Macedonian Journal of Chemistry and Chemical Engineering, Vol. **33**, No. 2, pp. 209–215 (2014)

MJCCA9 – 649
Received: February 14, 2014
Accepted: August 4, 2014

ISSN 1857-5552, e-ISSN 1857-5625
UDC: 547.972.2:615.453.4.074
Original scientific paper

SPECTROFLUORIMETRIC DETERMINATION OF QUERCETIN IN PHARMACEUTICAL DOSAGE FORMS

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A simple, accurate and precise method based on the fluorescence properties of the aluminum(III)–quercetin complex, for the determination of quercetin, has been developed and validated. The complex has strong emission at pH 3.30, λ_{em} = 480 nm, with λ_{ex} = 420 nm. The linearity range of quercetin determination was 1.5–60.5 ng ml^{-1} with LOD 0.09 ng ml^{-1} and LOQ 0.27 ng ml^{-1}. Recovery values in the range of 99.9–100.2% indicate a good accuracy of the method. The established method was applied for the determination of quercetin in capsules, with a recovery value of 98.3%, standard deviation of 0.22% and coefficient of variation of 0.09%.

The reliability of the method was checked by the newly developed RP-HPLC/UV method for capsules with the direct determination of quercetin after separation. The good agreement between the two methods indicates the applicability of the proposed spectrofluorimetric method for quercetin determination in pharmaceutical dosage forms, with high reproducibility, and enables the direct and simple determination without its prior extraction from samples.

The proposed spectrofluorimetric method has much better sensitivity and LOD and LOQ values that are about 1000 times lower than data reported in the literature.

Keywords: quercetin; flavonoids; spectrofluorimetric determination; RP-HPLC; capsules

СПЕКТРОФЛУОРОМЕТРИСКО ОПРЕДЕЛУВАЊЕ НА КВЕРЦЕТИН ВО ФАРМАЦЕВТСКИ ДОЗИРАНИ ФОРМИ

Развиен и валидиран е едноставен, точен и прецизен метод за определување на кверцетин заснован на флуоресцентните својства на комплексот кверцетин–алуминиум(III). Комплексот покажува силна емисија при pH 3,30, λ_{em} = 480 nm и λ_{ex} = 420 nm. Линеарниот опсег за определување на кверцетин е 1,5–60,5 ng ml^{-1} со LOD 0,09 ng ml^{-1} и LOQ 0,27 ng ml^{-1}. Вредностите на аналитичкиот принос од 99,9–100,2% покажуваат добра точност на методот. Воспоставениот метод е применет за определување на кверцетин во капсули со вредност на аналитичкиот принос од 98.3%, стандардна девијација од 0,22% и коефициент на варијација од 0,09%.

Веродостојноста на методот е проверена со новововедениот RP-HPLC/UV метод за капсули со директно определување на кверцетин по негова сепарација. Доброто согласување на двата метода укажува на применливост на предложениот спектрофлуорометриски метод за определување на кверцетин во фармацевтски дозирани форми со висока репродуцибилност и овозможува директно и едноставно определување без претходна екстракција од примероците.

Предложениот спектрофлуорометриски метод има многу подобра осетливост и вредностите LOD и LOQ се за околу 1000 пати пониски од оние дадени во литературата.

Клучни зборови: кверцетин; флавоноиди; спектрофлуорометриско определување; RP-HPLC; капсули

1. INTRODUCTION

Flavonoids are a large family of over 4000 ubiquitous secondary plant metabolites, which can be further divided into five sub-classes including flavonols, flavones, anthocyanins, catechins and flavononones [1]. Flavonoids and particularly quercetin derivatives have received more attention as dietary constituents during the last few years. Experimental studies have indicated that they possess numerous beneficial effects on human health, such as cardiovascular protection, anticancer activity, antiulcer effects and anti-allergic, antiviral, and anti-inflammatory properties [2].

