Optimization of solid–liquid extraction of antioxidants and saccharides from black mulberry fruit by response surface methodology

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Summary

The aim of this study was to examine the influence of solvent composition (ethanol/water, 40–80% (v/v)), temperature (40–80 °C) and time (20–60 min) on the extraction yield of phenolic compounds, flavonoids, saccharides and antioxidant activity of black mulberry (*Morus nigra* L.) fruit. Experimental values of total phenolic content were in the range from 15.38 g to 18.43 g of chlorogenic acid equivalents per kilogram of dry extract, and total flavonoids in the range from 7.74 g to 12.33 g of rutin eqivalents per kilogram of dry extract. Antioxidant activity expressed as IC_{50} value was in the range from 0.02 g to 0.04 g of mulberry extract per litre. The saccharide contents were in the range from 465.5 g to 502.2 g per kilogram of dry extract. Response surface methodology was used to determine the optimum extraction conditions and to investigate the effect of different variables on the properties of mulberry fruit extract. Optimal conditions within the experimental range of the studied variables were: solvent composition 58.7%, temperature 58.1 °C and extraction time 46.9 min. The experimental values agreed with those predicted, thus indicating suitability of response surface methodology for optimizing the extraction conditions.

Keywords

black mulberry fruit; response surface methodology; phenolics; flavonoids; antioxidant activity; saccharides

Phytochemicals are substances found naturally in plant foods, including fruits, vegetables and grains. Increased dietary consumption of fruits and vegetables rich in phytochemicals correlates with improved cardiovascular health as well as reduced cancer, stroke, degenerative diseases, loss of functionality associated with aging etc. [1–6]. Berries as a group of food rich in nutrients received great attention over the past few years particularly because of their health benefits for lowering the risk factors of chronic diseases [7, 8]. Because of this reason, black mulberry (*Morus nigra* L.) has gained an important position in the food industry due to the presence of phytochemicals. Today, due to its nutritive value, the mulberry fruits is consumed both in fresh and processed forms. Mulberry fruits can be utilized in various forms such as jam, syrup, vinegar, concentrate, ice-cream, alcohol. Recent studies revealed that mulberry fruits had essential effects in human diet and health with the help of its components such as organic acids, phenolics and saccharides [9–13]. Compared to other berries, the bioactive compounds and phytochemicals of mulberry extracts have not been extensively studied. Today many industries investigated, generated and applied natural bioactive compounds for the preparation of dietary supplements, nutraceuticals, functional food ingredients or cosmeceuticals [14]. Within this trend, we examined the extraction of black mulberry fruits and studied the influence of extraction parameters on the content of bioactive compounds and on the antioxidant activity, using response surface methodology (RSM). This methodology is a collection of statistical and mathematical techniques useful for developing, improving and optimizing

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processes. RSM is a critical technology in developing new processes and optimizing their performance. The objectives of quality improvement, including reduction of variability and improvement of process and production performance, can often be accomplished directly using RSM [15]. The central composite rotatable design (CCRD) was used to determine the optimal solid–liquid extraction conditions for the extraction of antioxidants and total saccharides from black mulberry fruit [16].

In the present study, phytochemicals were isolated by solid-liquid extraction with different parameters. Extraction parameters, namely, solvent composition, extraction temperature and time were optimized using RSM. The obtained information may help to substantiate the potential health benefits of the extracts of mulberry fruits as a potential functional food source for lowering the risk factors of common chronic diseases.

MATERIALS AND METHODS

Chemicals and reagents

The compound 1,1-diphenyl-2-picryl-hydrazylhydrate (DPPH) and Folin–Ciocalteu reagent were purchased from Sigma-Aldrich (St. Louis, Missouri, USA). Chlorogenic acid and rutin were purchased from Sigma-Aldrich. Aluminium chloride hexahydrate, anhydrous sodium carbonate, and sodium acetate trihydrate were purchased from Merck (Darmstadt, Germany). Commercial N_2 (Messer, Belgrade, Serbia) was used. All other chemicals and reagents were of analytical reagent grade.

Sample preparation

Dried plant material was used. Voucher specimens (*Morus nigra* L. No. 2-1753, Rimski Šančevi, Novi Sad, Serbia, UTM 34TDR2 01) were confirmed and deposited at the Herbarium of the Department of Biology and Ecology (BUNS Herbarium), Faculty of Natural Sciences, University of Novi Sad, Serbia [17]. Plant samples (10 g) were extracted using a solvent of different composition (ethanol/water, 40–80% (v/v)), different temperature (40–80 °C) and different time (20–60 min). The extraction process was carried out using a bath thermostat (Laborgerätebörse, Burladingen, Germany). The obtained extracts were stored in a flask filled with N_2 and kept at 4 °C to prevent oxidative damage until analysis.

