ISSN 1330-9862
(FTB-2854)

original scientific paper

Optimization of Solid-Liquid Extraction of Antioxidants from Black Mulberry Leaves by Response Surface Methodology

Marija Radojković[1], Zoran Zeković[1], Stela Jokić[2], Senka Vidović[1], Žika Lepojević[1] and Svetlana Milošević[1]*

[1]Faculty of Technology, Department of Biotechnology and Pharmaceutical Engineering, University of Novi Sad, Bulevar cara Lazara 1, RS-21000 Novi Sad, Serbia

[2]Faculty of Food Technology, Josip Juraj Strossmayer University of Osijek, F. Kuhača 20, HR-31000 Osijek, Croatia

Received: June 28, 2011
Accepted: April 6, 2012

Summary

The extraction of active components from natural sources depends on different factors. The knowledge of the effects of different extraction parameters is useful for the optimization of the process, as well for the ability to predict the extraction yield. The aim of this study is to examine the influence of solvent concentration (ethanol/water 40–80 %, by volume), temperature (40–80 °C) and solvent/raw material ratio (10–30 mL/g) on the extraction yield of phenolic compounds, flavonoids and antioxidant activity from black mulberry (*Morus nigra* L.) leaves. Experimental values of total phenolic content were in the range from 18.6 to 48.7 mg of chlorogenic acid equivalents per g of dried leaves and total flavonoids in the range from 6.0 to 21.4 mg of rutin eqivalents per g of dried leaves. Antioxidant activity expressed as the inhibition concentration at 50 % (IC_{50} value) was in the range from 0.019 to 0.078 mg of mulberry extract per mL. Response surface methodology (RSM) was used to determine the optimum extraction conditions and to investigate the effect of different variables on the observed properties of mulberry leaf extracts. The results show a good fit to the proposed model (R^2>0.90). The optimal conditions for obtaining the highest extraction yield of phenolics and flavonoids were within the experimental range. The experimental values agreed with those predicted, thus indicating suitability of the used model and the success of RSM in optimizing the investigated extraction conditions.

Key words: black mulberry leaves, response surface methodology, phenolics, flavonoids, DPPH

Introduction

Dietary antioxidants are important components because they protect against free radicals such as reactive oxygen species in the human body. Free radicals are known to be responsible for degenerative diseases of ageing and are recognized as major factors causing cancer, cardiovascular disorders and diabetes (*1*). In the last few decades, fruits, leaves, fruit and leaf extracts and seeds have received much attention as sources of bioactive substances such as antioxidants, antimutagens and anticarcinogens (*2-4*). The total antioxidant activity of plant food is the result of individual activities of each of the antioxidant compounds present such as vitamin C, tocopherols, flavonoids, antocyanins and phenolic acids, being the major phytochemicals responsible for antioxidant activity of plant material (*5,6*). Plant phenolics are struc-

*Corresponding author; Phone: ++381 21 485 3740; Fax: ++381 21 450 413; E-mail: ramarija@uns.ac.rs

turally diversified class of phytochemicals with high antioxidant activity. Naczk and Shahidi (*7*) reported in their review that this fact affects the difficult choice of standardized methods of extraction.

Extraction efficiency is commonly a function of process conditions. Quantitative extraction of active constituents is an important step before analysis. The quantity of analytes extracted from different matrices depends on the type of matrix, extraction techniques and conditions (*8*). Many factors, such as solvent concentration, extraction time, temperature, pH, liquid/solid ratio and particle size, may significantly influence the liquid-solid extraction (*9-14*). The positive or negative role of each factor in the mass transfer of the process is not always obvious; the chemical characteristics of the solvent and the diverse structure and composition of the natural products ensure that each material or solvent system shows different behaviour, which cannot be predicted (*15*). Response surface methodology (RSM) is a statistical method that uses quantitative data from appropriate experimental designs to determine and simultaneously solve multivariate equations. These equations can be graphically represented as response surfaces which can be used in three ways: (*i*) to describe how the test variables affect the response, (*ii*) to determine the interrelationships among the test variables, and (*iii*) to describe the combined effect of all test variables on the response (*16*).

Mulberry is a fast-growing deciduous plant that grows under different climatic conditions, *i.e.* tropical, subtropical and temperate (*17*). It is valued for its foliage, which constitutes the chief feed for silkworms. The leaves are nutritious, palatable and non-toxic, and are stated to improve milk yield when fed to dairy animals (*18*). Different kinds of research have investigated various properties of whole mulberry fruits, mulberry fruit extracts, mulberry leaves and mulberry seed oil. Gecgel *et al.* (*2*) reported that the black mulberry seed consisted of 27.5–33.0 % of crude oil, 20.2–22.5 % of crude protein, 3.5– 6.0 % of ash, 42.4–46.6 % of carbohydrates and 112.2–152.0 mg of total phenolics per 100 g of mulberry seeds. Arabshahi-Delouee and Urooj (*19*) studied the antioxidant properties of various solvent extracts of mulberry leaves and stated that the methanolic extract, with the highest amount of total phenolics, was the most potent antioxidant in all the assays used. In another study where the chemical compositions of white, red and black mulberries were compared, it was stated that the highest total phenolic and flavonoid yields were observed in black mulberry (*20*). However, to our knowledge, there are few reports on the optimization of mulberry leaf extraction (*21,22*), and this is the first study on the optimization of extraction conditions of phenolic compounds and antioxidant activity of mulberry plants grown in Serbia and central Balkan region. This information will be of considerable value to the commercial growers of mulberry trees or pharmaceutical industry for potential mulberry supplement production.

The aim of this study is to determine the effects of the investigated parameters (temperature, liquid/solid ratio and solvent concentration) on the antioxidant activity of the extracts and the extraction yield of phenolic compounds from black mulberry leaves, and to obtain a response surface model for the extraction yield of total phenolics and total flavonoids, as well as for the determination of antioxidant activity of mulberry extracts.