Quercetin is the major representative of the flavonoid subclass of flavonols [3], a group of flavonoids that occurs in foods as *O*- and *C*-glycosides (Fig. 1).

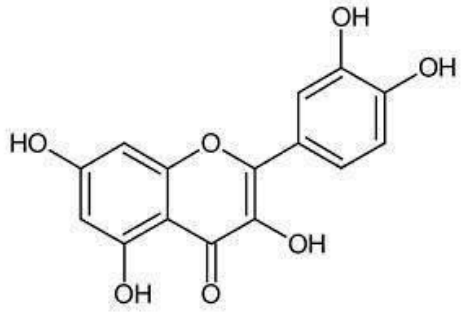

Fig. 1. Quercetin (3,3',4',5,7-pentahydroxyflavone)

Quercetin is a natural polyphenolic antioxidant, which is present in vegetables, fruits and juices. It is a strong antioxidant because it can chelate metal ions such as Pd(II) [4], Cu(II) [5, 6], Co(II) [7], Al(III) [8], scavenge oxygen free radicals [9, 10] and prevent the oxidation of low density lipoprotein (LDL) *in vitro* [11]. Oxidized LDL is hypothesized to be an intermediate in the formation of atherosclerotic plaques [12].

All of these activities indicate that quercetin could be a compound with potential clinical application. Since quercetin is one of the most common flavonols and one of the most powerful antioxidants, it was important to develop a simple, precise and accurate method for the determination of quercetin in different samples. Several methods were proposed in the literature to determine quercetin in the samples of apple and tomato juice and fruits, wines, teas, serums and pharmaceutical preparations. These include HPLC [13–15], HPTLC [16] LC-MS [17], spectroscopic methods [5, 18, 19], adsorptive stripping voltammetry [20], electrochemical analysis [21] and fluorimetric methods [22]. The majority of methods required some pretreatment of real samples, such as solid phase extraction [23 and references therein].

The aim of this study was to develop and validate a simple, rapid and sensitive spectrofluorimetric method for the determination of quercetin in pharmaceutical dosage form. The proposed method is based on the formation of the Al^{3+}-quercetin complex at pH 3.30, which has intensive fluorescence emission at 480 nm with excitation at 420 nm. The reliability of the method was checked by comparison with the results obtained by RP-HPLC/UV method.

2. EXPERIMENTAL

2.1. *Materials and solutions*

Aluminum-nitrate (Fluka AG, Buchs, Germany), quercetin-dihydrate ($C_{15}H_{10}O_7 \cdot 2H_2O$, Mr = 338.27 g mol^{-1}, CAS number 6151-25-3, Fluka AG, Buchs, Germany), acetonitrile (J.T. Baker, Deventer, Netherlands), methanol, NaOH, CH_3COOH (Merck, Darmstadt, Germany), all p.a. grade, have been used. All reagents were used without further purification. The stock solution of aluminum-nitrate was prepared by dissolving $Al(NO_3)_3$ in double-distilled water with the addition of an appropriate amount of nitric acid to prevent the initial hydrolysis of aluminum(III)-ion. The content of aluminum(III)-ion was determined gravimetrically by precipitation with ammonia. The solution of quercetin was prepared by dissolving a precisely measured mass of quercetin-dihydrate in 70% (v/v) of methanol.

Quercetin + C capsules were available from Twin Laboratories Inc., Ronkonkoma, New York, USA (nominal composition declared for two capsules: vitamin C 1400 mg, quercetin-dihydrate 500 mg excipients: gelatin, silica and vegetable-based stearic acid).

Working solutions were prepared by dilution of the stock solutions of aluminum nitrate (1.00×10^{-3} mol l^{-1} $Al(NO_3)_3$) and quercetin-dihydrate (1.00×10^{-4} mol l^{-1}).

