FRI-9.2-1-EC-01

DEVELOPMENT OF A NEW PRECISE AND SENSITIVE ANALYTICAL METHOD FOR QUERCETIN QUANTIFICATION

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Abstract: *Quercetin has been demonstrated to play an important role in the protection against environmental oxidative stress. The aim of the current study was to develop a new rapid, sensitive and precise analytical methodology for the determination and quantification of the natural antioxidant in pure form. Two direct spectrophotometric and a RP-HPLC-PDA methods for the bioflavonoid analyses were established. The influences of wavelength, pH, temperature and mobile phase on the biologically active compound determination were investigated. The proposed RP-HPLC-PDA procedure characterized by short retention time (5.9 min), high precision (RSD = 1.326%), and excellent linearity (R^2 = 0.9997), which together with the lowest determined values of the lower limit of detection (LOD) and lowesr limit of quantification, undoubtedly proved the undeniable advantages of the chromatographic method over the proposed spectrophotometric techniques.*

Keywords: *quercetin, RP-HPLC, UV-VIS spectrophotometry, quantitative analyses.*

INTRODUCTION

Oxidative stress is the most common mechanism in the toxicology of environmental agents, unifying the action of broad classes of physichochemically disparate environmental pollutants, including oxidant gases, organic compounds, particulate surfaces, metal ions, etc. As advanced redox biology investigations identify previously unrecognized targets for disruption by exposure to xenobiotics, redox toxicology has emerged as a new scientific field of study. Environmental contaminants can directly or indirectly induce oxidative stress on cells, which involves the disruption of metabolic or bioenergetic processes (Samet, M. & Wages, P., 2018). The search, identification and mechanism of action of veroius antioxidants in the protection against environmental oxidative stress has long been a subject of investigation (Bao, D., Wang, J., Pang, X. & Liu H., 2017).

In regard to the proven adverse side effects (liver damage, mutagenesis, etc.) of a number of synthetic antioxidants, there has been a recent scientific upsurge of interest in natural antioxidants (Gupta, A., Sheth, N, Pandey, S. and Yadav, J., 2015).

In this respect, current scientific investigations evidenced that quercetin, a natural polyphenolic flavonol, exibits a variety of biological activities including antioxidant, anti-carcinogenic, anti-inflammatory, cardioprotective and a susceptibility reduction to viral infections (Tejada, S., Nabavi, S., Capo, X., Martorell, M., del Mar Bibiloni, M., Tur, J., Pons, A. & Sureda, A., 2017).

The precise studies in this direction, however, require the application of reliable analytical methods for separation, identification and quantification of quercetin from plant extracts, food, body fluids, biological samples and phamaceutical dosage forms (Kuntic, V., Pejic, N., Micic, S., Vukojevic, V., Vujic, Z. & Malecev, D., 2005), which provoked the current investigations.

The aim of the present study was to develop a new rapid, sensitive and precise analytical methodology for the quantification of the natural antioxidant quercetin in pure form.

EXPOSITION

Chemicals

Quercetin, acetonitrile (ACN, ≥99.8 %), orthophosphoric acid (H_3PO_4, 85%), methanol (≥ 99.9 %), ethylacetate HPLC grade, were obtained from Sigma-Aldrich.

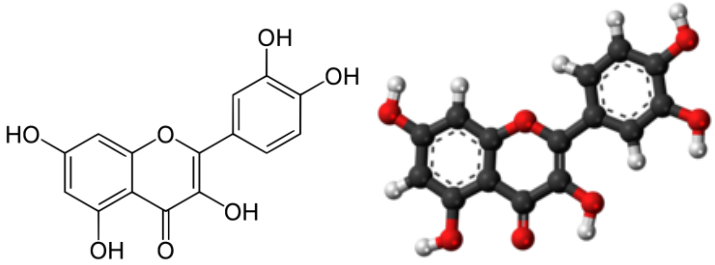


Fig. 1. Chemical structure of quercetin

UV/VIS Spectrophotometric Analyzes

Quercetin concentration was measured in 10 mm quartz cells on UV-VIS spectrophotometer DR 5000 Hach Lange (Germany). All spectra were recorded in the UV region at λ = 373 nm at 2 nm slit width, 900 nm/min scan speed, and very high smoothing.