Material and Methods

Chemicals and reagents

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

Sample preparation

In this research 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 (*23*). The samples of mulberry leaves were dried naturally (in shade and in a draught) during one month. Immediately after drying, the samples of leaves were processed, and then ground in a blender before the extraction. Particle size was determined using sieve sets (Erweka, Munich, Germany), and mean particle size was (0.31±0.03) mm.

Plant samples (10 g) were extracted using a solvent of different compositions (ethanol/water 40–80 %, by volume), at a temperature of 40–80 °C, and solvent/raw material ratio of 10–30 mL/g. The extraction process was carried out using a bath thermostat (Laborgerätebörse GmbH, 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 (*24,25*), and was expressed as mass (in mg) of chlorogenic acid equivalents (CAE) per mass (in g) of dried mulberry leaves. The reaction mixture was prepared by mixing 0.1 mL of ethanolic solution of liquid extract (5 mg of dried leaves per mL), 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, 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 colourimetric assay (*26*) using rutin as a standard, and it was expressed as mass (in mg) of rutin equivalents (RE) per mass (in g) of dried mulberry leaves. 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 %, by volume, methanol; 5 %, by volume, acetic acid and 25 %, by volume, distilled water) at room temperature for 60 min.

The resulting solution was filtered through Whatman paper no. 4 and the filtrate volume was adjusted to 10 mL. The probes 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 probe was prepared by replacing $AlCl_3$ solution with distilled water. The absorbance of the probes and blank probe was measured immediately at 430 nm. Triplicate tests were done for each sample.

DPPH· scavenging assay

The free radical scavenging activity of mulberry leaf extract was determined as described by Espin *et al.* (*27*). Briefly, the mulberry extract was mixed with methanol (96 %) and 90 μM DPPH to give a final concentration of 0.05, 0.075, 0.1 and 0.2 mg/mL. After 60 min at room temperature, the absorbance was measured at 517 nm and expressed as radical scavenging capacity (RSC/%). RSC was calculated using the following equation (*28*):

$$\mathrm{RSC}=(100-\frac{A_s}{A_b})\cdot 100 \qquad /1/$$

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

This activity was also expressed as the inhibition concentration at 50 % (IC_{50}), *i.e.* the concentration of the test solution required to scavenge 50 % of the initial radical. The values are presented as the mean of three measurements.

Experimental design

One of the common experimental designs used for engineering purposes is a Box-Behnken design that includes three variables and three factorial levels (*29*). The independent variables used in this study were solvent concentration, extraction temperature and liquid-solid ratio. Coded and uncoded levels of the independent variables and the experimental design are given in Table 1. Coded value 0 stands for centre point of the variables and is repeated for experimental error. Factorial points are coded as ±1.

Table 1. Coded and uncoded levels of independent variables used in the response surface methodology

Independent variable	Symbol	Level		
		Low (–1)	Middle (0)	High (+1)
solvent concentration/%	X_1	40	60	80
temperature/°C	X_2	40	60	80
liquid/solid ratio/(mL/g)	X_3	10	20	30

Second-order polynomial equation was used to express the investigated responses (Y), namely the total extraction yield of phenolics (in mg of CAE per g), total flavonoid yield (in mg of RE per g) and IC_{50} value (in mg/mL) of the mulberry extracts as a function of the coded independent variables, where X_1, $X_2 \ldots X_k$ are the independent variables affecting the responses Y; β_0, β_j (i=1, 2...k), β_{ii} (i=1, 2...k), and β_{ij} (i= 1, 2…k; j=1, 2…k) are regression coefficients for intercept, linear, quadratic, and interaction terms, respectively; k is the number of variables. Coded independent variables for our experiment are solvent concentration, temperature and liquid/solid ratio.

$$Y=\beta_0+\sum_{i=1}^{k}\beta_i X_i+\sum_{i=1}^{k}\beta_{ii}X_i^2+\sum_{\substack{i=1\\ i<j}}^{k-1}\sum_{j=2}^{k}\beta_{ij}X_iX_j \qquad /2/$$

Statistical analysis was performed using RSM software Design-Expert® v. 7 (Stat-Ease, Minneapolis, MN, USA). The results were statistically tested by the analysis of variance (ANOVA) at the 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, and keeping the other constant.

Results and Discussion

Analysis of the model

Effects of solvent concentration (ethanol/water 40–80 %, by volume), temperature (40–80 °C) and solvent/raw material ratio (10–30 mL/g) on antioxidant compounds (phenolics and flavonoids), and antioxidant activity of mulberry extracts in terms of IC_{50} value were studied by RSM. Experiments were performed according to the Box-Behnken design (Table 2). The results of the statistical analyses are presented in Table 3. The coefficients are related to coded variables. The ANOVA results for modelled responses are reported in Table 4. Joglekar and May (*30*) suggested that for a good fit of a model, R^2 should be at least 0.80. In our study, the R^2 values for these response variables were higher than 0.80, indicating the adequacy of the applied regression model. Fig. 1 shows the parity plot of the observed and predicted values for modelled responses. The best way of expressing the effect of any extraction parameter on antioxidant phenolic compounds and antioxidant activity within the investigated experimental range was to generate response surfaces of the model (Figs. 2–4). The final goal of response surface methodology is the process optimization. Thus, the developed models can be used for simulation and optimization.

