Modelling and optimization of quercetin extraction and biological activity of quercetin-rich red onion skin extract from Southeastern Serbia

IVANA M. SAVIC-GAJIC – IVAN M. SAVIC – VESNA D. NIKOLIC

Summary

Quercetin is a bioactive compound that has the beneficial effect on human health because of its antioxidant, anti-inflammatory, antimicrobial, antiviral, antiallergic, cardioprotective, vasodilatatory and anticancer activity. Due to high content of quercetin in the red onion skin, the aim of this study was modelling and optimization of quercetin extraction using central composite design. The extraction time (5–60 min), ethanol concentration (5–100%) and liquid-to-solid ratio (10–80 $cm^3 \cdot g^{-1}$) were varied to estimate their effect on the yield of quercetin. The optimal extraction conditions were obtained for 47.3 min using 80% ethanol adjusted to pH 1.0 at a liquid-to-solid ratio of 63.9 $cm^3 \cdot g^{-1}$. In the extract obtained under these conditions, the quercetin content was found to be 28.5 $g \cdot kg^{-1}$ of the dry plant material, while the content of total flavonoids and polyphenols was found to be 30.1 g rutin equivalent per kilogram of the dried extract and 53.82 g gallic acid equivalent per kilogram of the dried extract, respectively. The studies of the antioxidant activity indicated that quercetin had a higher activity than the extract enriched with quercetin. Quercetin was also isolated from the extract by the liquid-liquid extraction technique and structurally characterized using instrumental methods.

Keywords

central composite design; total flavonoid; total polyphenol; antioxidant activity

Red onion (*Allium cepa* L.) is a biennial herbaceous originating from the territory of western and central Asia [1]. In recent years, the production of onion has increased around the world at least by 25 %, reflecting its medicinal and nutritive value. According to FAOSTAT database (Food and Agriculture Organization, Rome, Italy), production of dried onions was around 88 million tons in 2014. In the European Union, 500 000 t of onion waste is produced annually (comprising stalk, skin, small and damaged onions), which represents an ecological problem [2]. The onion waste is disposed because it is not suitable to be use as animal feed. However, onion skin can be used as a source of dietary fibre and phenolic compounds. Also, the skin has a high content of quercetin, which is a strong antioxidant [3]. Having in mind that onions (onion, red onion, garlic) and their skins are a good source of various bioactive compounds, extraction procedures of quercetin and its glycosides from these plant materials were intensively developed and optimized [4–6]. In the literature, conventional solvent extraction [7], ultrasound-assisted extraction (UAE) [5] and microwave-assisted extraction (MAEx) [8] are commonly used for quercetin extraction from the plant materials. Various solvents, such as methanol, ethanol, ethyl acetate and dimethylformamide, were used to estimate their effect on the quercetin yield [7]. Supercritical extraction using water was also developed for quercetin extraction from onion skin, as an alternative to extraction by organic solvents [9]. Given that quercetin exists in the glycoside and aglycone forms, extraction needs to be performed in the presence of a mineral acid (e.g. hydrochloric acid) to assure hydrolysis of the glycoside bonds.

Antioxidant activity of the onion skin extracts was commonly determined using 2,2-diphenyl-1-picrylhydrazyl (DPPH) assay. Lee et al. [10] investigated the antioxidant activity of the extracts

Ivana M. Savic-Gajic, Ivan M. Savic, Vesna D. Nikolic, Department of Organic-technological Sciences, Faculty of Technology, University of Nis, Bulevar oslobodjenja 124, 16000 Leskovac, Serbia.

Correspondence author:
Ivana M. Savic-Gajic, tel.: +381 16 247 203; fax: +381 16 242 859, e-mail: vana.savic@yahoo.com

obtained by ethanol, hot water and by subcritical water extraction. The authors reported that the antioxidant activity of ethanol extract was the highest compared to the aqueous extract and subcritical aqueous extract at 165 °C. However, the extract obtained by subcritical water extraction at 110 °C had a similar antioxidant activity as the ethanol extract. Due to a high antioxidant activity [11], onion skin has already found application as a dietary supplement to improve the organoleptic quality and stability, as well as to extend the shelf-life of food [12].

The traditional optimization method (one-factor-at-a-time, OVAT) requires considerable amount of time and resources. This method investigates the effect of one factor at a time, while other factors are kept at a constant value. Using this approach, the analysis of interactions between independent variables is not possible. This limitation of OVAT method can be overcome using the Central Composite Design (CCD). CCD is an effective statistical technique suitable for optimization of various processes, characterized by a reduced number of experimental trials. making use of this benefit, the extraction process of bioactive compounds was optimized using CCD.

The objective of this study was to model and optimize the conditions for quercetin extraction from red onion skin (*Allium cepa* L.) using CCD. The extract was analysed to evaluate the antioxidative activity and to determine the content of total flavonoids and polyphenols. Quercetin was also isolated from the extract using liquid-liquid extraction technique and structurally characterized using ultraviolet–visible spectroscopy (UV-Vis) and Fourier transform infrared (FTIR) spectroscopy.

MATERIALS AND METHODS

Reagents and chemicals

Quercetin standard (Merck, Darmstadt, Germany), rutin trihydrate standard (purity 97%; Alfa Aesar–Johnson Matthey, Heysham, United Kingdom), aluminium(III)-cloride, potassium acetate, 2,2-diphenyl-1-picrylhydrazyl (DPPH), butyl hydroxy toluene (BHT), potassium bromide spectroscopic purity (Sigma Aldrich, St. Louis, Missouri, USA), Folin-Ciocalteu reagent (AppliChem, Darmstadt, Germany), gallic acid (purity > 97.5%), sodium carbonate, 96% ethanol (MosLab, Belgrade, Serbia), absolute ethanol (Alkaloid, Skopje, Macedonia), methanol HPLC grade (Avantor Performance Materials, Deventer, Netherlands) were used in this study. All other reagents were of analytical grade.

Plant material

Red onion (*Allium cepa* L.) skin was grown in Leskovac district in Southeastern Serbia (coordinates: N 42°58'25" E 22°04'31"). The skin was dried at room temperature in the dark for 7 days to the moisture content of 15 %. This value is in accordance with the requests of international Pharmacopoeias [13]. When dried under these conditions, the organoleptic characteristics of plant material are not significantly changed. Before standard extraction procedure, the plant material was cut in order to increase the surface of contact with the solvent.

Procedure for quercetin extraction

The cut plant material (1 g) was transferred to the flask of 100 cm^3 and treated with acidified ethanol solution (pH 1.0) in order to hydrolyse the glycoside bonds between quercetin aglycone and carbohydrate residue in the molecule of quercetin glycoside. The extraction of quercetin was performed under reflux at the boiling point of the solvent. A constant temperature was maintained using water bath. After extraction, the solid matrix was separated from liquid phase by vacuum filtration using Büchner funnel. About 2 cm^3 of the extract was dried in a laboratory dryer at 105 °C to the constant mass of samples in order to define the total extractive content. The content of quercetin in the extracts was determined by high-performance liquid chromatography (HPLC) as described previously [14].

