tbody>
</table>

* n = 6

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Table 5. Results of the robustness of the method

<table>
<thead>
<tr>
<th>Temperature °C</th>
<th>Flow 1,1 mL/min</th>
<th>257 nm</th>
<th>RSD (%)</th>
<th>Area</th>
<th>RSD (%)</th>
<th>375 nm</th>
<th>RSD (%)</th>
<th>Area</th>
<th>RSD (%)</th>
<th>257 nm</th>
<th>RSD (%)</th>
<th>Area</th>
<th>RSD (%)</th>
<th>375 nm</th>
<th>RSD (%)</th>
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</thead>
<tbody>
<tr>
<td>23</td>
<td></td>
<td></td>
<td>0.42</td>
<td>0.40</td>
<td>0.44</td>
<td>0.40</td>
<td>0.55</td>
<td>0.50</td>
<td>0.58</td>
<td>0.57</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>25</td>
<td></td>
<td></td>
<td>0.33</td>
<td>0.30</td>
<td>0.32</td>
<td>0.30</td>
<td>0.46</td>
<td>0.43</td>
<td>0.46</td>
<td>0.46</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>30</td>
<td></td>
<td>a</td>
<td>0.09</td>
<td>0.07</td>
<td>0.09</td>
<td>0.05</td>
<td>0.22</td>
<td>0.22</td>
<td>0.23</td>
<td>0.22</td>
<td>b</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>35</td>
<td></td>
<td>b</td>
<td>0.08</td>
<td>0.08</td>
<td>0.10</td>
<td>0.11</td>
<td>0.15</td>
<td>0.15</td>
<td>0.15</td>
<td>0.15</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

a Working flow rate in the proposed method
b Working column temperature in the proposed method

Robustness

Robustness is a measure of the ability of the analytical procedure to remain unchanged in small, but deliberate variations of the method parameters. Variations were made by changing the flow rate of the mobile phase (± 0.1 mL/min) and the column temperature (+5 °C; -2 °C; -5 °C). The obtained values were within acceptable limits (< 2%) (Bliesner, 2006; Ph. Eur., 10.0, 2019).

From the results obtained in the study of method robustness, we can conclude that the method small and deliberate variations in flow rate and column temperature have no significant effect on system stability (Table 5).

Conclusion

A simple, accurate, and reproducible high-performance liquid chromatography (HPLC) method has been developed and validated for the quantification of quercetin in dietary supplements. This proposed method enables fast, direct, and simple determination of quercetin, using isopropanol as a solvent, without prior extraction from the samples. The method exhibited good linearity (R² > 0.9999) over the studied concentration range (4.93 – 118.21 μg/mL). The proposed method is very sensitive as the established LOD and LOQ for quercetin were 0.00071 μg/mL and 0.00215 μg/mL at 257 nm, and 0.00078 μg/mL and 0.00236 μg/mL at 375 nm, respectively. It has demonstrated good intra-day and inter-day precision (relative standard deviations values were < 2%) and excellent method accuracy (with the recovery of 100.09% and 100.29% for quercetin, at 257 nm and 375 nm, respectively). Results from the system suitability test showed that the method performance is similar at both wavelengths (257 nm and 375 nm). The method was also successfully applied to determine quercetin in commercially available capsules containing quercetin and ascorbic acid concomitantly. It could be recommended for routine analysis of the dietary supplements containing quercetin in food quality control laboratories.

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Резиме

Опредељување на кверцетин во додатоци на исхрана со изократски RP-HPLC метод

Драгица Донева1*, Билјана Бауер2

1 Институт за јавно здравје на Република Северна Македонија, 50 Дивизија 6, 1000 Скопје, Република Северна Македонија
2 Институт за фармакоднозија, Фармацевтски Факултет, Универзитет „Св. Кирил и Методиј“, Мајка Тереза 47, 1000 Скопје, Република Северна Македонија

Ключни зборови: кверцетин, HPLC, додатоци во исхрана, флавоноид

Кверцетинот е природен флавоноид кој се наоѓа во зеленчуку и овошјето. Покажува антиоксидантна, антиинфламаторна, антихепатотоксична, антиалергична и антивирусна активност. Поради големата популярност на додатоците во исхраната со кверцетин, важно е да се контролира нивниот квалитет. Цел на овој труд беше да се развије едноставен и точен HPLC метод за определување на кверцетин во додатоци во исхрана. Хроматографското раздвојување беше постигнато со изократски метод, користејќи реверзно-фазна колона Purospher STAR® RP-18 (150 x 4,6 mm I.D., големина на честички 5 μm), со мобилна фаза составена од ацетонитрил и вода (закиселена со о-фосфорна киселина до pH 3,0), во сооднос од 30:70 (V/V) и проток од 1,1 mL/min. Температура на колоната беше 30 °C, а волумен на инјектирање 20 μL. DAD детекторот беше поставен на 275 nm и 375 nm. Како растворувач е користен изопропанол. Методот е валидиран со утврдување на соодветност на системот, специфичност, линеарност, опсег, лимит на детекција и квантификација, точност, прецизност и робустност. Се карактеризира со едноставна подготовка, добра прецизност (RSD < 2%) и добар аналитички принос (100,09% и 100,29% на 257 nm и 375 nm, соодветно). Границите на детекција и квантификација се: 0,00071 μg/mL и 0,00215 μg/mL на 257 nm, 0,00078 μg/mL и 0,00236 μg/mL на 375 nm, соодветно. Тестот на соодветност на системот покажа дека методот дава споредливи резултати на двете бранови должини. Методот беше успешно применет за определување на кверцетин во диететски додаток во форма на капсули и се препорачува за рутинска анализа на кверцетин во додатоци на исхрана во лабораториите за контрола на квалитетот на храната.