Effect of extraction conditions on the content of total phenolics in the extracts

Solubility of phenolics is governed by their chemical nature in the plant that may vary from simple to very highly polymerized substances. Plant material may contain various quantities of phenolic acids, phenylpropanoids, anthocyanins and tannins, among others. There is a possibility of interaction of phenolics with other plant components and this interaction may lead to the formation of complexes that may be quite insoluble. Solubility of phenolics is also affected by the polarity of the used solvent(s). Therefore, it is very difficult to develop an extraction procedure suitable for the extraction of all plant

Table 2. Experimental matrix and values of the observed response

Run	Solvent concentration %	Temperature °C	Liquid/solid ratio mL/g	TP	TF	IC_{50}	Coded solvent concentration variable	Coded temperature variable	Coded liquid/solid ratio variable
1	40	40	20	30.3	14.1	0.051	–1	–1	0
2	40	60	10	18.6	11.0	0.048	–1	0	–1
3	40	80	20	30.3	12.3	0.078	–1	1	0
4	40	60	30	19.0	12.0	0.038	–1	0	1
5	60	40	10	23.9	8.5	0.047	0	–1	–1
6	60	80	10	28.6	9.1	0.049	0	1	–1
7	80	60	10	32.0	6.0	0.051	1	0	–1
8	80	80	20	31.9	8.3	0.052	1	1	0
9	80	60	30	31.6	6.6	0.065	1	0	1
10	60	80	30	37.8	10.6	0.033	0	1	1
11	60	60	20	47.8	21.4	0.022	0	0	0
12	60	60	20	45.6	20.3	0.021	0	0	0
13	60	60	20	48.7	19.5	0.019	0	0	0
14	60	60	20	47.9	20.3	0.022	0	0	0
15	60	60	20	52.4	20.3	0.031	0	0	0
16	80	40	20	34.1	13.3	0.068	1	–1	0
17	60	40	30	32.1	13.2	0.060	0	–1	1

TP content of total phenolics in the extracts is expressed in mg of CAE per g of dried mulberry leaves
TF content of total flavonoid in the extracts is expressed in mg of RE per g of dried mulberry leaves
IC_{50} is expressed in mg of extract per mL

phenolics (*7*). Fig. 2 shows the 3D surface plots of the interactive effects of the independent variables corresponding to the response variable. It can be seen that the extraction yield of phenolic compounds of mulberry extracts increased with the increase of solvent concentration up to about 75 %. Furthermore, the TP increased also with the increase of extraction temperature up to about 65 °C, after which further increase in temperature did not cause a significant change in total phenolic yield. It can be seen that the TP increased with the increase of liquid/solid ratio and reached maximum at about 20 mL/g. Any further increase in liquid/solid ratio did not increase the yield of the extraction of phenolic compounds. The TP of mulberry extracts varied from 18.63 to 52.43 mg of CAE per g of dried leaves, according to different investigated parameter levels. Our research shows better results than previous studies based on mulberry leaves grown in other regions of the world. Mulberry leaves grown in Serbia showed higher content of phenolic compounds than mulberry leaves grown in Pakistan and India (*19,22*).

The influence of solvent concentration (45–80 % of ethanol) and temperature (30–70 °C) on the extractability of total phenolic content from the branch bark of black mulberry was published by Wu *et al.* (*21*). The authors reported that maximum content of total phenolics was obtained in terms of 77.95 % ethanol and 70 °C temperature, which is confirmed by our study. Thus, an increase in the temperature increased the diffusion coefficient, and hence the rate of diffusion and the content of total phenolics in the extracts. The same conclusion was confirmed by the study of Jokić *et al.* (*31*). Type of solvent is the most investigated factor. Rostango *et al.* (*32*) found that it is necessary to add a certain amount of water to the extraction solvent in order to improve the extraction of phenolic compounds. The water content higher than 60 % resulted in a reduction of the extraction yield of the same components. Syzdłowska-Czerniak *et al.* (*33*) reported that care must be taken when adjusting the parameters of temperature and solvent concentration, because higher temperatures reduce the polarity of solvents, thus increasing its capability to extract non-polar compounds.

The effect of the solvent/sample ratio has been investigated for different raw materials by many authors (*10,34,35*). Our research has shown that liquid/solid ratio has a positive effect; in fact, the higher the solvent/solid ratio, the higher the total amount of solids obtained. This is consistent with mass transfer principles; the driving force during mass transfer is the concentration gradient between the solid and the bulk of the liquid, which is greater when a higher solvent/solid ratio is used. Also, interactions of the extracted compounds with the solvent could have modified the activity coefficients and thus the solubility of the compounds. Similar results about the effect of temperature and solvent/solid ratio on the extraction of phenolic compounds were also reported for milled berries by Cacace and Mazza (*35*), and for grape pomace by Pinelo *et al.* (*15*), who also found similar relationship of temperature and liquid/solid ratio with extraction yields.

The response surface was generated based on the second-order equation, and for TP the equation was expressed as:

$$Y=48.49+3.92X_1+1.03X_2+2.18X_3-11.09X_1^2-5.76X_2^2-12.10X_3^2-0.57X_1X_2-0.19X_1X_3+0.23X_2X_3 \quad /3/$$

where Y represents the dependent variable (content of total phenolics in the extracts) and X_1, X_2 and X_3 repre-

Table 3. Regression equation coefficients for the selected responses

Variable	Coefficient	Standard error	F-value	p-value
Total phenolic content				
Intercept	48.49	1.98		
X_1	3.92	1.56	6.31	0.0403
X_2	1.03	1.56	0.44	0.5302
X_3	2.18	1.56	1.95	0.2054
X_1^2	–11.09	2.15	26.51	0.0013
X_2^2	–5.76	2.15	7.16	0.0317
X_3^2	–12.10	2.15	31.55	0.0008
X_1X_2	–0.57	2.21	0.066	0.8042
X_1X_3	–0.19	2.21	0.007	0.9327
X_2X_3	0.23	2.21	0.011	0.9186
R^2=0.9203				
Total flavonoid content				
Intercept	20.34	0.57		
X_1	–1.90	0.45	17.72	0.0040
X_2	–1.10	0.45	5.95	0.0448
X_3	0.95	0.45	4.46	0.0726
X_1^2	–4.89	0.62	61.66	0.0001
X_2^2	–3.42	0.62	30.17	0.0009
X_3^2	–6.54	0.62	110.27	<0.0001
X_1X_2	–0.79	0.64	1.54	0.2549
X_1X_3	–0.10	0.64	0.025	0.8792
X_2X_3	–0.82	0.64	1.64	0.2414
R^2=0.9733				
IC_{50} value				
Intercept	0.023	0.0035		
X_1	0.0027	0.0028	0.91	0.3729
X_2	–0.0017	0.0028	0.38	0.5569
X_3	–0.00004	0.0028	0.0002	0.9897
X_1^2	0.024	0.0039	29.99	0.0009
X_2^2	0.015	0.0039	22.01	0.0022
X_3^2	0.0087	0.0039	2.60	0.1510
X_1X_2	–0.010	0.0040	7.01	0.0330
X_1X_3	0.0059	0.0040	2.26	0.1763
X_2X_3	–0.0073	0.0040	3.38	0.1085
R^2=0.9131				

X_1=solvent, X_2=temperature, X_3=liquid/solid ratio
$p<0.01$ highly significant, $0.01\leq p<0.05$ significant, $p\geq0.05$ not significant

sent the independent variables (solvent concentration, temperature and liquid/solid ratio).