Determination of antioxidant compounds

The content of total phenolics (TP) in the extracts was determined by the Folin–Ciocalteu method [18], and was expressed as mass (in grams) of chlorogenic acid equivalents (CAE) per mass (in kilograms) of dried extract. The reaction mixture was prepared by mixing 0.1 ml of ethanolic solution of liquid extract, 7.9 ml of distilled water, 0.5 ml of the Folin–Ciocalteu reagent and 1.5 ml of 20% sodium carbonate. After 1 h, the absorbance at 750 nm was measured against a blank using spectrophotometer Jenway 6300 (Bibby Scientific, Stone, United Kingdom), which had been prepared in a similar manner, by replacing the extract with distilled water. Triplicate tests were conducted for each sample.

The content of total flavonoids (TF) was determined by aluminium chloride colorimetric assay [19] using rutin as a standard, and it was expressed as mass (in grams) of rutin equivalents (RE) per mass (in kilograms) of dried extract. Flavonoids from mulberry extracts were extracted using the following procedure: 1 ml of mulberry ethanolic liquid extract was evaporated and dissolved in 2 ml of extraction medium (70% (v/v), methanol, 5% (v/v), acetic acid and 25% (v/v), distilled water) at room temperature for 60 min. The resulting solution was filtered through Whatman paper No. 4 (Whatman, Dassel, Germany) and the filtrate volume was adjusted to 10 ml. The samples were prepared by mixing 5 ml of extract, 1 ml of distilled water and 2.5 ml of $AlCl_3$ solution (26.6 mg $AlCl_3 \cdot 6H_2O$ and 80 mg of CH_3COONa dissolved in 20 ml of distilled water). A blank sample was prepared by replacing $AlCl_3$ solution with distilled water. The absorbance of the samples and blank sample was measured immediately at 430 nm using spectrophotometer Jenway 6300. Triplicate tests were done for each sample.

DPPH• scavenging assay

The free radical-scavenging activity of mulberry fruits extract was determined as described by Espin et al. [20]. Samples were prepared by mixing mulberry extract, methanol (96%) and DPPH (90 μmol·l^{-1}) to give a final concentration of 0.05 mg·ml^{-1}, 0.075 mg·ml^{-1}, 0.1 mg·ml^{-1} and 0.2 mg·ml^{-1}. After 60 min at room temperature, the absorbance was measured at 517 nm using spectrophotometer Jenway 6300 and expressed as radical-scavenging capacity *RSC* in percent. *RSC* was calculated using the following Eq. 1:

$$RSC = 100 - \left(\frac{A_s}{A_b} \times 100\right) \quad (1)$$

where A_s is the absorbance of sample solution, and A_b is the absorbance of control.

This activity was also expressed as 50% inhibi-

Tab. 1. The uncoded and coded levels of independent variables used in the RSM design.

Independent variables	Symbols	Levels		
		Low (–1)	Middle (0)	High (+1)
Solvent composition [%]	X_1	40	60	80
Temperature [°C]	X_2	40	60	80
Time [min]	X_3	20	40	60

tory concentration (IC_{50}), i.e. the concentration of the test solution required to scavenge 50% of the initial radicals. The values are presented as a mean of three measurements.

High performance liquid chromatography

Saccharides of the dried mulberry extract were dissolved in ultrapure water, and galactose solution was added. Aqueous sample extract was passed through a 0.45 μm syringe filter, just before high performance liquid chromatography (HPLC) analyses. The analysis of saccharides was carried out by Perkin-Elmer HPLC system series 200 (Perkin-Elmer, Shelton, Washington, USA) equipped with degasser, isocratic pump, oven, refractive index detector and TotalChrom Navigator software. The separation was performed on Meta-Carb Ca Plus column (Agilent Technologies, Santa Clara, California, USA; 300 mm × 7.8 mm), thermostated at 90 °C. A 20 ml aliquot was injected onto the column and eluted with deionized water at a flow rate of 0.5 ml·min^{-1}. A standard solution was composed of saccharose, glucose, galactose and fructose at a concentration of 1 mg·ml^{-1}; 2 mg·ml^{-1}; 2.5 mg·ml^{-1} and 3 mg·ml^{-1}, respectively. Saccharides from the aqueous sample extract were identified on the basis of their retention time and quantified on the basis of peak area using internal standard procedure.

