Macedonian Journal of Chemistry and Chemical Engineering, Vol. **41**, No. 1, pp. 119–128 (2022)

MJCCA9 – 853

ISSN 1857-5552
e-ISSN 1857-5625

Received: January 21, 2021
Accepted: April 5, 2022

DOI: 10.20450/mjcce.2022.2238
Original scientific paper

OPTIMIZATION OF THE EXTRACTION OF ANTIOXIDANTS FROM STINGING NETTLE LEAF USING RESPONSE SURFACE METHODOLOGY

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The aim of this study was to optimize the parameters for the extraction of total flavonoids from stinging nettle leaf. Comparison of the effects of different solvents on total flavonoid content showed that, regardless of extraction time, aqueous methanolic extracts had higher total flavonoid content than did aqueous ethanolic extracts. So, full factorial design and response surface methodology (RSM) were employed to estimate the effects of methanol content (50, 75 and 100 %) and extraction time (30, 60 and 90 min) on the total flavonoid content and antioxidant capacities of the extracts. RSM analysis showed that methanol content in the solvent influenced significantly total flavonoid content and FRAP (ferric-reducing antioxidant power) antioxidant capacity, while extraction time had no significant effect on either of these responses. Extraction parameters for maximal total flavonoid content were estimated to be 69 % aqueous methanol and 67 min, and 65 % aqueous methanol and 83 min for maximal FRAP antioxidant capacity. DPPH (2,2-diphenyl-1-picrylhydrazyl) antioxidant capacity was not significantly affected by extraction time or methanol percentage in the solvent.

Keywords: *Urtica dioica* L.; flavonoids; antioxidant capacity; phenolic compounds; response surface methodology

ОПТИМИЗАЦИЈА НА ЕКСТРАКЦИЈАТА НА АНТИОКСИДАНСИ ОД ЛИСТОТ НА КОПРИВА СО ПРИМЕНА НА МЕТОДОЛОГИЈАТА НА ПОВРШИНА НА ОДГОВОР

Целта на ова истражување е да се оптимизираат параметрите за екстракција на вкупните флавоноиди од листот на коприва. Споредбата на влијанијата на различните растворувачи врз вкупната содржина на флавоноиди покажува дека, независно од времето на екстракција, водните метанолни екстракти содржат поголемо количество вкупни флавоноиди од водните етанолни екстракти. Затоа беше применет целосен факторијален приод и методологија на површини на одговор (RSM), за да се проценат влијанијата на содржината на метанол (50, 75 и 100 %) и времето на екстракција (30, 60 и 90 min) врз вкупната содржина на флавоноиди и антиоксидацискиот капацитет на екстрактите. RSM-анализата покажа дека содржината на метанол во растворувачот значајно влијае врз вкупната содржина на флавоноиди и фериредукциската антиоксидациска способност (FRAP), додека времето на екстракција нема значајно влијание врз овие одговори. Екстракциските параметри за максимална содржина на флавоноиди беа проценети на 60 % воден метанол и 67 min, како и 65 % воден метанол и 83 min за максимален антиоксидациски капацитет на FRAP. Времето на екстракција или уделот на метанол во растворувачот не влијаеја значајно врз антиоксидацискиот капацитет на DPPH (2,2-дифенил-1-пикрилхидразил).

Клучни зборови: *Urtica dioica* L.; флавоноиди; антиоксидациски капацитет; фенолни соединенија; методологија на површина на одговор

1. INTRODUCTION

Flavonoids are secondary plant metabolites ubiquitous in the plant kingdom with multiple functions in plant physiology.[1] With a characteristic three-ring nucleus, flavonoids also contain several hydroxyl groups, which are considered crucial for their antioxidant activity.[2] Ever since the 'French paradox', fruit and vegetable-rich diet-related positive effects on human health have been largely attributed to phenolic compounds, especially flavonoids.[3] There is a growing body of evidence that long-term dietary intake of flavonoid-rich food has favorable effects in numerous chronic diseases in humans, and these effects have usually been attributed to the antioxidant action of flavonoids.[3,4] Most flavonoids fulfill the basic requirements of a good antioxidant agent: they can prevent radical-mediated oxidation by scavenging free radicals (superoxide, peroxyl, alkoxyl, and hydroxyl radicals) producing much more stable aroxyl radicals.[1,2]