In this paper, the extraction time, ethanol concentration and liquid-to-solid ratio were defined as the independent variables of CCD. The CCD method consisted of the experimental trials, which belonged to the factor design, and the sets of the axial and center points. These points actually corresponded to the exactly defined combination of the process parameters. The analysis of each independent variable at two levels was possible based on the application of the factor design, while the center point considered the combination of mean levels of the process parameters of the factor design. The axial points were almost identical with the center point, except that one factor varied above and below the mean level of the factorial design. The levels of process parameters were coded according to Eq. 1 and they are presented in Tab. 1.

$$X_i = \frac{x_i - x_0}{\Delta x_i} \quad (1)$$

where X_i are the coded values (–1.68, –1, 0, +1, +1.68) of the independent variables, x_i are the actual values of the process parameters, x_0 is the

Tab. 1. Actual and coded levels of process parameters for quercetin extraction from red onion skin.

Process parameters	Uncoded variable	Coded variable	levels				
			–1.68	–1	0	+1	+1.68
Extraction time [min]	τ	X_1	5.0	16.2	32.5	48.9	60.0
Ethanol concentration [%]	C_e	X_2	5.0	24.3	52.5	80.7	100.0
Liquid-to-solid ratio [$cm^3 \cdot g^{-1}$]	ω	X_3	10.0	24.2	45.0	65.8	80.0

actual value of the center point, Δx_i is the step of change.

For modelling of process using CCD, it is necessary to perform $2^f + 2f + C$ experiments, where f is the number of process parameters and C is the number of repetitions of the center point. In this paper, the effect of three process parameters on the yield of quercetin was analysed, while the number of repetitions of the center point was 6. In order to model the extraction procedure using this approach, it was enough to perform 20 experiments. The repetition of the center point is performed to obtain the statistical parameters of the proposed model.

After determination of the content of quercetin in the extracts, the data were modelled using a second order polynomial equation. A general equation of the second order polynomial model can be presented in the following way (Eq. 2):

$$Y = a_0 + a_1x_1 + a_2x_2 + a_3x_3 + \\ + a_{11}{x_1}^2 + a_{22}{x_2}^2 + a_{33}{x_3}^2 + \\ + a_{12}x_1x_2 + a_{13}x_1x_3 + a_{23}x_2x_3 + \varepsilon \quad (2)$$

where x_1, x_2, x_3 are independent variables; a_0 is intercept; a_1, a_2, a_3, a_{11}, a_{22}, a_{33}, a_{12}, a_{13}, a_{23} are regression coeficients, Y is response and ε is residual term.

Statistical analysis

The statistical analysis, i.e. modelling and optimization of the extraction procedure, was done using the software packages Design Expert 7.0.0 (Stat Ease, Minneapolis, Minnesota, USA) and Statistica 12.0 (StatSoft, Tulsa, Oklahoma, USA). The experimental data were analysed by multiple regression analysis using the method of least squares. The regression coefficients in the polynomial equation, as well as the effect of process parameters on the quercetin yield, were analysed using analysis of variance (ANOVA). The terms of the polynomial equation were analysed and statistically verified using F-distribution ($p < 0.05$) at a 95% confidence level. Adequacy of the model was estimated based on the calculation of the coefficient of determination (R^2), the adjusted correlation coefficient (R^2_{adj}) and the predicted correlation coefficient (R^2_{pre}). After fitting the data, three-dimensional diagrams were constructed for easier observation of the effect of independent variables on the defined response of system.

The root mean square error (*RMSE*) was used as a measure of CCD model error, which was calculated as follows (Eq. 3).

$$RMSE = \sqrt{\frac{\left(y_i^p - y_i^m\right)^2}{n}} \quad (3)$$

where y_i^p is experimental value, y_i^m is predicted value and n is the number of experiments.

The mean squared error (*MSE*) was calculated according to Eq. 4:

$$MSE = \frac{\sum\left(y_i^p - y_i^m\right)^2}{n} \quad (4)$$

The mean absolute error (*MAE*), which represents the mean value of the subtraction between the predicted and experimental values, was obtained using Eq. 5:

$$MAE = \frac{\left|y_i^p - y_i^m\right|}{n} \quad (5)$$

Optimization of quercetin extraction

Derringer's desirability function in the Design–Expert software (Stat-Ease, Minneapolis, Minnesota, USA) was used for definition of the optimal conditions for quercetin extraction from red onion skin. When using this function, the general approach is to convert the response (Y_i) into a dimensionless partial desirability function (d_i) using Eq. 6 [15]:

$$d_i = h_n(Y_i) \quad (6)$$

The various desirability functions d_i were used depending on whether a response of the system (Y_i) had to be maximized or minimized. Maximization of the response depends on the partial desirability function, which depends on the exponent s value. When the value of exponent s is 1,

the desirability function increases linearly toward T_i representing the sufficiently large value of the response. The function is convex for $s < 1$, while the function is concave for $s > 1$ (Eq. 7):

$$d_i(Y_i) = \begin{cases} 0 & Y_i(x) < L_i \\ \left(\frac{Y_i(x)-L_i}{T_i-L_i}\right)^s & L_i \leq Y_i(x) \leq T_i \\ 1 & Y_i(x) > L_i \end{cases} \quad (7)$$

where L_i is the lower value, U_i is the upper value and T_i is the target value obtained for the response, respectively.

If the response of the system should be reduced to the minimum value, then its partial desirability function is marked as T_i and has a sufficiently small value of the response (Eq. 8):

$$d_i(Y_i) = \begin{cases} 1 & Y_i(x) < U_i \\ \left(\frac{Y_i(x)-U_i}{T_i-U_i}\right)^s & T_i \leq Y_i(x) \leq U_i \\ 0 & Y_i(x) > U_i \end{cases} \quad (8)$$

where U_i is the upper value of the response.

The quercetin content in the extracts of red onion skin was maximized in this study.

Validation of the optimal conditions and proposed models

The developed model equation for prediction of quercetin content in the extracts of red onion skin was tested at the optimal conditions. In order to verify the adequacy of the model, the experimental value of quercetin yield was compared with the predicted value.

Determination of total flavonoid content

The content of total flavonoid in the optimal extract was determined using spectrophotometric method with aluminium(III) chloride [16]. The amount of total flavonoid was expressed as grams of rutin equivalent (RE) per kilogram of the dried extract. A series of rutin solutions was prepared in the concentration range of 1–100 μg·cm^{-3} by dilution of the stock solution (100 μg·cm^{-3} in 96% ethanol). The samples were prepared by addition of 1.5 cm^3 of ethanol (96%), 0.1 cm^3 of aluminium(III)-chloride (10%, w/w), 0.1 cm^3 of potassium acetate (1 mol·dm^{-3}) and 2.8 cm^3 of distilled water to 0.5 cm^3 of the solution of rutin standard. Instead of aluminium chloride, the blank solution contained an equivalent amount of distilled water. After construction of the calibration curve, the complex between aluminium(III)-chloride and the extract was also prepared by taking 0.5 cm^3 of the solution of the extract. The samples were incubated at room temperature in quartz cuvettes (1 cm × 1 cm base) for 30 min. The absorbance was measured at 415 nm in a Varian Cary 100 spectrophotometer (Mulgrave, Victoria, Australia).