HPLC system and conditions

RP-HPLC system comprising of a Hypersil BDS C18 (5 μM, 4.6 x 150 mm) column, Surveyor LC Pump Plus, PDA detector, and Surveyor Autosampler Plus (Thermo Fisher Scientific) was used.

Data Processing

All UV/VIS spectrophotometric and HPLC analyses were made in triplicate. Experimental data was analyzed by regression analyses with determination of the corresponding correlation coefficients (R^2) and relative standard deviation (RSD, %). The efficiency and accuracy of the developed UV/VIS and HPLC methods was estimated based on the calculated limit of detection (LOD, mg/L) and limit of quantification (LOQ, mg/L).

UV/VIS Spectrophotometric Analyses

The UV/VIS spectra of quercetin in acidic and neutral ethanoic solutions at pH 4 and 7 displayed maximum absorbance peaks in the longwave UVA region at λ = 373 nm for the entire concentration range of 0.5 – 200 mg/L (Fig 2).

The obtained standard calibration curves quercetin at both pH values were linear over the tested range of initial concentrations (Fig. 3A, B) and the values of both correlation coefficients have approximatey equal values R^2 = 0.998. Though the UV absorption peaks were well resolved, those obtained at pH 4 characterized with higher absorbances.

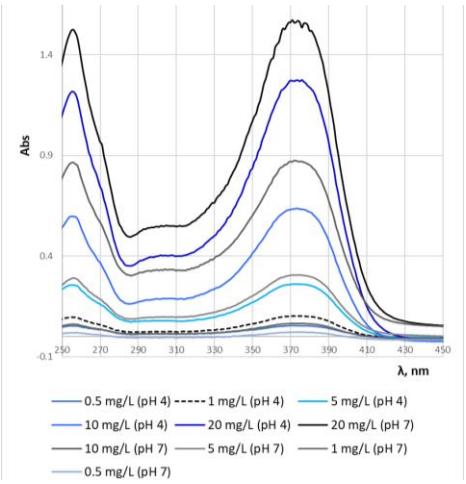
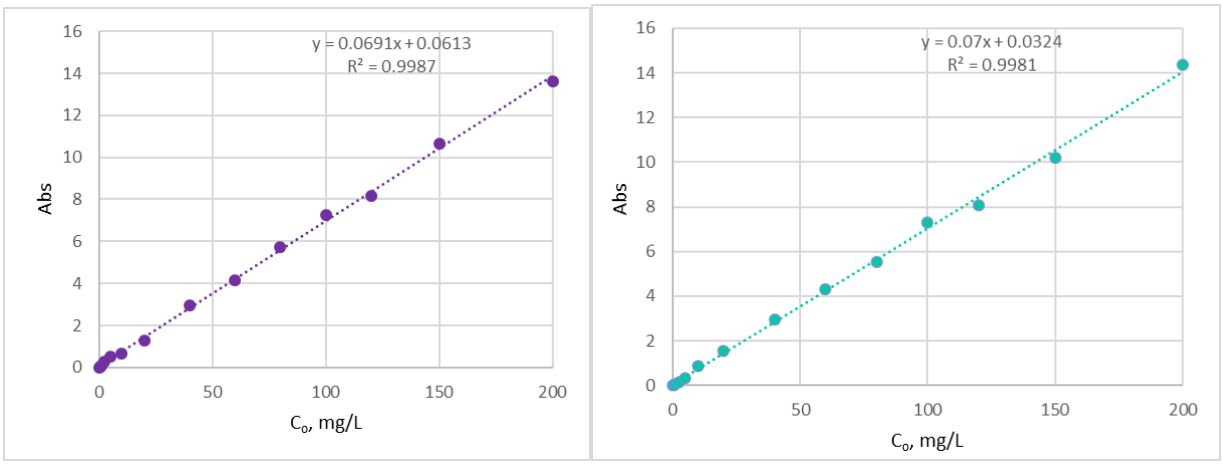