Stinging nettle (*Urtica dioica* L.) is a low-requirement, globally present, perennial plant rich in phytochemicals and plant fibers.[5,6] With low demands, this plant can be used as a source of phytochemicals such as flavonoids and other phenolic compounds.[5] Flavonoids detected in stinging nettle leaves are usually flavonols, mostly glycosides of quercetin, kaempferol, and isorhamnetin, which have been proven to have a strong antioxidant capacity.[7,8]

The qualitative and quantitative efficacy of the extraction of flavonoids is influenced by numerous extraction parameters.[9] Flavonoids are a structurally diverse subclass of phenolic compounds consisting of more than 4000 compounds which are glycosylated, methylated, or in a form of aglycon, often bound to other cell constituents.[10] This is why there is no universal protocol for the extraction of flavonoids and why extraction parameters should be optimized for each extraction.[9] So, lately, response surface methodology (RSM) has been proven to be a useful tool for the optimization of extractions.[11] Using multiple regression analysis, RSM provides a mathematical model of the relationship between process factors and response variables as well as insight into the effects of process factors and their interactions on response variables.[12,13] RSM coupled with experimental design has been extensively used in chemistry, biochemistry, and industry as an effective and rational approach to optimization as opposed to a time-consuming one-variable-at-a-time technique.[13,14]

The aim of this study was to evaluate the effects of extraction parameters such as solvent composition and extraction time on the total flavonoid content and antioxidant capacity of stinging nettle leaf extracts. Full factorial experimental design with two factors and three levels, as well as RSM, were used to obtain the optimal conditions for the extraction of antioxidants from stinging nettle leaves.

2. EXPERIMENTAL

2.1. *Plant material*

Air-dried and chopped stinging nettle leaves were provided by the Institute for Medicinal Plant Research "Dr. Josif Pančić", Belgrade, Serbia. Granulometric characteristics of the material were: mesh 0.71 = 28.0 %, mesh 0.3 = 70.8 %, and mesh 0.15 = 0.50 %.

2.2. *Extraction*

Maceration was used as an extraction technique, carried out at room temperature with varying extraction times (30, 60, and 90 min). Solid/liquid ratio was 1:20 (1.25 g of plant material in a glass vial with 25 ml of 50, 75, and 100 % aqueous methanol or 50, 75, and 96 % aqueous ethanol). The extracts were then filtered through a 0.45-μm cellulose filter (Millipore, Billerica, MA, USA), and the filtrates were diluted with appropriate solvent up to the required volume. All extracts were prepared in triplicate.

2.3. *Total flavonoid content*

The total flavonoid content of extracts was measured spectrophotometrically according to a previously described method.[15] Briefly, 200 μl of the extract was mixed with 60 μl of 5 % $NaNO_2$ (Superlab®, Belgrade, Serbia) aqueous solution. After 5 min incubation, 60 μl of 10 % $AlCl_3$ water solution (Sigma-Aldrich, Saint Louis, MO, USA) was added, followed by the addition of 400 μl of 1 M NaOH (Carlo Erba, Milan, Italy). The mixture was vortexed vigorously and absorbance measured at 510 nm (Perkin Elmer Lambda Bio UV/VIS). Total flavonoid content was expressed as milligrams of (+)-catechin equivalent per gram of plant material (mg CTE/g D.W.).

2.4. *Ferric-reducing antioxidant power*

One of the methods used for the assessment of the antioxidant potential of the extracts was ferric-reducing antioxidant power (FRAP) assay.[16] In brief, the working FRAP reagent was prepared by mixing 25 ml of 300 mM acetate buffer (pH 3.6), 2.5 ml of 10 mM TPTZ (2,4,6-tri(2-pyridyl)-s-triazine; Sigma-Aldrich, Saint Louis, MO, USA) in 40 mM HCl, and 2.5 ml of 20 mM $FeCl_3$ (Zorka Pharma a.d., Šabac, Serbia). A 70-µl sample was added to 2.1 ml of FRAP reagent. After 5 min incubation period, absorbance was measured at 593 nm (Perkin Elmer Lambda Bio UV/VIS). A standard curve was prepared using 0–100 µM $FeSO_4 \cdot 7H_2O$ aqueous solution (Sigma-Aldrich, Saint Louis, MO, USA). Results were expressed in micromoles of Fe^{2+} per gram of plant material (µmol Fe^{2+}/g D.W.).