Experimental design

Solvent composition (X_1), extraction temperature (X_2) and time (X_3) were independent variables involved in optimization of the extraction process in terms of achieving the maximum contents of antioxidants and saccharides. Investigated factors and levels tested are summarized in Tab. 1. Experimental daata were fitted with second order

Tab. 2. Experimental matrix and values of the observed responses.

Run	Solvent composition [%]	Temperature [°C]	Time [min]	Phenolics content [g·kg^{-1}]	Flavonoids content [g·kg^{-1}]	IC_{50} [g·l^{-1}]	Saccharides content [g·kg^{-1}]
1	40 (-1)	40 (-1)	40 (0)	16.77	8.82	0.026	486
2	40 (-1)	60 (0)	20 (-1)	15.41	8.95	0.031	481
3	40 (-1)	80 (1)	40 (0)	17.12	10.09	0.027	486
4	40 (-1)	60 (0)	60 (1)	18.24	9.73	0.030	498
5	60 (0)	40 (-1)	20 (-1)	15.38	8.88	0.031	485
6	60 (0)	80 (1)	20 (-1)	17.20	9.99	0.035	492
7	80 (1)	60 (0)	20 (-1)	17.19	10.01	0.035	483
8	80 (1)	80 (1)	40 (0)	16.49	7.74	0.025	484
9	80 (1)	60 (0)	60 (1)	18.48	9.05	0.029	485
10	60 (0)	80 (1)	60 (1)	17.31	11.13	0.028	466
11	60 (0)	60 (0)	40 (0)	17.52	12.33	0.024	495
12	60 (0)	60 (0)	40 (0)	17.99	11.77	0.024	502
13	60 (0)	60 (0)	40 (0)	18.21	11.56	0.025	502
14	60 (0)	60 (0)	40 (0)	18.30	11.56	0.025	496
15	60 (0)	60 (0)	40 (0)	17.99	11.36	0.024	501
16	80 (1)	40 (-1)	40 (0)	16.08	7.97	0.024	498
17	60 (0)	40 (-1)	60 (1)	17.91	10.48	0.030	499

Phenolics content is expressed in grams of chlorogenic acid equivalents per kilogram. Flavonoids content is expressed in grams of rutin equivalents per kilogram.

response surface model with the following Eq. 2:

$$y = \beta_0 + \sum_{j=1}^{k} \beta_j X_j + \sum_{j=1}^{k} \beta_{jj} X_j^2 + \sum\sum_{i<j} \beta_{ij} X_i X_j \quad (2)$$

where y is response (total phenolics, total flavonoids, antioxidant activity and saccharides content); β_0, β_j, β_{jj}, β_{ij} are constant coefficients of intercept, linear, quadratic and interaction terms, respectively; and X_i and X_j are coded independent variables (solvent composition, extraction temperature and time).

Statistical analysis

Statistical analysis was performed using RSM software Design-Expert v.7 (Stat-Ease, Minneapolis, Minnesota, USA). The results were statistically tested by the analysis of variance (ANOVA) at a significance level of $p = 0.05$. The adequacy of the model was evaluated by the coefficient of determination (R^2) and model p-value. A mathematical model was established to describe the influence of single process parameter and/or interaction of multiple parameters on each investigated response. Response surface plots were generated with the same software and drawn by using the function of two factors, keeping the other constant.

RESULTS AND DISCUSSION

Response surface methodology (RSM) is an effective statistical technique for optimization of complex processes [21]. It was successfully demonstrated that RSM can be used to optimize the extraction yields of compounds from many fruits and medicinal plants [22–26]. To our knowledge, there are few reports on the optimization of mulberry leaves extraction [24–27], and this is the first study on the optimization of extraction conditions of phenolic compounds, saccharides and antioxidant activity of mulberry plants grown in Serbia and Balkan region.

We used RSM to optimize the solid–liquid extraction of antioxidants and saccharides from black mulberry fruit (*Morus nigra* L.). The influence of solvent composition (ethanol/water, 40–80% (v/v)), temperature (40-80 °C) and time (20–60 min) on the extraction yield of phenolic compounds, flavonoids, saccharides and antioxidant activity were investigated. The ranges of variables were selected on the basis of our preliminary studies. Tab. 2 shows the obtained experimental data for the investigated responses. The effect of linear, quadratic or interaction coeffi-

Tab. 3. Regression equation coefficients for selected responses.