2.2. *Instruments*

Fluorescence spectra were collected using a Fluorolog-3 spectrofluorimeter (Jobin Yvon Horiba, Paris, France) equipped with a 450 W xenon lamp and a photomultiplier tube. Samples were placed in a 1 cm optical path length quartz cuvette for spectral recording. The slits on the excitation and emission beams were both set at 5 nm. The spectra were corrected for the dark counts. In each measurement, three scans with a one-second-integration time were averaged. The emission

spectrum of the solvent (70% v/v methanol) was subtracted. All measurements were performed at 24 °C controlled by a Peltier element. Measurements of pH were carried out using a Metler Toledo mp 120 pH-meter (precision ± 0.01 pH unit) equipped with a combined glass electrode. All spectrofluorimetric measurements were taken in acetate buffers at pH 3.30 (in 70% v/v methanol) which had been prepared according to Perrin [24].

Chromatographic measurements were carried out using the HPLC system (Shimadzu, Kyoto, Japan), composed of a quaternary pump LC-20AT, an autosampler (injection volume 20 µl) and equipped with an RP AquaGold aQ column (150 × 4.6 mm, 5 µm, Thermo, USA), degasser, DGU-20A$_3$, a column thermostat CTO-20A and variable UV-Vis diode array SPD-M20A. Acquisition and data analysis were performed with the manufacturer software LC Solution. The mobile phase consisted of 2% acetic acid and acetonitrile 95:5% (v/v); flow-rate: 1 ml min^{-1} at 30 °C; injection volume 20 µl. The wavelength of detection was 254 nm.

2.3. *Sample preparations for determination of quercetin in capsules*

The following procedure was used to determine the quercetin content in capsules. The mass of ten capsules was determined and used to calculate the average mass of one capsule. The inside contents of the ten combined capsules were weighed on an analytical balance and powdered. A mass of powder equal to the medium mass of one capsule content was weighed out, dissolved in 100 ml 70% methanol and treated in an ultrasonic bath at 25 °C for 15 min. The obtained solution was filtered through a millipore membrane filter with a pore size of 0.45 µm. The filtrate (0.25 ml) was transferred into a 10 ml volumetric flask, then 0.5 ml of aluminum(III)-nitrate solution was added at a concentration of 1×10^{-3} mol l^{-1}. Volumetric flasks were filled with acetic buffer pH 3.30 prepared in 70% (v/v) methanol.

The obtained data were used to calculate analytical validation parameters of both spectrofluorimetric and chromatographic methods with the aid of Origin v. 7 software [25, 26].

3. RESULTS AND DISCUSSION

3.1. *Spectrofluorimetric determination of quercetin in methanol-water solutions*

The composition of the solvent influences the fluorescence intensity and the solubility of the complex as well. The optimal composition of the solvent was methanol : water 70 : 30% (v/v), because the maximum intensity of fluorescence and solubility of the complex was observed in this solvent. The fluorescence spectra were recorded using 70% (v/v) methanol as a blank, and excitation and emission wavelengths were λ_{ex} = 420 nm and λ_{em} = 480 nm, respectively. Excitation (1') and emission (1) spectra of aluminum(III)–quercetin solution and excitation (2') and emission (2) spectra of quercetin are shown in Figure 2.

The composition of the aluminum(III)-ion and quercetin complex (2:1) was confirmed by the molar ratio method. The fluorescence intensities were measured for solutions with a constant concentration of aluminum(III)-ions (1.0×10^{-8} mol l^{-1}), while the concentration of quercetin varied in the range 5.0×10^{-9} to 2.0×10^{-7} mol l^{-1}. The measurements were taken in acetic buffer pH 3.30 prepared in 70% (v/v) methanol, with λ_{ex} = 420 nm, λ_{em}= 480 nm, and slits set on 5 nm.