Determination of the total polyphenolic content

The total polyphenolic content in the optimal extract was determined according to Folin-Ciocalteu's method [17]. The amount of total polyphenols was expressed as grams of gallic acid equivalent (GAE) per kilogram of the dried extract. A series of solutions for determination of gallic acid was prepared in the concentration range of 5–50 μg·cm^{-3} from the stock solution (1 mg·cm^{-3} prepared in 96% ethanol). The samples were obtained by mixing 0.25 cm^3 of the sample solution, 2.25 cm^3 redistiled water, 0.25 cm^3 Folin-Ciocalteu's reagent and 2.5 cm^3 sodium carbonate (7%, w/w). The absorbance was measured at 740 nm after incubation of 30 min. The recordings were performed on the Varian Cary 100 spectrophotometer in the quartz cuvettes (1 cm × 1 cm base).

Determination of antioxidant activity

Antioxidant activity of the optimal extract enriched with quercetin was determined using DPPH assay. The percentage of inhibition of DPPH radicals can be defined based on the change in the absorbance at 517 nm. The stock solution of BHT (250 μg·cm^{-3}), quercetin (200 μg·cm^{-3}) and the extract were diluted in order to prepare a series of the solutions. The samples (2.5 cm^3) were treated with 1 cm^3 of the solution of DPPH radicals (3×10^{-4} mol·dm^{-3}), and then incubated in the dark at laboratory temperature for 30 min. The absorbance was measured at 517 nm in relation to 96% ethanol. The control solution was obtained by dilution of the ethanolic solution of DPPH radicals (1 cm^3) with 96% ethanol (2.5 cm^3). The inhibition of DPPH radicals was caluculated using Eq. 9 [18]:

$$I = \frac{A_C - (A_S - A_B)}{A_C} \quad (9)$$

where I is inhibition of DPPH radicals (in percent), A_S is the absorbance of the samples treated with DPPH radicals, A_B is the absorbance of the samples without addition of DPPH radicals, A_C is the absorbance of the control solution.

Isolation of quercetin

The extract obtained under optimal conditions for quercetin extraction (150 cm^3) was evaporated to dryness under vacuum by a rotatory evaporator at 60 °C. After that, the solid extract was dissolved in 100 cm^3 of boiling distilled water under reflux. In order to isolate quercetin from the complex mixture, thus obtained aqueous

extract was washed with four portion of diethyl ether (50 cm^3) in a separatory funnel. The upper layer of the organic phase was separated from the aqueous phase, and then it was evaporated under typical atmospheric conditions in order to remove diethyl ether. The native quercetin was pre-crystallized from distilled water (10 cm^3). The undissolved residue was filtered through a cellulose membrane filter with the pore size of 0.45 μm (Econofilters, Agilent Technologies, Santa Clara, California, USA). The filtrate was kept in a refrigerator at 4 °C, and the obtained yellow crystals were separated from water by filtration. The crystals of quercetin were dried in a desiccator to the constant mass and structurally characterized using instrumental methods.

Structural characterization of isolated quercetin

The samples of standard and isolated quercetin were dissolved in methanol and adjusted to the concentration of 5 $\mu g \cdot cm^{-3}$. The UV-Vis spectra were recorded in the Varian Cary 100 spectrophotometer at laboratory temperature in quartz cells (1 cm × 1 cm × 4.5 cm) using methanol as a blank solution.

FTIR spectra of the standard and isolated quercetin were recorded in the range of 4000–400 cm^{-1} at the resolution of 2 cm^{-1} in Bomem MB-100 FTIR spectrometer (Hartmann and Braun, Quebec, Canada). The apparatus was equipped with a standard DTGS/KBr detector (Hartmann and Braun). Homogenization of the sample (1 mg) and potassium bromide (150 mg) was performed in a mortar. After that, the mixture was pressured under vacuum in order to obtain transparent KBr pellets.

RESULTS AND DISCUSSION

Modelling of quercetin extraction

The quercetin content in the plant extracts was defined using the validated HPLC method. The calibration curve of quercetin (Eq. 10):

$$A_{370} = 87.84 C_Q - 53.83 \quad (R^2 = 0.996) \qquad (10)$$

(where A_{370} is the absorbance at 370 nm, C_Q is the concentration of quercetin expressed in micrograms per volume centimeters) was linear in the concentration range of 5–150 $\mu g \cdot cm^{-3}$. The obtained coefficient of determination indicated that 99.6 % of the variation in the peak area of quercetin should be explained using the regression model. Limit of detection (*LOD*) and limit of quantification (*LOQ*) were found to be 5.02 $\mu g \cdot cm^{-3}$ and 15.21 $\mu g \cdot cm^{-3}$, respectively.

Extraction of quercetin from red onion skin was performed under reflux at the boiling point of the used solvent, because this bioactive compound is well soluble in boiling ethanol solution [19]. Given that the extraction process depends on a great number of process parameters, the effect of extraction time, ethanol concentration and liquid-to-solid ratio was investigated. CCD was used as a mathematical approach for process modelling, because the optimal conditions are possible to be obtained after a lower number of the performed experiments compared to the OVAT method. In this way, the consumption of all available resources was reduced. The main disadvantage of the conventional method (OVAT) is optimization of one variable at a time, while all other parameters are constant. Another serious drawback of OVAT approach is obtaining of local optimal conditions, which are different than global optimal conditions. Using CCD, a great number of the process parameters are optimized simultaneously. Also, interactions between process parameters can be considered using this advanced mathematical model. In order to optimize the three extraction parameters using CCD method, 20 experiments are necessary to be performed. The combination

Tab. 2. Experimental runs for mathematical modelling of the extraction process of quercetin from red onion skin according to central composite design model.

Std	τ [min]	C_e [%]	ω [$cm^3 \cdot g^{-1}$]	Y_{exp}	Y_{pred}
				[$g \cdot kg^{-1}$]	
5	16.1	24.3	65.8	9.7	9.8
10	60.0	52.5	45.0	21.4	20.5
8	48.9	80.7	65.8	28.5	29.2
2	48.9	24.3	24.2	8.7	9.0
17	32.5	52.5	45.0	17.5	17.4
19	32.5	52.5	45.0	16.8	17.4
15	32.5	52.5	45.0	16.5	17.4
1	16.1	24.3	24.2	6.5	6.4
12	32.5	100.0	45.0	25.6	25.0
18	32.5	52.5	45.0	18.0	17.4
20	32.5	52.5	45.0	17.8	17.4
14	32.5	52.5	80.0	20.3	19.6
9	5.0	52.5	45.0	9.9	9.9
16	32.5	52.5	45.0	17.7	17.4
4	48.9	80.7	24.2	22.6	23.1
6	48.9	24.3	65.8	11.6	12.1
13	32.5	52.5	10.0	11.8	11.6
7	16.1	80.7	65.8	18.9	19.2
11	32.5	5.0	45.0	5.5	5.3
3	16.1	80.7	24.2	12.6	12.7

Std – standard order, τ – extraction time, C_e – ethanol concentration, ω – liquid-to-solid ratio, Y_{exp} – experimental value of quercetin yield, Y_{pred} – predicted value of quercetin yield.