Table 4 shows the test statistics for the model (F-test and probability) of TP. The probability (p-value) of regression model was 0.0043, which means that there was a statistically significant multiple regression relationship between the independent variables and the response variable. The R^2 value for this response (Table 3) was 0.9203, which means that the response surface model could explain more than 92 % of the variation of the studied response variables, thus indicating that the variability of responses was explained well by the model. The lack of fit, which measures the fitness of the model, resulted in no significant F-value ($p>0.05$) in terms of the studied response variables, indicating that the model was sufficiently accurate for predicting the response variations. The coefficients of the regression equation and p-values for TP are shown in Table 3. The second-order terms of solvent concentration, temperature and liquid/solid ratio (X_1^2, X_2^2, X_3^2), and the first-order term of solvent concentration (X_1) had significant effects ($p<0.05$) on the TP of mulberry extracts. The interactions between the investigated variables had no significant ($p>0.05$) effect on the extraction yield of phenolics.

Effect of extraction conditions on total flavonoid yield

Flavonoids are considered as phenolic compounds with the highest antioxidant activity due to their chemical structure (*36*). The effects of solvent concentration, temperature and liquid/solid ratio on the flavonoid yield of black mulberry extracts, as well as their interactions, are shown in Fig 3. It can be seen that TF in the extracts increased with the increase of ethanol concentration from 40 to 60 %. Further increase of ethanol concentration led to the decrease in the flavonoid yield. The polarity of the solvent plays an important role in the selective extraction of different flavonoid families. Furthermore, the TF increased with the increase of liquid/solid ratio up to about 20 mL/g. Similar results were published by Cacace and Mazza (*37*). The TF also increased with the increase of extraction temperature from 40 to 60 °C, while further increase of temperature led to the decrease in TF. Depending on the different investigated parameters, the TF of black mulberry extracts varied from 6.0 to 21.4 of mg RE per g of dried mulberry leaves (Table 2). Also, the extracts obtained in our study have a higher content of total flavonoids in the extracts compared to the extracts obtained in previous studies (*19*,*22*). For one group of authors (*12*,*38*,*39*), who investigated plant extraction, the operational conditions were: ethanol concentration 50 % and temperature above 50 °C, like in our study. All authors agree that an increase in the extraction temperature improves extraction process, enhancing both the solubility of the solute and the diffusion coefficient, but also beyond a certain temperature, phenolic and flavonoid compounds can be denaturised (*10*,*11*).

The quadratic term for all three investigated parameters had a significant effect on the TF of the extracts (Table 3). As for the significance of the polynomial coefficients, their p-values suggest that the most important factor influencing the extraction yield of flavonoids was solvent concentration. Analysis of the results showed that all the investigated interactions had no statistically significant effect on the TF. From Fig. 1, a good agreement between the observed and predicted values for TF in mulberry extracts can be seen. Polynomial model tested for the selected response was significant at 95 % confidence level ($p=0.05$; Table 4). The model F-value of 28.35 implies that models for selected responses are significant. The goodness of fit of the model was checked by the determination coefficient that was found to be 0.9733 for the response of the extraction yield of flavonoids, which indicates that less than 3 % of the variations could not be explained by the model. Analysis of variance also shows that the regression models for TF had no significant lack of fit ($p>0.05$).

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

Source	Sum of squares	Degree of freedom	Mean square	F-value	p-value
Total phenolic content					
Recovery					
model	1578.47	9	175.39	8.98	0.0043
residual	136.69	7	19.53		
lack of fit	111.82	3	37.27	6.00	0.0582
pure error	24.87	4	6.22		
total	1715.16	16			
Total flavonoid content					
Recovery					
model	416.53	9	46.28	28.35	0.0001
residual	11.43	7	1.63		
lack of fit	9.50	3	3.17	6.55	0.0505
pure error	1.93	4	0.48		
total	427.96	16			
IC_{50} value					
Recovery					
model	0.0046	9	0.0005	8.17	0.0056
residual	0.0004	7	0.00006		
lack of fit	0.0004	3	0.0001	5.54	0.0658
pure error	0.00008	4	0.00002		
total	0.0050	16			

The mathematical model representing the TF (Y) as a function of the independent variables (in terms of coded values) within the region under investigation was expressed by the following equation:

$$Y=20.34-1.90X_1-1.10X_2+0.95X_3-4.89X_1^2-3.42X_2^2-6.54X_3^2-0.79X_1X_2-0.10X_1X_3-0.82X_2X_3 \quad /4/$$

where Y is the flavonoid yield, X_1 is solvent concentration, X_2 is temperature and X_3 is liquid/solid ratio.

Effect of extraction conditions on the antioxidant activity of mulberry extracts

The IC_{50} values were used to report the DPPH scavenging capacity of mulberry extracts. The IC_{50} value 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 (*27*).

The estimated constants, coefficients of linear, quadratic and interaction effects calculated by multivariable linear regression are shown in Table 3. Fitting the constants and coefficients into Eq. 2, the following equation was obtained:

$$Y=0.023+0.003X_1-0.002X_2-0.00004X_3+0.021X_1^2+0.018X_2^2+0.006X_3^2-0.010X_1X_2+0.006X_1X_3-0.007X_2X_3 \quad /5/$$

where Y is the IC_{50} value, X_1 is the solvent concentration, X_2 is temperature and X_3 is liquid/solid ratio. Among the three independent variables tested, only the quadratic term for all three investigated parameters had a significant effect on the antioxidant activity of mulberry extracts.