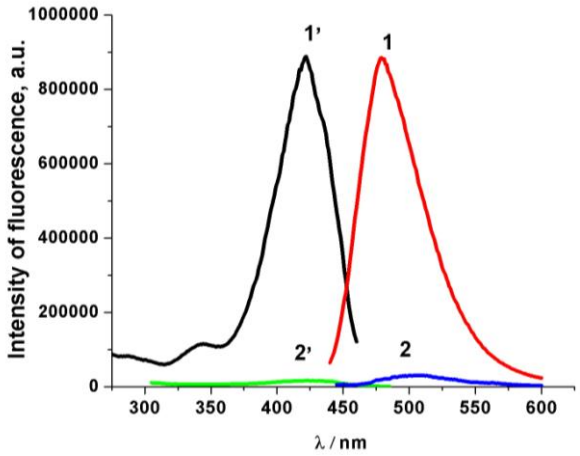

Fig. 2. Excitation (1′) and emission (1) spectra of aluminum(III)–quercetin complex and excitation (2′) and emission (2) spectra of quercetin

The influence of pH on the intensity of fluorescence of the aluminum(III)–quercetin complex was examined in the range 2.0–5.5, as shown in Figure 3. The pH dependence of fluorescence intensity exhibits a complex shape. The optimal pH value was around 3.3, which is used for all further experiments.

The stability constant of the complex in pH 3.30 was estimated according to the procedure based on the modified Bjerrum method [27, 28] and the (conditional) stability constant was found to be log$K$ = 27.79 ± 0.02. Such a high stability constant enables the determination of quercetin based on the formation of the aluminum(III)–quercetin complex, at pH 3.30 and in the presence of 70% (v/v) methanol.

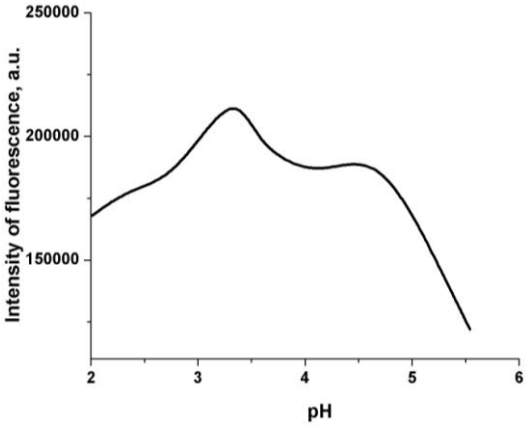

Fig. 3. Effect of pH on the intensity of fluorescence of aluminum(III)–quercetin complex

Spectrofluorimetric determination is based on the calibration curve, where the intensity of fluorescence (I_F, in % and obtained with λ_{ex} = 420 nm and λ_{em} = 480 nm) is linearly dependent on the quercetin concentration (c_{Querc}, expressed as ng ml^{-1}), according to the equation:

$$I_F = (1.47 \pm 0.01)\, c_{Querc} + (0.56 \pm 0.04),$$

where N = 9 and correlation coefficient r^2 = 0.9999.

Linear dependence of the intensity of fluorescence of the complex was obtained in the concentration range 1.5–60.5 ng ml^{-1} quercetin. The limit of detection (LOD) was found to be 0.09 ng ml^{-1}, while the limit of quantification (LOQ) is 0.27 ng ml^{-1}.

The accuracy of the method was determined for three different quercetin concentrations (Table 1). The high accuracy and repeatability of the method are indicated by good recovery and low values of SD.

T a b l e 1

The spectrofluorimetric determination of quercetin in aqueous-methanolic solutions, N=5

Added quercetin (ng ml^{-1})	Found quercetin (ng ml^{-1})	Recovery (%)	RSD (%)
6.06	6.07 ± 0.021	100.17	0.35
12.12	12.11 ± 0.020	99.92	0.17
24.24	24.23 ± 0.018	99.96	0.08

3.2. *Spectrofluorimetric determination of quercetin in capsules*

The established method was applied for the determination of quercetin in pharmaceutical preparations of *Quercetin + C capsules* (content declared on two capsules: 1400 mg vitamin C and 500 mg quercetin-dihydrate), manufactured by Twinlab, USA. Samples were prepared according to the procedure described in the *Experimental* section, and the results of the spectrofluorimetric determination of quercetin in capsules are presented in Table 2.