Tab. 3. ANOVA test for the second-order polynomial model.

	SS	*df*	*MS*	*F*-value	*p*-value
Model	735.65	9	81.74	174.6	< 0.0001
X_1	135.65	1	135.65	289.7	< 0.0001
X_2	467.51	1	467.51	998.5	< 0.0001
X_3	77.80	1	77.80	166.1	< 0.0001
X_1X_2	30.03	1	30.03	64.1	< 0.0001
X_1X_3	0.06	1	0.06	0.1	0.7251
X_2X_3	4.65	1	4.65	9.9	0.0103
X_{12}	8.63	1	8.63	18.4	0.0016
X_{22}	9.44	1	9.44	20.2	0.0012
X_{32}	5.77	1	5.77	12.3	0.0056
Residual	4.68	10	0.47		
Lack-of-fit	2.89	5	0.58	1.6	0.3051
Pure error	1.79	5	0.36		

X_1 – extraction time, X_2 – ethanol concentration, X_3 – liquid-to-solid ratio, Lack-of-fit – model error, Pure error – experimental error, *SS* – sum of squares, *df* – degree of freedom, *MS* – mean sum of squares.

of the levels of the extraction parameters are given in Tab. 2 according to the CCD matrix. Otherwise, a random order of experiments was generated by the software. The quercetin yield was in the range of 5.5–28.5 g·kg^{-1} of the dried plant material (dpm) for these extraction conditions. The obtained values were fitted using a second-order polynomial equation.

The significance of equation terms can be estimated based on *F*-distribution (*F*-test) and *p*-value. The terms were considered significant when the *p*-value was lower than 0.05. The sum of squares (*SS*), degree of freedom (*df*), mean sum of squares (*MS*), *F*-value and *p*-value are given in Tab. 3. Mathematical relations that are used

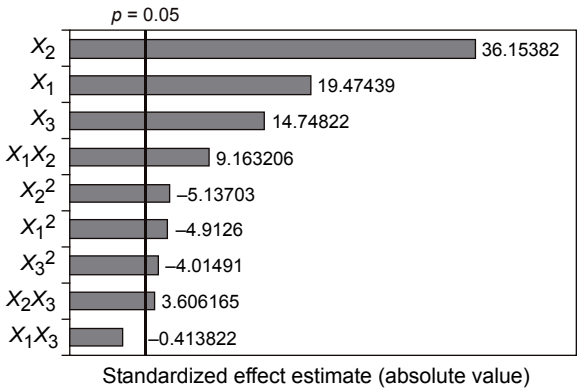

Fig. 1. Pareto chart of the standardized effects.

X_1 – extraction time, X_2 – ethanol concentration, X_3 – liquid-to-solid ratio.

at ANOVA test (*SS, MS, F*-value, R^2, R^2_{adj}) are commonly applied in the literature [20, 21]. The *F*-value of lack-of-fit (1.6) was not significant relative to the pure error (1.79), because its value was lower than the critical *F*-value of 5.05. There was a 30.51% chance that this *F*-value occured, due to noise. The predicted determination of coefficient $R^2_{pred} = 0.967$ is in good agreement with the adjusted determination of coefficient $R^2_{adj} = 0.988$. The adequate precision that defined the signal-to-noise ratio was found to be 49.43. It is desirable that this value should be greater than 4, which was the case in this study. The *MSE* value for the proposed model was 0.234, while the *RMSE* value was 0.484. The calculated *MAE* for the regression model was 0.406. These parameters of error should be lower during the generation of the model.

Based on the obtained statistical parameters, it can be concluded that the proposed model can predict the quercetin yield without a significant deviation from the actual value. In other words, this model can be considered as adequate for modelling of quercetin extraction from red onion skin.

Pareto chart of the standardized effects, generated by the Statistica 12.0 software, is presented in Fig. 1. Based on this chart, it can be easier to note the most important factor and interaction effects. Each term is significant in the polynomial equation, if its value is greater than the reference line ($p < 0.05$). The higher absolute value of the standardized effect indicates the higher effect on the system response. As can be seen, the linear terms of the polynomial equation have the greatest impact on the quercetin yield. Ethanol concentration has the greatest impact compared to other extraction parameters. Also, the impact of extraction time was almost twice lower than that of the ethanol concentration. The smallest effect had the liquid-to-solid ratio compared to all other linear terms of the equation. The quadratic and interaction effects were also statistically significant terms, except for the interaction between the extraction time and liquid-to-solid ratio.

The polynomial equation, which describes the procedure of quercetin extraction from red onion skin, can be presented in the form of the coded variables as follows (Eq. 11):

$$Y = 17.41 + 3.15X_1 + 5.85X_2 + \\ + 2.39X_3 + 1.94X_1X_2 \quad (11)$$

This model, converted into the empirical model with the actual variables, can be presented in the following way (Eq. 12):

$$Y = -3.86 + 0.17\tau + 0.12C_e + \\ + 0.19\omega + 4.20 \times 10^{-3}\tau C_e + \\ + 1.30 \times 10^{-3}C_e\omega - 2.90 \times 10^{-3}\tau^2 - \\ - 1.01 \times 10^{-3}{C_e}^2 - 1.46 \times 10^{-3}\omega^2 \quad (12)$$

Only the statistically significant terms determined by analysis of variance (ANOVA test) are given in the equations. The negative sign of regression coefficient in the polynomial equation indicates that this term has a negative effect on the quercetin yield. The effect of a term of the polynomial equation is higher with increasing the absolute value of the regression coefficient.

The normal probability plot of residuals is presented in Fig. 2. The obtained functional dependency indicates that the residues have a normal (or Gaussian) distribution, since the plot follows a straight line with a minimum deviation. This behaviour confirms the adequacy of the proposed model for the design space.

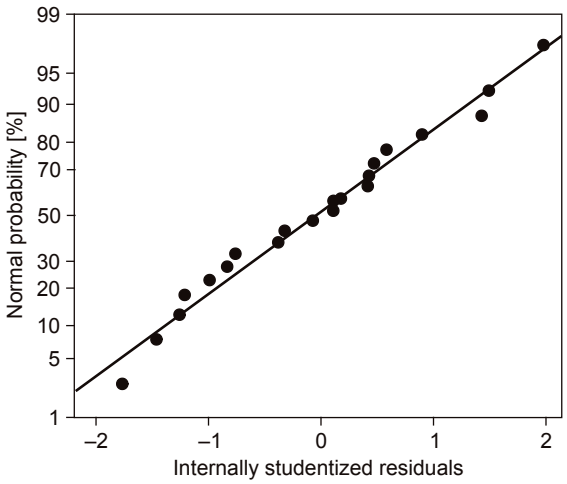

Fig. 2. Normal probability plot of the standardized residuals.