Fig. 2. UV-VIS spectra of quercetin at pH 4 and pH 7



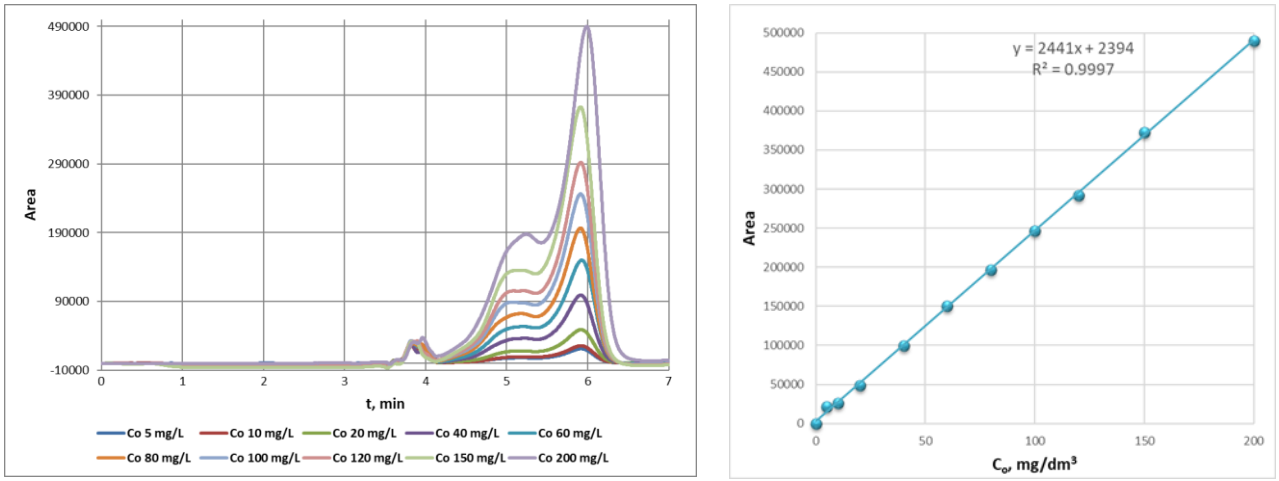
A. **B.**

Fig. 3. Calibration curve of quercetin at **A.** pH = 4 and **B.** pH = 7

RP-HPLC analyses

Two series of experiments with ten quercetin standard solutions with concentrations 5, 10, 20, 40, 60, 80, 100, 120 150 and 200 mg/L with two mobile phases were conducted. The first tested mobile phase consisted of ACN:Ethylacetate: 0.05 % H_3PO_4 (12:2:86, v/v/v), and the second - of MeOH:ACN:H_2O:CH_3COOH glacial (40:15:45:1, v/v/v/v). The effects of flow rate (ν, ml/min), pH and column temperature (T) on the separation process were investigated in the range of ν = 0.5 – 1.2 ml/min and T = 20 – 30°C. Optimum performance was obtained with the second mobie phase at a flow rate 0.5 mL/min at 30°C. Satisfactory peak resolution and optimum analyses time were established at a wavelength of λ = 370 nm. The peaks obtained at pH 4 characterized with approximately 1.2 times greater area, thus this value was selected as the optimum one. The investgated flavonoid was successfully detected within 5.9 min (Fig. 4A).

The obtained HPLC chromatograms did not contain interference peaks, which could influence the quantitative results. The standard calibration curve plotted based on the spectral peak areas is presented in Fig. 4B. It is characterized by a high correlation coefficient of R^2 = 0.9997.



A. B.

Fig. 4. **A.** HPLC chromatograms and **B.** calibration curve of quercetin solutions in the concentration range C_o = 5 – 200 mg/L

Accuracy and effectiveness of the applied analytical methods

To assess the feasibility of the three analytical methods investigated, the RSD, LOD and LOQ values were determined based on the obtained in the recent study experimental data.

The values of LOD and LOQ were estimated according to the guidelines of the International Conference on Harmonization of Technical Requirements for Registration of Pharmaceuticals for Human Use (ICH) based on the standard deviation of the response and the slope of the calibration curve of the analyte. The values of these parameters are presented in Table 1.