In the present experiment, the model with the p= =0.0056 was statistically significant, which implies that the model was suitable for this experiment. Meanwhile, the lack of fit of this model was insignificant with the p=0.0658. The response equation fitted the experimental data with R^2=0.9131, indicating that 91 % of the variability in IC_{50} value can be explained by the model presented in Eq. 4. The plot of the observed *vs*. the predicted values of responses Y is shown in Fig. 1. The plot shows a close fit of the observed values with the predicted ones. The complete quadratic model showed a good fit and appeared to reasonably represent the data.

Some researchers reported that the antioxidant activity was correlated well with TP, suggesting that polyphenols have a major influence on the antioxidant activity (*11,12*). The response surface of IC_{50} value as a function of independent variables within the experimental range was generated by using the empirical model presented in Eq. 4. The generated response surfaces (Fig. 4) show that the minimum IC_{50} value of the extracts was at about 60 °C, which means that the antioxidant activity of the extracts was higher at this temperature. Furthermore, it can be seen that solvent concentration, temperature and liquid/solid ratio showed a quadratic effect on the investigated response. IC_{50} value decreased up to about 60 % ethanol concentration, followed by further increase of IC_{50} value. Temperature of the extraction is important parameter in the process optimization; high temperature degraded antioxidant compounds (*11*). The study of Azizah *et al.* (*9*) reported that the compounds of the extract were stable at all temperatures (except higher than 90 °C), which is the opposite of our findings. In some studies, decreasing the liquid/solid ratio resulted in an increase in the antioxidant activities to a

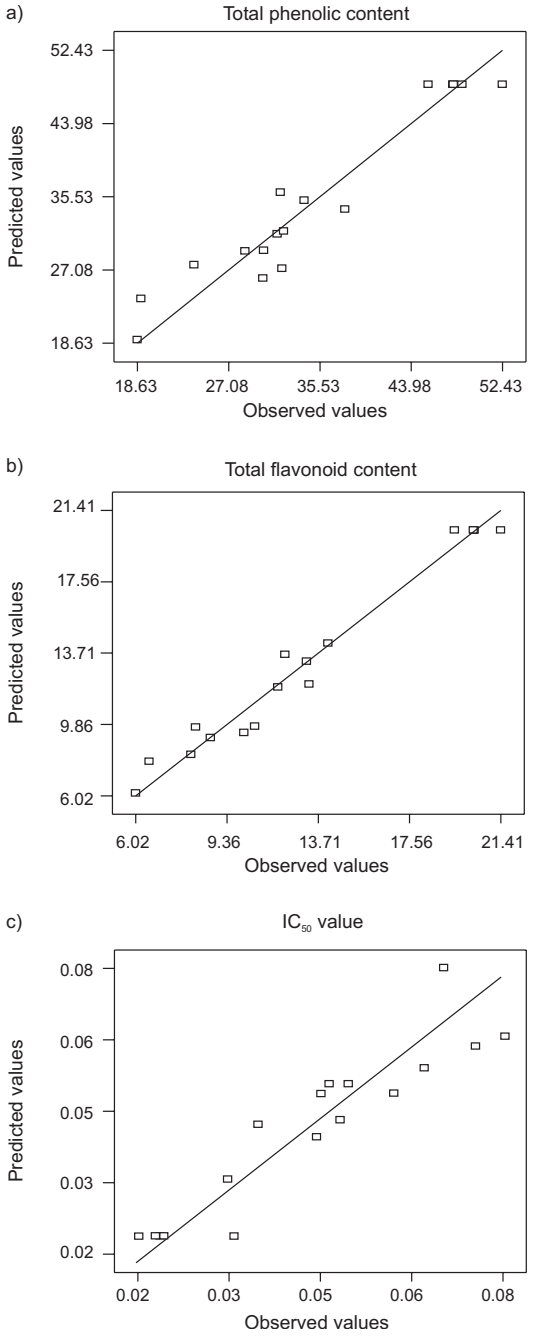

Fig. 1. Parity plot showing observed *vs*. predicted values for modelled responses

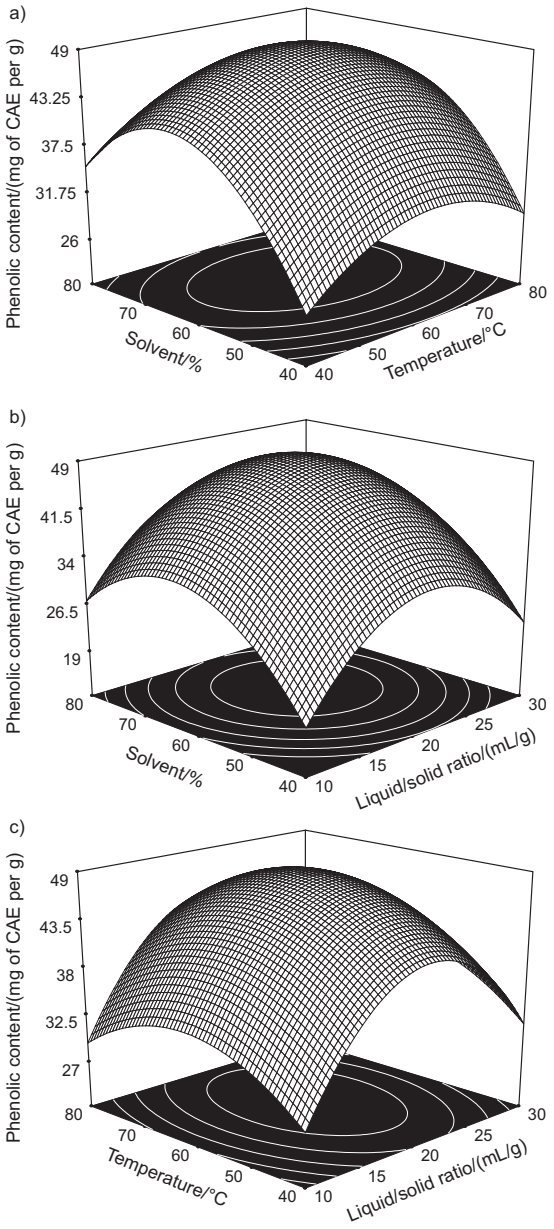

Fig. 2. Response surface plots showing the effects of investigated parameters on the extraction yield of phenolics and their interactions: a) liquid/solid ratio was constant at 20 mL/g, b) temperature was constant at 60 °C, c) solvent concentration was constant at 60 %