Three-dimensional diagrams are of significant importance for consideration of the effects of the process parameters on the system response. The three-dimensional diagrams, representing the interaction effect between the process parameters, were generated in Statistica 12.0 software. The effect of extraction time and ethanol concentration on the quercetin yield at the liquid-to-solid ratio of 45 $cm^3 \cdot g^{-1}$ is shown in Fig. 3A. It can be seen that the quercetin yield was higher for longer extraction times and higher ethanol concentrations. The effect of extraction time was especially evident at ethanol concentrations higher than 60 %. The area of the highest quercetin yield was noticed for the extraction times longer than 35 min using ethanol concentrations higher than 60 %. This behaviour was expected because quercetin is a bioactive compound well soluble in alcohol [22].

The impact of extraction time and liquid-to-solid ratio using 52.5% ethanol is shown in Fig. 3B. The quercetin content in the extract increased with increasing the extraction time and liquid-to-solid ratio. Based on the ANOVA test, the interaction between these process parameters was presented as a statistically insignificant term of the polynomial equation. This justified omission of this statistically insignificant term from the polynomial equation in order to improve the prediction ability of the model. The impact of this interaction on the quercetin content in the red onion skin extract was insignificant. The surface shape indicates that a strong interaction exists between the observed process parameters. The trend of increasing the quercetin yield with increasing the liquid-to-solid ratio is almost identical for shorter and longer extraction times. Otherwise, the impact of extraction time on the quercetin yield was similar at the lower and higher liquid-to-solid ratios. In this study, the higher liquid-to-solid ratios were used in order to improve better soaking the plant material. Saturation in the increase of quercetin content was achieved at the extraction time of 50 min and liquid-to-solid ratio of 60 $cm^3 \cdot g^{-1}$.

The three-dimensional response surface in Fig. 3C illustrates the interaction effects of ethanol concentration and liquid-to-solid ratio on the extraction yield of quercetin when the extraction time is 32.5 min. The effect of ethanol concentration was expressed at liquid-to-solid ratios higher than 50 $cm^3 \cdot g^{-1}$. The change of liquid-to-solid ratio had no significant effect at lower ethanol concentrations. This influence was more pronounced for ethanol solutions with concentrations greater than 80 %.

Optimization of the procedure for quercetin extraction

The procedure for quercetin extraction from red onion skin was optimized using numerical optimization method. The obtained optimal conditions for quercetin extraction were: extraction time of 47.3 min, 80% ethanol acidified by hydrochloric acid to pH 1.0, and liquid-to-solid ratio of 63.9 $cm^3 \cdot g^{-1}$. The quercetin content in the optimal extract predicted by CCD model was found to be 28.5 $g \cdot kg^{-1}$ dpm, while the experimental value was 29.0 $g \cdot kg^{-1}$ dpm. The model error during prediction of quercetin yield under these conditions was 1.7 %. Agreement between the predicted and experimental values of the response indicated a good

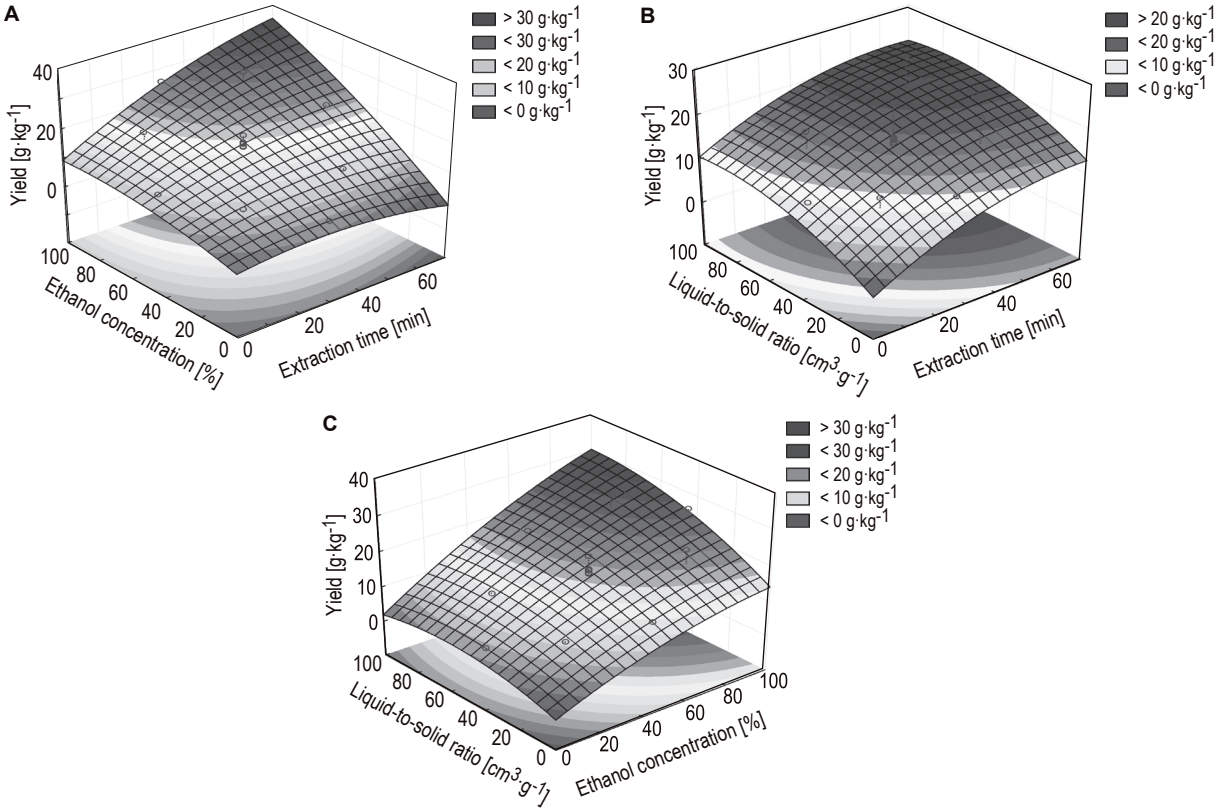

Fig. 3. Effect of extraction parameters on quercetin yield.

A – interaction between extraction time and ethanol concentration at the liquid-to-solid ratio of 45 $cm^3 \cdot g^{-1}$, B – interaction between extraction time and liquid-to-solid ratio using 52.5% ethanol, C – interaction between ethanol concentration and liquid-to-solid ratio for 32.5 min of extraction.
Quercetin yield is expressed per kilogram of the dried plant material.

ability of the model to predict the quercetin yield for the given experimental conditions.