Term	Coefficients	Standard error	*F*-value	*p*-value
Total phenolics content				
Intercept	18.00	0.24		
X_1	0.088	0.19	0.21	0.6615
X_2	0.25	0.19	1.67	0.2376
X_3	0.85	0.19	19.45	0.0031
X_1^2	–0.51	0.26	3.66	0.0974
X_2^2	–0.89	0.26	11.23	0.0122
X_3^2	–0.17	0.26	0.39	0.5509
X_1X_2	0.015	0.27	0.0029	0.9588
X_1X_3	–0.38	0.27	2.01	0.1994
X_2X_3	–0.61	0.27	4.99	0.0607
$R^2 = 0.8648$				
Total flavonoids content				
Intercept	11.72	0.31		
X_1	–0.35	0.24	2.11	0.1893
X_2	0.35	0.24	2.10	0.1910
X_3	0.32	0.24	1.74	0.2286
X_1^2	–1.87	0.33	31.56	0.0008
X_2^2	–1.19	0.33	12.65	0.0093
X_3^2	–0.41	0.33	1.49	0.2614
X_1X_2	–0.37	0.34	1.2	0.3098
X_1X_3	–0.44	0.34	1.63	0.2425
X_2X_3	–0.11	0.34	0.11	0.7487
$R^2 = 0.8929$				
IC_{50} value				
Intercept	0.025	0.0004613		
X_1	–0.000025	0.0003647	0.0047	0.9473
X_2	0.000662	0.0003647	3.30	0.1121
X_3	–0.002038	0.0003647	31.22	0.0008
X_1^2	0.0008375	0.0005027	2.78	0.1396
X_2^2	0.0003625	0.0005027	0.52	0.4942
X_3^2	0.005763	0.0005027	131.42	<0.0001
X_1X_2	0.00005	0.0005157		0.9255
X_1X_3	–0.0015	0.0005157		0.0227
X_2X_3	–0.001575	0.0005157		0.0185
$R^2 = 0.9646$				
Saccharides content				
Intercept	499.24	2.67		
X_1	–0.065	2.11	0.00095	0.9763
X_2	–5.03	2.11	5.67	0.0488
X_3	0.97	2.11	0.21	0.6613
X_1^2	–4.50	2.91	2.39	0.1661
X_2^2	–6.21	2.91	4.55	0.0703
X_3^2	–7.84	2.91	7.26	0.0309
X_1X_2	–3.35	2.99	1.26	0.2995
X_1X_3	–3.71	2.99	1.54	0.2540
X_2X_3	–10.06	2.99	11.34	0.0120
$R^2 = 0.8364$				

X_1 – solvent; X_2 – temperature; X_3 – time.
$p < 0.01$ – highly significant, $0.01 \leq p < 0.05$ – significant, $p \geq 0.05$ – not significant.

cients on the response was tested for significance by analysis of variance. Regression coefficients of intercept, linear, quadratic and interaction terms of the model were calculated using least square method. Tab. 3 summarizes the ANOVA (*F*-test) and *p*-value that are used as a means to check the significance of each coefficient and indicate the interaction strength of each parameter. The lack of fit, which measures the fitness of models, resulted in no significant *F*-value ($p > 0.05$) in terms of the response variable studied, indicating that the models were sufficiently accurate for predicting the response variations (Tab. 4). The fitted model represented the experimental data well with high correlation coefficients, R^2, varying from 0.8364 to 0.9646, depending on the investigated responses. The best results were obtained in the case of IC_{50} value, where the model was statistically significant with $p = 0.0003$ and with a high correlation coefficient ($R^2 = 0.9646$).

Total phenolics content of mulberry extracts

Phenolic compounds have been associated with the health benefits derived from consuming high levels of fruits and vegetables [28–30]. Polyphenols are one of the phytochemical groups whose "protective" properties include antioxidant, antimicrobial, anticancer and cardiovascular-protective activities [28–31]. In this study, the total phenolics content of mulberry extracts varied from 15.38 g·kg^{-1} to 18.48 g·kg^{-1} (expressed as CAE) according to different investigated parameter levels. Total phenolics content was significantly influenced by linear term of extraction time and by quadratic term of temperature (Tab. 3). Furthermore, the interaction between the investigated parameters had no significant effect on total phenolics content ($p > 0.05$). Fig. 1 shows that total phenolics content in mulberry extracts increased with increasing the temperature up to about 60 °C. Further increase in temperature caused a decrease

Tab. 4. Analysis of variance (ANOVA) of the modelled responses.