certain level, like in our study. On the other hand, the effect of solvent concentration on the antioxidant activity of the extracts was investigated in some studies (*12, 13*,*35*,*37*). All authors reported that the interaction between temperature and ethanol concentration mainly affected the antioxidant activity, like in our research.

Optimization of the extraction of black mulberry leaves

Optimization is an essential tool in food engineering for the efficient operation of different processes to yield a highly acceptable product. During optimization of extraction process, several response variables describe the quality characteristics of the obtained extracts. Some of these variables need to be maximized, while others need to be minimized. To optimize the process with two or more output responses, it is helpful to use the concept of desirability function (Fig. 5). The desirability function is one of the most widely used methods for optimization of multiple response processes in science and engineering. Desirability ranges from zero to one for any given response. A value of one represents the ideal case, while zero indicates that one or more responses fall outside the desirable limits (*40*).

The main goal of this research was to find the best settings for the extraction, *i.e.* the most optimal solvent

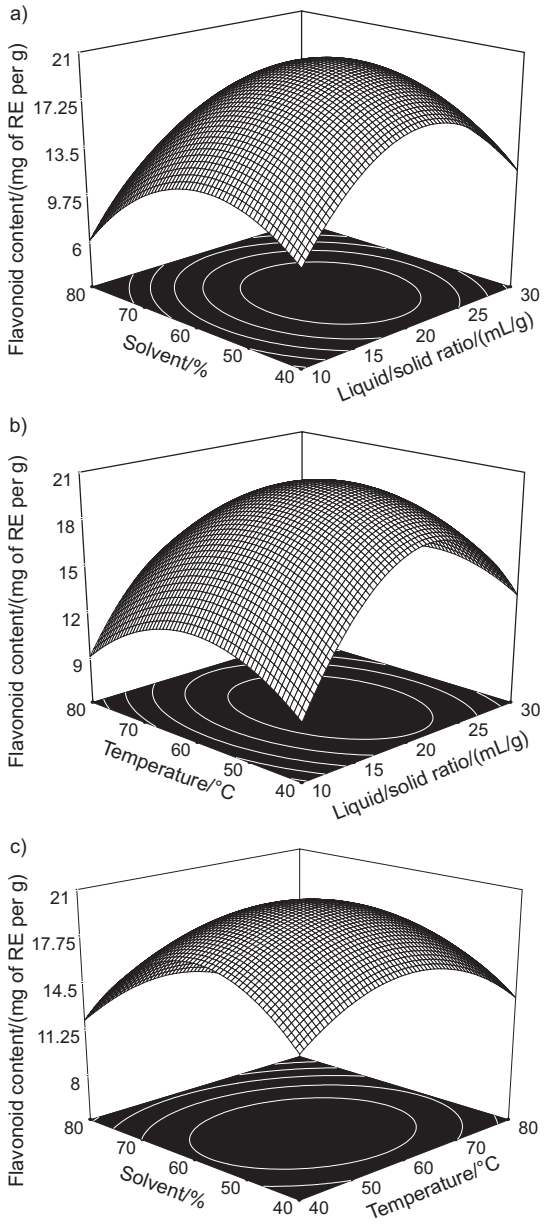

Fig. 3. Response surface plots showing the effects of investigated parameters on the extraction yield of flavonoid and their interactions: a) temperature was constant at 60 °C, b) solvent concentration was constant at 60 %, c) liquid/solid ratio was constant at 20 mL/g

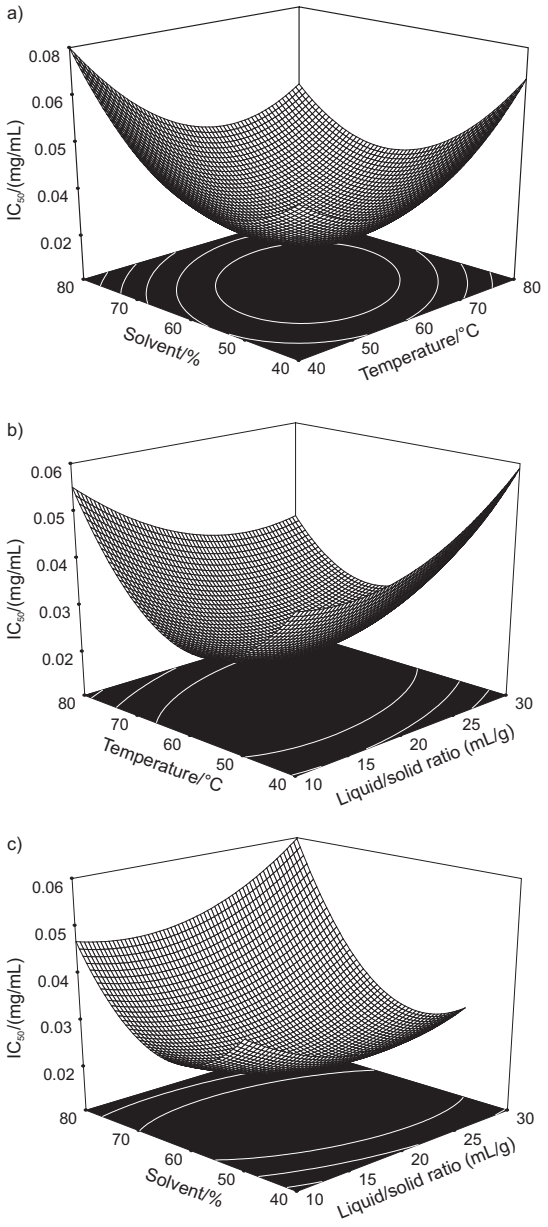

Fig. 4. Response surface plots showing the effect of the investigated parameters on the IC_{50} value and their interactions: a) liquid/solid ratio was constant at 20 mL/g, b) solvent concentration was constant at 60 %, c) temperature was constant at 60 °C