Table 1. Values of *RSD* %, *LOD* (mg/L) and *LOQ* (mg/L) for the UV/VIS and HPLC methods

Method	**R^2**	***RSD*, %**	***LOD*, mg/L**	***LOQ*, mg/L**
UV/VIS Method I (pH = 4)	0.9987	1.949	2.639	8.796
UV/VIS Method II (pH = 7)	0.9981	3.614	3.148	10.496
RP-HPLC Method	0.9997	1.326	1.768	5.894

The comparative analyses of the obtained experimental results revealed that the developed and applied HPLC method characterized with the highest correlation coefficient and the lowest RSD, LOD and LOQ values. Thus, it could be suitable for analyses of samples with quercetin concentrations of approximately 5 mg/L. According to the HPLC chromatograms presented in Fig. 4A the sample with 5 mg/L concentration characterized with a well-resolved peak, i.e. the accuracy of the method at and above this limit is satisfactory. Besides, the chromatograms in the entire tested antioxidant concentration range were significantly pronounced. Another advantage of the HPLC method is the fact that dilution of the samples with high concentrations (up to 200 mg/L) was not necessary as they were directly detected and quantified.

Regarding the developed UV-VIS methodologies, and based on the data from Table 1, it could be concluded that undoubtedly UV-VIS method I at pH = 4 displayed higher accuracy and efficiency as the values of the determined R^2 were higher and the RSD – lower, as compared to these of the spectrophotometric technique at pH = 7. The comparative estimation of the UV-VIS spectral data of quercetin obtained by both spectrophotometric methods (Fig. 2) revealed that the spectral peaks of Method I characterized with approximately 1.5 times greater absorbances. Other advantages of this methology are the significantly lower values of the calculated LOD and LOQ parameters, which prove the applicability of Method I for the accurate quantification of samples with the lowest quercetin concentration 8.8 mg/L.

However, both spectrophotometric methods required additional dilution of the smaples with flavonoid concentrations $\geq$ 30 mg/L, as the obtained absorbance peaks were not well-resolved and even reduced, which complicates the analytical procedure.

Despite of the undisputable advantages of the developed RP-HPLC method for analytical determination and quantification of quercetin, UV-VIS method I could be applied in some cases when cost-effectiveness and rapidity are required.

CONCLUSION

RP-HPLC-PDA analytical methodology appropriate for the quantitative determination of the natural antioxidant quercetin was developed in the present study. Desirable chromatographic separation was achieved on a C_{18} column employing as a mobile phase a mixture of MeOH:ACN:H_2O:CH_3COOH glacial (40:15:45:1, v/v/v/v). The obtained HPLC chromatograms were well pronounced and did not contain any interference peaks, which could influence the quantitative results. The applied method offered short analysis time (5.9 min), high precision (*RSD* 1.326 %) and high linearity (R^2 0.9997). It characterized with satisfactory *LOD* and *LOQ* values. The simple and rapid method developed enhances the capabilities for the accurate and selective determination and quantification of quercetin in various biological samples, pharmaceutical formulations for ecological and medical purposes as studying the pharmacokinetics of the flavonoid in living organisms.

ACKNOWLEDGEMENTS:

The study was supported financially by Scientific Project No. 13/18 VMF, Faculty of Veterinary Medicine, Trakia University, Stara Zagora, Bulgaria.

REFERENCES

Bao, D., Wang, J., Pang, X. & Liu H. (2017). Protective effect of quercetin against oxidative stress-induced cytotoxicity in rat pheochromocytoma (PC-12) cells. *Molecules*, 22(7), 1-14.

Gupta, A., Sheth, N, Pandey, S. and Yadav, J. (2015). Determination of quercetin a biomarker in hepatoprotective polyherbal formulation through high performance thin layer chromatography. *Journal of Chromatography & Separation Techniques*, 6(6), 1-9.

International Conference on Harmonization of Technical Requirements for Registration of Pharmaceuticals for Human Use, ICH Harmonized Tripartite Guideline, *Validation of Analytical Procedures: Text and methodology*, Q2 (R1), p. 1-13 (2005).

Kuntic, V., Pejic, N., Micic, S., Vukojevic, V., Vujic, Z. & Malecev, D. (2005). Determination of the stability constants of the quercetin titanyloxalato complex. *J. Serb. Chem. Soc*., 70(5), 753–763.

Samet, M. & Wages, P. (2018). Oxidative stress from environmental exposures. *Current Opinion in Toxicology*, 7, 60-66.

Tejada, S., Nabavi, S., Capo, X., Martorell, M., del Mar Bibiloni, M., Tur, J., Pons, A. & Sureda, A. (2017). Quercetin effects on exercise induced oxidative stress and inflammation. *Current Organic Chemistry*, 21(4), 348-356.