Jin et al. [4] optimized various procedures (conventional solvent extraction, UAE and MAEx) for quercetin extraction from red onion skin using response surface methodology (RSM). The highest quercetin yield was obtained under the following extraction conditions: the extraction time of 16.5 min, the temperature of 59.2 °C and 59.3% ethanol for the conventional solvent extraction; the extraction time of 117 s, 69.7% ethanol in MAEx; the extraction time of 21.7 min, the power of 606.4 W and 43.8% ethanol for UAE. The most productive extraction procedure for quercetin extraction was the MAEx with the yield of 4.84 $g \cdot kg^{-1}$, while the slightly lower yield was achieved at the ultrasonic-assisted (3.76 $g \cdot kg^{-1}$) and conventional solvent extraction (3.42 $g \cdot kg^{-1}$). Jang et al. [5] also optimized the procedure of UAE of quercetin from the solid waste of onion using ethanol solution. They showed that the ethanol concentration (40–80 %) and extraction temperature (40–60 °C) had the highest effect on the quercetin yield, while the effects of pH (2–6) and extraction time (15–35 min) were significantly lower. Under the optimal extraction temperature of 49 °C using 59% ethanol, the quercetin yield was found to be 11.08 $g \cdot kg^{-1}$ with the solid waste of onion.

Comparing the literature data with the obtained quercetin yield, it can be concluded that the acidified ethanol solution and red onion skin are the right choice for the extraction of this bioactive compound. In this study, the quercetin yield was several fold higher than the data obtained in other available studies. The main contribution of this paper is the simple extraction procedure and the use of the plant material, which has a higher content of quercetin.

Determination of total flavonoids and polyphenols

The calibration curve of rutin was constructed in order to determine the content of total flavonoid in the extract of red onion skin. The equation

of calibration curve (Eq. 13):

$$A_{415} = 0.008C_R + 0.07 \ (R^2 = 0.999) \qquad (13)$$

(where A_{415} is the absorbance at 415 nm expressed in absorption units, C_R is the concentration of rutin expressed in micrograms per volume centimetres) was linear in the concentration range of 1–100 μg·cm^{-3}. *LOD* for this method was found to be 1.89 μg·cm^{-3}, while *LOQ* was found to be 5.71 μg·cm^{-3}. The total flavonoid content of 30.1 g·kg^{-1} with the dried extract (expressed as RE) was determined using this calibration curve.

The equation of calibration curve for gallic acid (Eq. 14):

$$A_{740} = 0.05C_{GA} - 0.12 \ (R^2 = 0.997) \qquad (14)$$

(where A_{740} is the absorbance at 740 nm, C_{GA} is the concentration of gallic acid expressed in micrograms per volume centimetres) was used for determination of the polyphenol content in the extract. *LOD* was 2.21 μg·cm^{-3}, while *LOQ* was 7.36 μg·cm^{-3}. A linear calibration curve for gallic acid was obtained in the concentration range of 5–50 μg·cm^{-3}. The determined content of polyphenols was 53.82 g·kg^{-1} with the dried extract (expressed as GAE). Makris and Kefalas [23] confirmed that the extraction of polyphenols from onion solid wastes follows the second order kinetics. Also, the yield of total polyphenols was 21.10 g·kg^{-1} the dry weight in the extract obtained using 0.1% HCl in 60% (v/v) aqueous ethanol at 40 °C. Based on this results, it can be concluded that the polyphenolic content in the dried extract obtained in this paper was higher compared with the literature data.

Determination of antixidant activity

The antioxidant activity of the quercetin standard, synthetic antioxidant BHT and extract obtained under the optimal conditions was deteremined using DPPH assay. The functional dependency between the inhibition of free DPPH radicals and concentration of the samples is presented in Fig. 4.

The investigation of antioxidant activity of BHT was done in the concentration range of 0.25–0.0078 mg·cm^{-3}. The half-maximal inhibitory concentration (IC_{50}) value of 36.6 μg·cm^{-3} for this substance was determined by interpolation. Ethanol solutions of the red onion skin extract were prepared in the concentration range of 6.1–98.4 μg·cm^{-3}, and the calculated IC_{50} value was found to be 24.6 μg·cm^{-3}. The activity of quercetin was investigated in the concentration range of 0.003–0.200 mg·cm^{-3}, while its IC_{50} value was 10.4 mg·cm^{-3}. The lower IC_{50} value of the investigated sample indicated the better ability to "scavenge" free radicals or the better antioxidant activity. The IC_{50} value was the lowest for quercetin standard compared to other samples. The extract of red onion skin had a slightly lower activity. The concentration necessary to inhibit 50% of the initial concentration of free DPPH radicals was almost two times lower than the quercetin concentration. The lowest antioxidant activity had the synthetic antioxidant BHT. The highest percentage of inhibition of 80 % was achieved at the BHT concentration of 0.12 mg·cm^{-3}. The percentage of DPPH radicals inhibition of 93 % was the highest for quercetin standard at the concentration of 0.05 mg·cm^{-3}. The total inhibition of DPPH radicals was achieved using the extract at the concentration of 0.1 mg·cm^{-3}.

The ability of quercetin to "scavenge" the free DPPH radicals can be attributed to: (1) ortho-catechol group in the B ring, which provides a high stability of the formed radicals, (2) conjugation of the B ring to the 4-oxo group via 2,3-double bond, which provides delocalization of the electrons and (3) the hydrogen bonds between the 3- and 5-OH groups and the 4-oxo group, which provide delocalization from 4-oxo group to both substituents. These functional groups provide better delocalization of the electrons, and therefore provide a higher stability of aroxyl radicals.

Structural characterization of isolated quercetin

The isolated quercetin was structurally characterized using UV-Vis and FTIR spectroscopy methods. The maximum absorption for both samples was determined in the ranges 210–220 nm, 250–255 nm and 360–370 nm. The absorption between 210–220 nm (primary band) and 250–255 nm (secondary band) originated from

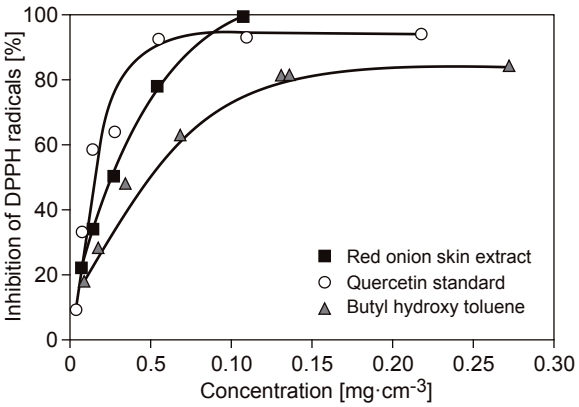

Fig. 4. Antioxidant activity of the red onion skin extract, quercetin standard and butyl hydroxy toluene.