Source	Sum of squares	Degree of freedom	Mean square	*F*-value	*p*-value
Total phenolics content					
The recovery					
Model	13.18	9	1.46	4.97	0.0230
Residual	2.06	7	0.29		
Lack of fit	1.69	3	0.56	6.13	0.0562
Pure error	0.37	4	0.092		
Total	15.24	16			
Total flavonoids content					
The recovery					
Model	27.31	9	3.03	6.48	0.0111
Residual	3.28	7	0.47		
Lack of fit	2.72	3	0.91	6.55	0.0505
Pure error	0.55	4	0.14		
Total	30.59	16			
IC_{50} value					
The recovery					
Model	0.0002	9	0.00002	21.22	0.0003
Residual	0.000007	7	0.000001		
Lack of fit	0.000006	3	0.000002	6.08	0.0569
Pure error	0.000001	4	0.0000003		
Total	0.0002	16			
Saccharides content					
The recovery					
Model	1277.36	9	141.93	3.98	0.0412
Residual	249.93	7	35.70		
Lack of fit	201.88	3	67.29	5.60	0.0647
Pure error	48.05	4	12.01		
Total	1527.29	16			

$p < 0.01$ – highly significant; $0.01 \leq p < 0.05$ – significant; $p \geq 0.05$ – not significant.

in total phenolics content. It can be seen also that the total phenolics content increased significantly with increasing the extraction time. This observation was understandable because an extended extraction time favours the extraction of phenolic compounds [32]. Ethanol concentration had also a significant influence on total phenolic content. As the extraction of phenolic compounds depends largely on the polarity of solvents and compounds, single solvent might not be effective for the extraction of a bioactive compound. Hence, a combination of alcohol with water is more effective in extracting phenolic compounds than alcohol alone [33]. The total phenolics content increased with an increase in ethanol concentration from 40% to 65% (Fig. 1). This was probably due to the increased solubility of phenolic compounds in the mixture of ethanol and water. The findings obtained in our study are in good agreement with ROSTANGO et al. [34], where the total phenolics content decreased when the ethanol concentration was above 60%.

Total flavonoid content of mulberry extracts

Temperature, time and composition of the solvent were important parameters of the total flavonoids extraction from different materials [35–38]. In our experimental conditions, the total flavonoid content of mulberry extracts varied from 7.74 $g \cdot kg^{-1}$ to 12.32 $g \cdot kg^{-1}$ (expressed as RE). It is evident from Tab. 3 that quadratic terms of ethanol concentration and extraction temperature were the most predominant factors influencing total flavonoid content. Effects of individual parameters on total flavonoid content of mulberry extracts is described by data in Tab. 3, which could be used for calculation of optimal extraction parameters considering the output yield. Fig. 1 shows that total flavonoid content increased with an increase of the extraction time. It can be expected that after a certain time of extraction the yield declined. This might be due to decomposition of active compounds during a prolonged extraction [38–40].

The increase of extraction temperature up to around 60 °C led to an increase in total flavonoid content, while with further increase of temperature, flavonoid content decreased. This phenomenon can be explained by a decrease in solvent viscidity and accelerated movement of molecules, which promoted a faster release of bioactive compounds from plant cells. However, much higher temperature may promote degradation of some thermo–sensitive compounds [36, 38]. Therefore, the best extraction temperature was considered to be 58 °C in our study. A similar trend was also seen with an increase of ethanol concentration