concentration, temperature and liquid/solid ratio. Desirability function was developed for the following criteria: maximum content of TP and TF, and maximum antioxidant activity (minimum IC_{50} concentration) in dried leaves. By applying desirability function method, the optimum extraction conditions were obtained: ethanol concentration of 59.47 %, temperature of 59.92 °C and liquid/solid ratio of 20.73 mL/g. The total extraction yield of mulberry phenolic extracts was calculated to be 48.5 mg of CAE per g of dried leaves, the flavonoid yield was 20.4 mg of RE per g of dried leaves and IC_{50} value was 0.023 mg/mL. The good correlation between these results confirmed that the response model was adequate for the intended optimization.

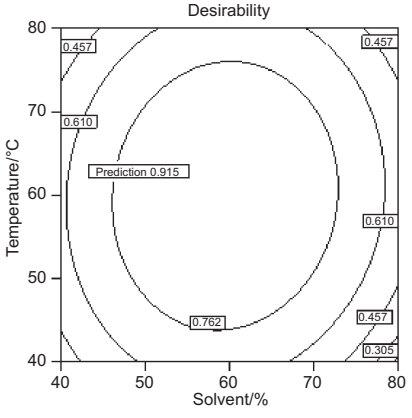

Fig. 5. The contour graph of overall desirability function

Conclusion

High correlation of the mathematical model indicated that a quadratic polynomial model could be employed to optimize the solid–liquid extraction of antioxidants from black mulberry leaves. From response surface plots, three factors (ethanol concentration, operating temperature and liquid/solid ratio) significantly influenced the phenolic and flavonoid content of black mulberry leaf extracts. The optimal conditions to obtain the highest extraction yield of phenolics and flavonoids from mulberry leaf extracts, as well as maximum antioxidant activity were ethanol concentration of 59.47 %, temperature of 59.92 °C and liquid/solid ratio of 20.73 mL/g. Under optimal conditions, the experimental values were in agreement with the predicted values.

Acknowledgements

Financial support of this work by the Serbian Ministry of Education and Science, Project no. TR 31013 is gratefully acknowledged. We are grateful to Dr Goran Anačkov, Department of Biology and Ecology, Faculty of Natural Sciences, University of Novi Sad, Serbia, for depositing *Morus nigra* L. at the BUNS Herbarium.