T a b l e 2

The spectrofluorimetric determination of quercetin in capsules, N=5

Quercetin + C capsules	Found quercetin-dihydrate (mg)	Recovery (%)	RSD (%)
Declared 250 mg of quercetin-dihydrate per capsule	245.7 ± 0.22	98.28	0.09

To confirm the applicability of the spectrofluorimetric method for determination of quercetin in some pharmaceutical formulations, the possible effect of excipients and other active compounds on the intensity of fluorescence of the aluminum(III)–quercetin complex was investigated. It was necessary to examine the influence of vitamin C and excipients on the fluorescence of the solution obtained after sample preparation *Quercetin + C capsules* following the previously explained procedure. The mixture, containing vitamin C and other declared excipients, was treated using the same procedure proposed for the determination of quercetin in capsules. The emission spectrum was recorded under the same conditions, λ_{ex} = 420 nm and λ_{em} = 480 nm, while slits were set to 5 nm. The emission spectrum of the aluminum(III)–quercetin complex in *Quercetin + C capsules* (1) and vitamin C and excipient mixture (2) are presented in Figure 4.

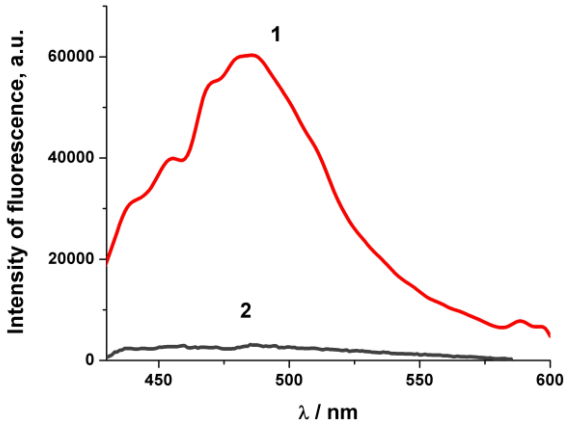

Fig. 4. Emission spectrum of aluminum(III)–quercetin complex in *Quercetin + C capsules* (1) and vitamin C and excipient mixture (2)

As can be seen, there is no considerable significant interference in the spectrofluorimetric determination of quercetin in capsules under the examined conditions, such as pH and methanol content.

3.3. *HPLC determination of quercetin in capsules*

To compare the performance of the proposed spectrofluorimetric method for the determination of quercetin in capsules (pharmaceutical dosage form), a comparative method of RP-HPLC with UV-Vis detection, was developed. Figure 5 presents the chromatogram of quercetin recorded under the optimal conditions.

Samples of *Quercetin + C capsules* for RP-HPLC analyses were prepared according to the procedure described for the spectrofluorimetric method. The different aliquots of prepared solutions were analyzed by the HPLC system with an AquaGold aQ column and UV detection.

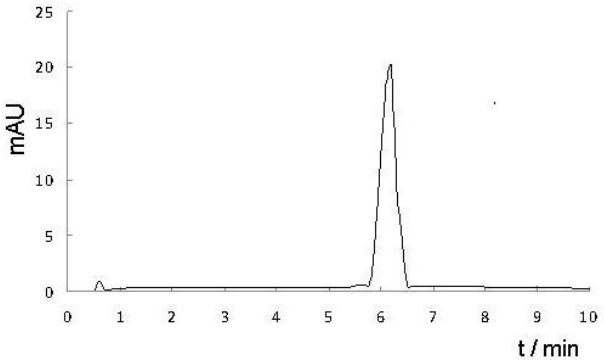

Fig. 5. Chromatogram of quercetin

A chromatogram of a sample prepared from *Quercetin + C capsules* is given in Figure 6. The peak of quercetin appears on the chromatogram with a retention time of 6.18 min (which was established comparing the chromatogram of the standard, quercetin, and confirmed by UV and mass spectra of the peak).