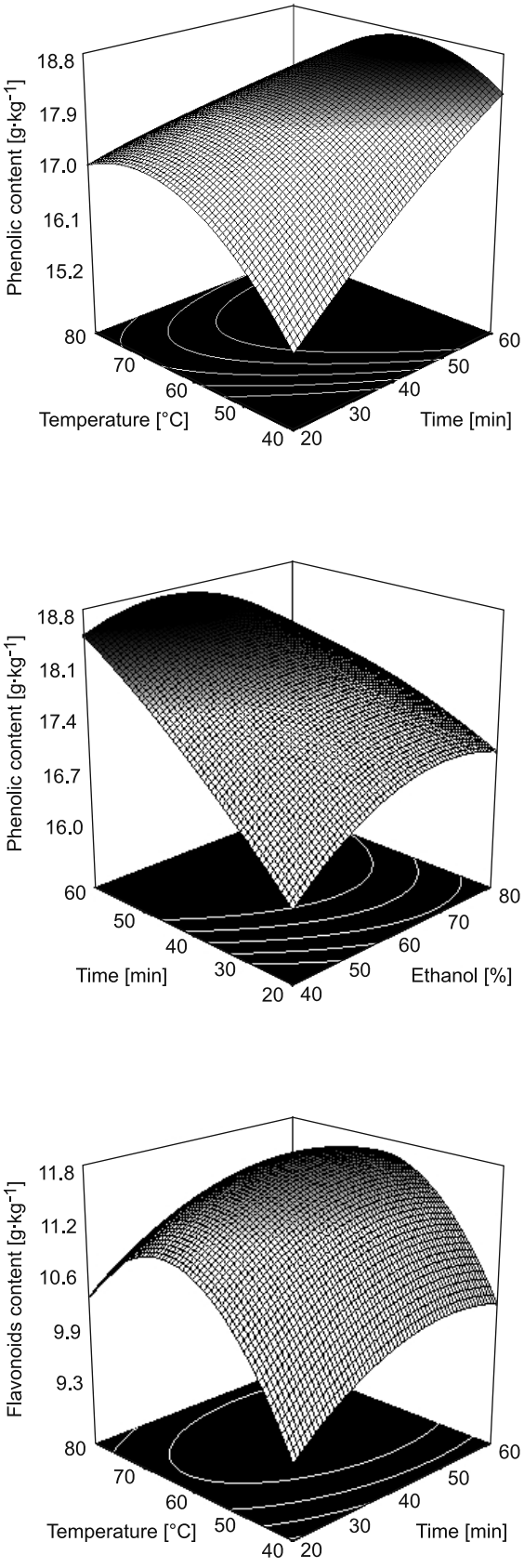

Fig. 1. Response surface plots showing the effects of investigated parameters on the phenolics and flavonoids content.

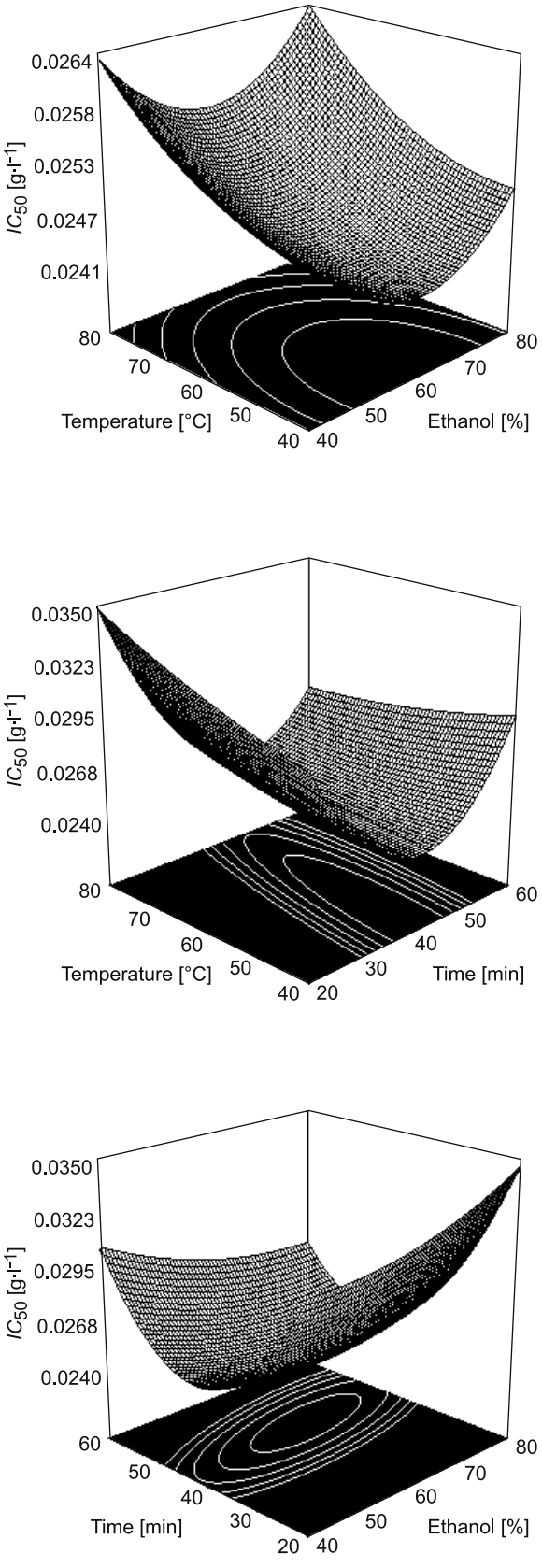

Fig. 2. Response surface plots showing effects of investigated parameters on the IC_{50} value and their interactions.