References

1. Dini, G.C. Tenore, A. Dini, Chemical composition, nutritional value and antioxidant properties of *Allium cepa* L. var. *tropeana* (red onion) seeds, *Food Chem. 107* (2008) 613–621.
2. U. Gecgel, S.D. Velioglu, H.M. Velioglu, Investigating some physicochemical properties and fatty acid composition of native black mulberry (*Morus nigra* L.) seed oil, *J. Am. Oil Chem. Soc. 88* (2011) 1179–1187.
3. M. Alothman, R. Bhat, A.A. Karim, Antioxidant capacity and phenolic content of selected tropical fruits from Malaysia, extracted with different solvents, *Food Chem. 115* (2009) 785–788.
4. P. Maisuthiasakul, S. Pasuk, P. Ritthiruangdej, Relationship between antioxidant properties and chemical composition of some Thai plants, *J. Food Compos. Anal. 21* (2008) 229–240.
5. J. Javanmardi, C. Stushnoff, E. Locke, J.M. Vivanco, Antioxidant activity and total phenolic content of Iranian *Ocimum* accessions, *Food Chem. 83* (2003) 547–550.
6. L. Pizzale, R. Bartolomeazzi, S. Vichi, E. Überegger, L.S. Conte, Antioxidant activity of sage (*Salvia officinalis* and *S. fruticosa*) and oregano (*Origanum onites* and *O. indercedents*) extracts related to their phenolic compound content, *J. Sci. Food Agric. 82* (2002) 1645–1651.
7. M. Naczk, F. Shahidi, Phenolics in cereals, fruits and vegetables: Occurrence, extraction and analysis, *J. Pharm. Biomed. Anal. 41* (2006) 1523–1542.
8. C.D. Stalikas, Extraction, separation, and detection methods for phenolic acids and flavonoids, *J. Sep. Sci. 30* (2007) 3268–3295.
9. A.H. Azizah, N.M.N. Ruslawati, T.S. Tee, Extraction and characterization of antioxidant from cocoa by-products, *Food Chem. 64* (1999) 199–202.
10. M. Pinelo, P. Del Fabbro, L. Manzocco, M.J. Nuñez, M.C. Nicoli, Optimization of continuous phenol extraction from *Vitis vinifera* byproducts, *Food Chem. 92* (2005) 109–117.
11. G. Spigno, L. Tramelli, D.M. De Faveri, Effects of extraction time, temperature and solvent on concentration and antioxidant activity of grape marc phenolics, *J. Food Eng. 81* (2007) 200–208.
12. D.R. Pompeu, E.M. Silva, H. Rogez, Optimisation of the solvent extraction of phenolic antioxidants from fruits of *Euterpe oleracea* using response surface methodology, *Bioresour. Technol. 100* (2009) 6076–6082.
13. H.H. Wijangaard, N. Brunton, The optimisation of solid-liquid extraction of antioxidants from apple pomace by response surface methodology, *J. Food Eng. 96* (2010) 134–140.
14. E.M. Silva, H. Rogez, Y. Larondelle, Optimization of extraction of phenolics from *Inga edulis* leaves using response surface methodology, *Sep. Purif. Technol. 55* (2007) 381–387.
15. M. Pinelo, M. Rubilar, M. Jerez, J. Sinerio, M.J. Nuñez, Effect of solvent, temperature and solvent-to-solid ratio on the total phenolic content and antiradical activity of extracts from different components of grape pomace, *J. Agric. Food Chem. 53* (2005) 2111–2117.
16. M. Giovanni, Response surface methodology and product optimization, *Food Technol. 37* (1983) 41–45.
17. S. Srivastava, R. Kapoor, A. Thathola, R.P. Srivastava, Mulberry (*Morus alba*) leaves as human food: A new dimension of sericulture, *Int. J. Food Sci. Nutr. 54* (2003) 411–416.
18. *The Wealth of India – Raw Materials, Vol. 6*, B.N. Sastri (Ed.), Council of Scientific and Industrial Research, New Delhi, India (1962) pp. 429–439.
19. S. Arabshahi-Delouee, A. Urooj, Antioxidant properties of various solvent extracts of mulberry (*Morus indica* L.) leaves, *Food Chem. 102* (2007) 1233–1240.
20. S. Ercisli, E. Orhan, Chemical composition of white (*Morus alba*), red (*Morus rubra*) and black (*Morus nigra*) mulberry fruits, *Food Chem. 103* (2007) 1380–1384.
21. C. Wu, L. Xu, J.C. Liu, X.Z. Huang, Y.M. Maimaiti, Process optimization for total polyphenol extraction from the tree branch bark of Xinjiang black mulberry (*Morus nigra* L.) by response surface methodology, *Food Sci. 32* (2011) 104–107.
22. A.A. Memon, N. Memon, D.L. Luthria, M.I. Bhanger, A.A. Pitafi, Phenolic acids profiling and antioxidant potential of mulberry (*Morus laevigata* W., *Morus nigra* L., *Morus alba* L.) leaves and fruits grown in Pakistan, *Pol. J. Food Nutr. Sci. 60* (2010) 25–32.
23. P.K. Holmgren, N.H. Holmgren, Additions to Index Herbariorum (Herbaria), edition 8 – fourteenth series, *Taxon, 52* (2003) 385–389.
24. V.L. Singleton, J.A. Rossi Jr., Colorimetry of total phenolics with phosphomolybdic-phosphotungstic acid reagents, *Am. J. Enol. Vitic. 16* (1965) 144–158.
25. M.P. Kähkönen, A.I. Hopia, H.J. Vuorela, J.P. Rauha, K. Pihlaja, T.S. Kujala, T. Heinonen, Antioxidant activity of plant extracts containing phenolic compounds, *J. Agric. Food Chem. 47* (1999) 3954–3962.
26. K.R. Markham: Flavones, Flavanols, and Their Glycosides. In: *Methods in Plant Biochemistry, Vol. 1: Plant Phenolics*, J.B. Harborne, P.M. Dey (Eds.), Academic Press, London, UK (1989) pp. 197–235.
27. J.C. Espin, C. Soler-Rivas, H.J. Wichers, Characterization of the total free radical scavenger capacity of vegetable oils and oil fractions using 2,2-diphenyl-1-picrylhydrazyl radical, *J. Agric. Food Chem. 48* (2000) 648–656.
28. S.S. Vidović, I.O. Mujić, Z.P. Zeković, Z.D. Lepojević, V.T. Tumbas, A.I. Mujić, Antioxidant properties of selected *Boletus* mushrooms, *Food Biophys. 5* (2010) 49–58.
29. R.H. Myers, D.C. Montgomery: *Response Surface Methodology: Process and Product Optimization Using Designed Experiments*, Jonh Wiley & Sons, Inc., New York, NY, USA (1995).
30. A.M. Joglekar, A.T. May, Product excellence through design of experiments, *Cereal Foods World, 32* (1987) 857–868.

31. S. Jokić, D. Velić, M. Bilić, A. Bucić-Kojić, M. Planinić, S. Tomas, Modelling of the process of solid-liquid extraction of total polyphenols from soybeans, *Czech J. Food Sci. 28* (2010) 206–212.

32. M.A. Rostango, M. Palma, C.G. Barroso, Pressurized liquid extraction of isoflavones from soybeans, *Anal. Chim. Acta, 522* (2004) 169–177.

33. Szydłowska-Czerniak, R. Amarowicz, E. Szłyk, Antioxidant capacity of rapeseed meal and rapeseed oils enriched with meal extract, *Eur. J. Lipid Sci. Technol. 112* (2010) 750–760.

34. Š. Stangler Herodež, M. Hadolin, M. Škerget, Ž. Knez, Solvent extraction study of antioxidants from balm (*Melissa officinalis* L.) leaves, *Food Chem. 80* (2003) 275–282.

35. J.E. Cacace, G. Mazza, Mass transfer process during extraction of phenolic compounds from milled berries, *J. Food Eng. 59* (2003) 379–389.

36. B.H. Havsteen, The biochemistry and medical significance of the flavonoids, *Pharmacol. Therapeut. 96* (2002) 67–202.

37. J.E. Cacace, G. Mazza, Extraction of anthocyanins and other phenolics from black currants with sulfured water, *J. Agric. Food Chem. 50* (2002) 5939–5946.

38. J. Shi, J. Yu, J. Pohorly, J.C. Young, M. Bryan, Y. Wu, Optimization of the extraction of polyphenols from grape seed meal by aqueous ethanol solution, *Food Agric. Environ. 1* (2003) 42–47.

39. L. Wang, B. Yang, X. Du, C. Yi, Optimisation of supercritical fluid extraction of flavonoids from *Pueraria lobata*, *Food Chem. 108* (2008) 737–741.

40. C. Cojocaru, M. Khayet, G. Zakrzewska-Trznadel, A. Jaworska, Modeling and multi-response optimization of pervaporation of organic aqueous solutions using desirability function approach, *J. Hazard. Mater. 167* (2009) 52–63.