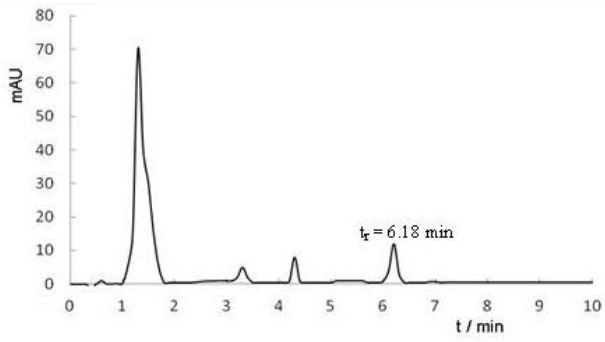

Fig. 6. Chromatogram of *Quercetin + C capsules* after sample preparation

Based on the obtained calibration curve for quercetin determination by the RP-HPLC method, the regression equation is as follows:

$$A = (4.38 \pm 0.05) \times 10^3 c_{\text{Querc}} + (0.05 \pm 0.002) \times 10^3 \quad (N = 7,\ r^2 = 1.0000)$$

where A is peak area and c_{Querc} is quercetin concentration expressed in μg ml^{-1}. Linearity is achieved for the quercetin concentration range of 0.05–200.0 μg ml^{-1}. The limit of detection (LOD) and limit of quantification (LOQ), were calculated from the calibration curve parameters, and were 0.066 μg ml^{-1} and 0.02 μg ml^{-1}, respectively.

The accuracy of the method was checked for three different concentration levels of quercetin (80, 100 and 120%), using the standard addition method. All measurements were performed in triplicate. The results of standard deviation and "recovery" values, shown in Table 3, confirmed the precision of the developed RP-HPLC method for quercetin determination.

T a b l e 3

Results of determination of quercetin by RP-HPLC/UV method, N=3

%	Added quercetin (μg ml^{-1})	Found quercetin (μg ml^{-1})	Recovery (%)
80	73.00	75.4 ± 0.8	103.37
100	91.26	90.46 ± 0.7	99.12
120	109.51	108.77 ± 0.6	99.32

The developed RP-HPLC/UV method was applied for the determination of quercetin in capsules; the results are presented in Table 4.

T a b l e 4

The RP-HPLC/UV determination of quercetin in Quercetin + C capsules, N=5

Quercetin + C capsules	Found quercetin-dihydrate (mg)	Recovery (%)	RSD (%)
Declared 250 mg of quercetin-dihydrate per capsule	243.0 ± 0.31	97.2	0.15

In comparison to methods reported in literature, this method is very fast and simple to perform, with very high sensitivity, a wide linear range and good operational stability.

The proposed spectrofluorimetric method has much better sensitivity, precision and (LOD) and (LOQ) values that were about 1000 times lower compared to the HPLC method developed for the simultaneous determination of quercetin and luteolin in capsules [15]. The spectrofluorimetric method exhibits the same advantages over the spectrophotometric methods suggested for the determination of quercetin in bulk drug and pharmaceutical formulations as well [5, 18, 19].

4. CONCLUSIONS

The proposed spectrofluorimetric method for quercetin determination in pharmaceutical dosage forms is simple, accurate, and precise, with high reproducibility; it enables direct and simple determination without its prior extraction from samples. The established spectrofluorimetric determination has much lower (LOD) and (LOQ) compared to those obtained by the RP-HPLC/UV method. There was no interference in excipients and ascorbic acid in the examined products, thus no additional extraction or separation procedures were required.

However, it is possible to determine quercetin content in pharmaceutical dosage forms if there is no presence of compounds which could react with aluminum(III)-ions and cause considerable fluorescence on λ_{em} = 480 nm, with λ_{ex} = 420 nm.

Acknowledgements. This work was partly supported by the Ministry of Education and Science of the Republic of Serbia, Projects #172016, #173017 and #172043.

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