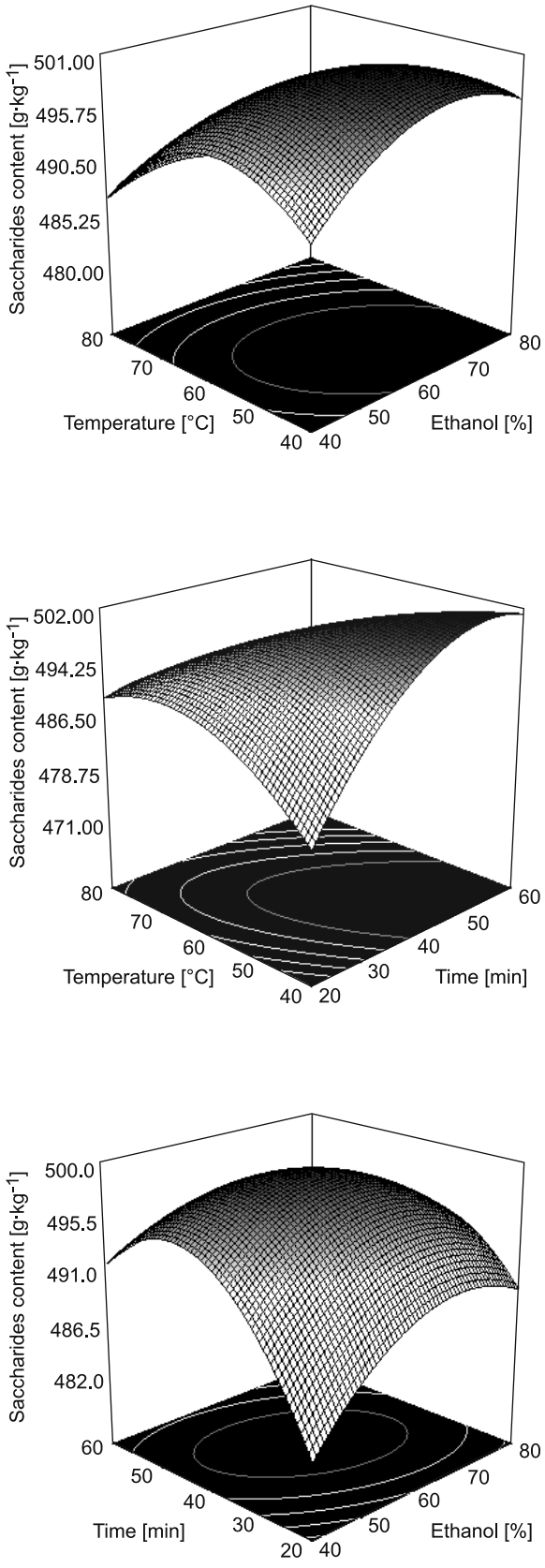

Fig. 3. Response surface plots showing effects of investigated parameters on the saccharides content and their interaction.

from 40% to 60%, while further increase in ethanol concentration led to a decrease in total flavonoid content in the extracts.

Antioxidant activity of mulberry extracts

The IC_{50} values were used to describe the DPPH scavenging capacity of mulberry extracts. IC_{50} is the required initial concentration of a selected antioxidant sample to quench 50% of the free radicals initially present in the reaction system; therefore, a higher IC_{50} value corresponds to a lower antioxidant activity in the sample [20]. From data in Tab. 3 it can be seen that linear term of extraction time ($p = 0.0008$), quadratic term of time ($p < 0.0001$) and interactions between solvent composition and time ($p = 0.0227$), as well as between extraction temperature and time ($p = 0.0185$), had significant influence on the response. The fitted model (Tab. 3) represents the experimental data very well with a high correlation coefficient, $R^2 = 0.9646$. Visualization of the influence of individual parameters is presented in Fig. 2. From this figure it can be seen that IC_{50} value increase when the extraction temperature is increased. Extraction temperature is an important parameter in process optimization, as a high temperature is known to degrade antioxidant compounds [41]. On the other hand, when the effect of solvent composition on the antioxidant activity of the extracts was investigated [25, 26, 42, 43], all authors reported that the interaction between temperature and ethanol concentration mainly affected the antioxidant activity, like in our research. With an increase of the extraction time to about 40 min, IC_{50} value decreased. From 40 min to 60 min of extraction, an increase of IC_{50} can be seen. Fig. 2 shows also that with increase of ethanol concentration up to around 55%, IC_{50} values of the investigated mulberry extracts decreased, which means that the antioxidant activity increased. Further increase of solvent composition led to a decrease of the antioxidant activity of mulberry extracts.