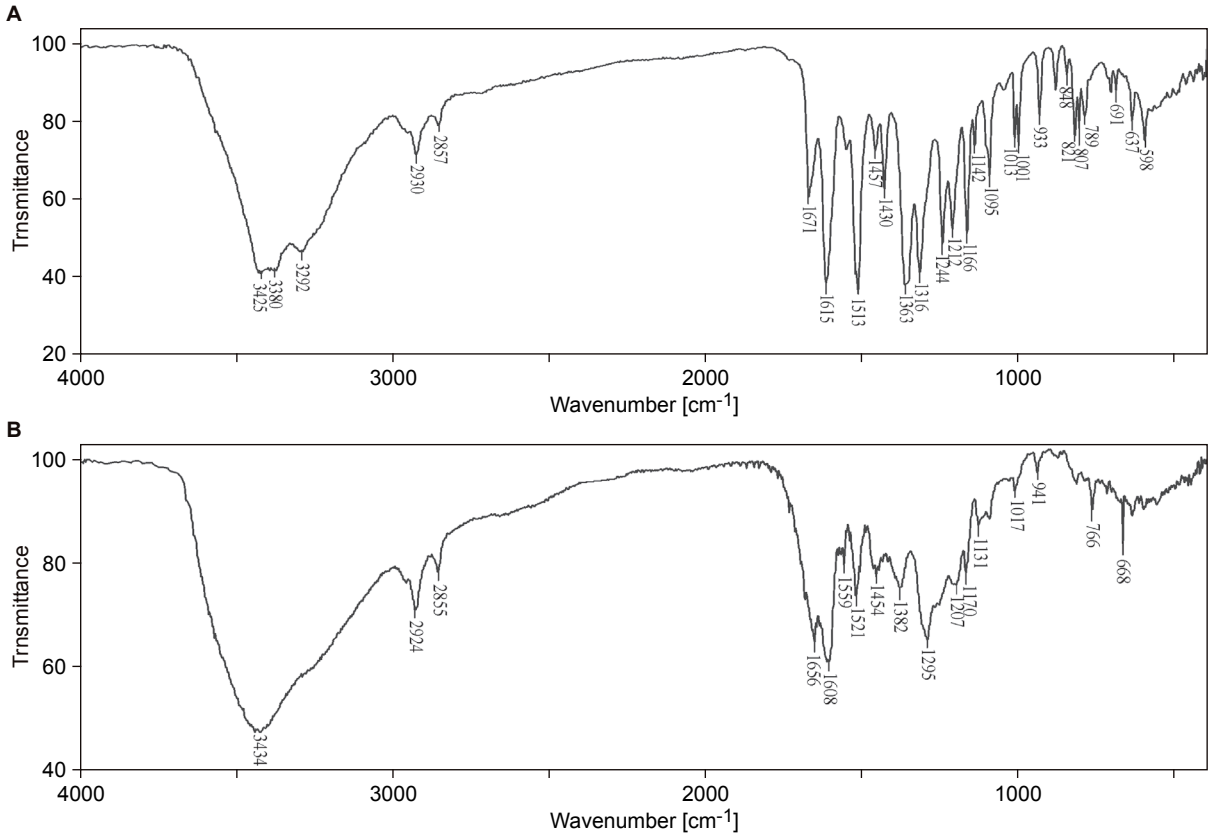

Fig. 5. Fourier transform infrared spectra of standard and isolated quercetin.

A – standard, B – isolated quercetin.

forbidden $\pi \rightarrow \pi^*$ transitions. The $n \rightarrow \pi^*$ transition in the structure of quercetin was also noticed between 360 nm and 370 nm. The obtained results indicated that these spectra are in good agreement with each other.

The FTIR spectrum of quercetin standard is presented in Fig. 5A. The broad bands at 3425 cm^{-1}, 3380 cm^{-1} and 3292 cm^{-1} originate from the valence vibrations ν(O-H). The intensive band at 1671 cm^{-1} is the result of valence vibration of free ketone group (C=O) [24]. The band at 1615 cm^{-1} belongs to the ν(C=O) vibration. This group is included in the formation of hydrogen bonds with OH groups in the position 3 and 5, which results in the frequency decrease of C=O and OH valence vibrations. Due to the formation of hydrogen bonds, quercetin has two forms in the solid state. The structure of quercetin with intra hydrogen bonds is more abundant than the structure with the free ketone group. In the spectrum, bands of valence vibration ν(C-O) as well as deformation vibration δ(C-OH) at 1363 cm^{-1}, 1316 cm^{-1} and 1244 cm^{-1} can be also noticed.

The spectrum of isolated quercetin (Fig. 5B) has the same bands as the spectrum of quercetin standard. The main difference between these spectra is in the intensity of the bands. The spectrum of quercetin standard has higher band intensity compared to the spectrum of isolated quercetin. The characteristic bands of quercetin at 3434 cm^{-1} of ν(O-H), at 1656 cm^{-1} of free ketone group, 1608 cm^{-1} of ν(C=O), 1382 cm^{-1} and 1295 cm^{-1} of ν(C-O) and δ(C-OH) can be also noticed in this spectrum.

CONCLUSION

The procedure of quercetin extraction from red onion skin was modelled using the reduced second order polynomial equation. Based on the ANOVA test, it can be concluded that the highest effect had ethanol concentration, followed by the extraction time and liquid-to-solid ratio. The content of total flavonoids and polyphenols was determined in the extract obtained under the optimal conditions for quercetin extraction. The contents of total flavonoid (30.1 $g \cdot kg^{-1}$ with the dried extract, expressed

as RE) and total polyphenol (53.82 g·kg^{-1} the dried extract, expressed as GAE) were determined by spectrophotometric methods with alumiunium chloride and Folin-Ciocalteu reagents, respectively. Based on the DPPH assay, it was confirmed that the extract of red onion skin enriched with quercetin had better antioxidant activity compared to the synthetic antioxidant BHT. The structure of isolated quercetin was confirmed using UV-Vis and FTIR spectrophotometry methods based on the comparison with quercetin standard.

Acknowledgements

The authors acknowledge the financial support for this study by Grant No. TR-34012 from the Ministry of Education, Science and Technological Development of the Republic of Serbia.

REFERENCES

1. Kumar, K. S. – Bhowmik, D. – Tiwari, P.: *Allium cepa*: a traditional medicinal herb and its health benefits. Journal of Chemical and Pharmaceutical Research, *2*, 2010, pp. 283–291. ISSN: 0975-7384 (print). <http://www.jocpr.com/articles/allium-cepa-a-traditional-medicinal-herb-and-its-health-benefits.pdf>
2. Benítez, V. – Mollá, E. – Martín-Cabrejas, M. A. – Aguilera, Y. – López-Andréu, F. J. – Cools, K. – Terry, L. A. – Esteban, R. M.: Characterization of industrial onion wastes (*Allium cepa* L.): dietary fibre and bioactive compounds. Plant Foods for Human Nutrition, *66*, 2011, pp. 48–57. DOI: 10.1007/s11130-011-0212-x.
3. Choi, I. S. – Cho, E. J. – Moon, J. H. – Bae, H. J.: Onion skin waste as a valorization resource for the by-products quercetin and biosugar. Food Chemistry, *188*, 2015, pp. 537–542. DOI: 10.1016/j.foodchem.2015.05.028.
4. Jin, E. Y. – Lim, S. – oh Kim, S. – Park, Y. S. – Jang, J. K. – Chung, M. S.– Park, H. – Shim, K. S. – Choi, Y. J.: Optimization of various extraction methods for quercetin from onion skin using response surface methodology. Food Science and Biotechnology, *20*, 2011, pp. 1727–1733. DOI: 10.1007/s10068-011-0238-8.
5. Jang, M. – Asnin, L. – Nile, S. H. – Keum, Y. S. – Kim, H. Y. – Park, S. W.: Ultrasound-assisted extraction of quercetin from onion solid wastes. International Journal of Food Science and Technology, *48*, 2013, pp. 246–252. DOI: 10.1111/j.1365-2621.2012.03180.x.
6. Katsampa, P. – Valsamedou, E. – Grigorakis, S. – Makris, D. P.: A green ultrasound-assisted extraction process for the recovery of antioxidant polyphenols and pigments from onion solid wastes using Box–Behnken experimental design and kinetics. Industrial Crops and Products, *77*, 2015, pp. 535–543. DOI: 10.1016/j.indcrop.2015.09.039.
7. Wach, A. – Pyrzyńska, K. – Biesaga, M.: Quercetin content in some food and herbal samples. Food Chemistry, *100*, 2007, pp. 699–704. DOI: 10.1016/j.foodchem.2005.10.028.
8. Kumar, B. – Smita, K. – Kumar, B. – Cumbal, L. – Rosero, G.: Microwave-assisted extraction and solid phase separation of quercetin from solid onion (*Allium cepa* L.). Separation Science and Technology, *49*, 2014, pp. 2502–2509. DOI: 10.1080/01496395.2014.933982.
9. Ko, M.-J. – Cheigh, C.-I. – Cho, S.-W. – Chung, M.-S.: Subcritical water extraction of flavonol quercetin from onion skin. Journal of Food Engineering, *102*, 2011, pp. 327–333. DOI: 10.1016/j.jfoodeng.2010.09.008.
10. Lee, K. A. – Kim, K.-T. – Kim, H. J. – Chung, M.-S. – Chang, P.-S. – Park, H. – Pai, H.-D.: Antioxidant activities of onion (*Allium cepa* L.) peel extracts produced by ethanol, hot water, and subcritical water extraction. Food Science and Biotechnology, *23*, 2014, pp. 615–621. DOI: 10.1007/s10068-014-0084-6.
11. Joung, E. M. – Jung, K. H.: Antioxidant activity of onion (*Allium cepa* L.) peel extracts obtained as onion byproducts. Korean Journal of Food Science and Technology, *46*, 2014, pp. 364–368. DOI: 10.9721/KJFST.2014.46.3.364.
12. Gawlik-Dziki, U. – Świeca, M. – Dziki, D. – Baraniak, B. – Tomiło, J. – Czyż, J.: Quality and antioxidant properties of breads enriched with dry onion (*Allium cepa* L.) skin. Food Chemistry, *138*, 2013, pp. 1621–1628. DOI: 10.1016/j.foodchem.2012.09.151.
13. The International Pharmacopoeia. 4th edition. Geneva : World Health Organization, 2007. ISBN: 978-9241563017.
14. Savic, I. M. – Nikolic, V. D. – Savic, I. M. – Nikolic, L. B. – Stankovic, M. Z.: Development and validation of a new RP-HPLC method for determination of quercetin in green tea. Journal of Analytical Chemistry, *68*, 2013, pp. 906–911. DOI: 10.1134/S1061934813100080.
15. Derringer, G. – Suich, R.: Simultaneous optimization of several response variables. Journal of Quality Technology, *12*, 1980, pp. 214–219. ISSN: 0022-4065.
16. Savić, I. M. – Nikolić, V. D. – Savić, I. M. – Nikolić, L. B. – Stanković, M. Z. – Moder, K.: Optimization of total flavonoid compound extraction from *Camellia sinensis* using the artificial neural network and response surface methodology. Hemijska industrija, *67*, 2013, pp. 249–259. DOI: 10.2298/HEMIND120313066S.
17. Singleton, V. L. – Orthofer, R. – Lamuela-Raventós, R. M.: Analysis of total phenols and other oxidation substrates and antioxidants by means of Folin-Ciocalteu reagent. Methods in Enzymology, *299*, 1999, pp. 152–178. DOI: 10.1016/S0076-6879(99)99017-1.
18. Cvetković, Ž. S. – Nikolić, V. D. – Savić, I. M. – Savić-Gajić, I. M. – Nikolić , L. B.: Development and validation of an RP-HPLC method for quantification of trans-resveratrol in the plant extracts. Hemijska industrija, *69*, 2015, pp. 679–687. DOI: 10.2298/

HEMIND140917004C.
19. Savic, I. M. – Nikolic, V. D. – Savic-Gajic, I. M. – Nikolic, L. B. – Moder, K. – Hopkins, M.: Optimization of quercetin extraction from green tea (*Camellia sinensis*) using central composite design, and the pharmacological activity of the extract. Chemical and Biochemical Engineering Quarterly, *30*, 2016, pp. 103–115. DOI: 10.15255/CABEQ.2015.2166.
20. Bezerra, M. A. – Santelli, R. E. – Oliveira, E. P. – Villar, L. S. – Escaleira, L. A.: Response surface methodology (RSM) as a tool for optimization in analytical chemistry. Talanta, *76*, 2008, pp. 965–977. DOI: 10.1016/j.talanta.2008.05.019.
21. Savic, I. M. – Nikolic, V. D. – Savic-Gajic, I. M. – Nikolic, L. B. – Ibric, S. R. – Gajic, D. G.: Optimization of technological procedure for amygdalin isolation from plum seeds (*Pruni domesticae* semen). Frontiers in Plant Science, *6*, 2015, article 276. DOI: 10.3389/fpls.2015.00276.
22. Razmara, R. S. – Daneshfar, A. – Sahraei, R.: Solubility of quercetin in water + methanol and water + ethanol from (292.8 to 333.8) K. Journal of Chemical and Engineering Data, *55*, 2010, pp. 3934–3936. DOI: 10.1021/je9010757.
23. Makris, D. P. – Kefalas, P.: Kinetic modelling for polyphenol extraction from onion (*Allium cepa*) solid wastes using acidified water/ethanol mixture. Acta Alimentaria, *44*, 2015, pp. 482–492. DOI: 10.1556/066.2015.44.0020.
24. Pralhad, T. – Rajendrakumar, K.: Study of freeze-dried quercetin–cyclodextrin binary systems by DSC, FT-IR, X-ray diffraction and SEM analysis. Journal of Pharmaceutical and Biomedical Analysis, *34*, 2014, pp. 333–339. DOI: 10.1016/S0731-7085(03)00529-6.

Received 27 June 2017; accepted 2 November 2017; published online 11 February 2018.