Content of saccharides

Saccharides as major components of fruits are present in the structure of plant tissues and act as energy source. Fruits contain from 3% to 30% saccharides. Glucose and fructose are the main hexoses. Plants produce saccharides by photosynthesis [44]. After water (75–82%), saccharides are the next most abundant constituents of mulberry. The total soluble saccharides content in the investigated mulberry fruits varied between 11.5% (*M. nigra*) and 13.9% (*M. alba*). These values are in agreement with those reported by Elmaci and Altug [22] for black mulberry cultivars from Aegean region of Turkey. In most mulberries, the main saccharides are saccharose, glucose and fructose. Eating quality of mulberry depends to a great extent on sweetness, which is related to the content of saccharides. In term of sweetness, if saccharose rated 1, then glucose is rated 0.75 and fructose 1.75. Ozgen at al. [23] reported that *M. nigra* and *M. rubra* contained glucose in the range of 55–71.2 $g \cdot l^{-1}$ and 28.5–49.6 $g \cdot l^{-1}$, fructose (48.6–64.1 $g \cdot l^{-1}$ and 27.7–46.6 $g \cdot l^{-1}$) and saccharose (0.1–0.7 $g \cdot l^{-1}$ and 0.4–1 $g \cdot l^{-1}$), respectively. Because glucose and fructose are main saccharides at harvest, the composition of these two saccharides dominates the taste of mulberry fruit.

The content of saccharides in the black mulberry fruit extract was found to be between 466 $g \cdot kg^{-1}$ and 502 $g \cdot kg^{-1}$ (Tab. 2). From Tab. 3 it can be seen that the saccharides content was significantly influenced by linear term of extraction temperature and by quadratic term of time. Furthermore, the interaction between temperature and time (X_2X_3) had a significant effect on the saccharides content ($p = 0.0120$). Fig. 3 shows that saccharides content in the black mulberry fruit extract increased with increasing the ethanol concentration and with longer extraction. The increase of extraction temperature up to about 65 °C led to an increase in the content of saccharides, while further increase of temperature did not show any significant effect on saccharides content.

Optimization of black mulberry fruit extraction process

Optimization is an essential tool in food engineering for the efficient operation of different processes yielding a highly acceptable product, using several response variables to describe the quality characteristics of the product. The main goal of this research was to find the best settings for extraction parameters, ethanol composition, extraction temperature and extraction time in the investigated experimental range given in Tab. 1.

In the present study, desirability function was developed for the following criteria: maximum content of total phenols, total flavonoids and saccharides, and minimum IC_{50} concentration (maximum antioxidant activity) in black mulberry fruit extracts. Second order polynomial models obtained in this study were utilized for each response in order to determine the specified optimum extraction condition. These regression models were valid only in the selected experimental domain.

By applying desirability function method, the optimum conditions within the experimental range of the studied variables were 58.7%, 58.1 °C and

46.9 min. At this point, the investigated responses were calculated as total phenols 18.27 $g \cdot kg^{-1}$ (expressed as CAE), total flavonoids 11.76 $g \cdot kg^{-1}$ (expressed as RE), IC_{50} of 0.025 $g \cdot l^{-1}$ and total saccharides 499.5 $g \cdot kg^{-1}$. The experimental values agreed with those predicted, thus indicating suitability of the response surface methodology in optimizing the investigated extraction conditions.

CONCLUSION

Mulberry (*Morus nigra* L.) fruit is a potential rich source of antioxidants and saccharides. Total phenolics content was in the range from 15.38 g to 18.43 g of chlorogenic acid equivalents per kilogram of dry extract, and total flavonoids in the range from 7.74 g to 12.33 g of rutin equivalents per kilogram of dry extract. The saccharides content was in the range from 465.5 g to 502.2 g per kilogram of dry extract, depending on extraction parameters (solvent composition, temperature and time). The use of RSM facilitated the selection of the best experimental conditions for extraction of the constituents. The analysis of variance (ANOVA) showed that the regression models were statistically good with a significance level of $p < 0.05$ for all investigated responses.

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