2.5. *DPPH method*

Another method used for the assessment of the antioxidant potential of the extracts was the DPPH method, which is based on the reduction of the stable DPPH (2,2-diphenyl-1-picrylhydrazyl) radical by antioxidants present in the extracts.[17] So, 100 µl of each extract diluted to five different concentrations was added to a set of five test tubes containing 1.4 ml of DPPH solution (Sigma-Aldrich, Saint Louis, MO, USA) whose absorbance was set to 0.8 at 517 nm. The mixture was kept in the dark for 20 min and the absorbance measured at 517 nm (Perkin Elmer Lambda Bio UV/VIS). Results were expressed as an IC_{50} value, which is the concentration of extract required to scavenge 50 % of DPPH radicals (mg/ml).

2.6. *Experimental design*

A full factorial design with two factors, three levels (3^2), and nine experimental points was used for evaluation of the effects of independent variables (factors) on the dependent variable (response). Factors were the content of methanol in the solvent (X_1: 50, 75, and 100 % aqueous methanol) and extraction time (X_2: 30, 60, and 90 min). Each factor was coded using the following equation:

$$X_i = (x_i - x_0)/\Delta x_i, \quad (1)$$

where X_i is the coded variable value, x_i is a real value of the variable, x_0 is a real value at the central point, and Δx_i is the distance between the real value at the central point and the maximum or minimum value of a variable (Table 1).

T a b l e 1

Real and coded values of factors: methanol content (c_{MeOH}) an extraction time (t)

Factor	Real value	Coded value
	c_{MeOH} / %	
	50	-1
X_1	75	0
	100	1
	t / min	
	30	-1
X_2	60	0
	90	1

Responses were total flavonoid content and antioxidant capacity (DPPH and FRAP); these values were measured at each experimental point. A second-order polynomial equation was used for fitting the data and predicting the response:

$$Y = \beta_0 + \sum_{i=1}^{n} \beta_i X_i + \sum_{i=1}^{n} \beta_{ii} X_i^2 + \sum_{\substack{i=1 \\ i<j}}^{n-1} \sum_{j=2}^{n} \beta_{ij} X_j, \quad (2)$$

where X_1, X_2…, X_n are factors, Y is the response, while β_0, β_i, β_{ii}, and β_{ij} are regression coefficients for the intercept, linear, quadratic, and cross-product terms, respectively. The quality of the fit was evaluated using a coefficient of determination R^2 and absolute average deviation (AAD).

2.7. *Statistical analysis*

Statistical analysis, experimental design, RSM, and prediction and verification of the model were performed using Statistica 7.0 (StatSoft Inc., Tulsa, OK, USA). Differences between groups of data were analyzed using one-way ANOVA and Fisher LSD test. Results were expressed as mean ± standard error with a significant p-value of less than 0.05.

3. RESULTS AND DISCUSSION

3.1. *Preliminary selection of the extraction solvent*

Qualitatively and quantitatively, extraction yield depends on the polarity of the compounds being extracted, which is why the extraction solvent is a major factor influencing the composition and the yield of the extracts.[18,19] So, we first compared the capacity of aqueous methanol and aque-

ous ethanol to extract flavonoids from stinging nettle leaves, as well as the antioxidant capacities of the extracts. The results are presented in Table 2. Comparisons were made between extracts with the same methanol or ethanol content in the solvent and the same extraction time. Regardless of the extraction time, 75 and 100 % aqueous methanolic extracts resulted in significantly higher total flavonoid content ($p < 0.001$) and significantly higher FRAP antioxidant capacity than did the 75 and 96 % aqueous ethanolic extracts. The only exception was 75 % aqueous methanolic extract prepared with 90 min extraction time, which had FRAP antioxidant capacity similar to 75 % aqueous ethanolic extract prepared with the same extraction time. Furthermore, 100 % aqueous methanolic extracts had significantly higher DPPH antioxidant capacity ($p < 0.001$) than 96 % aqueous ethanolic extracts, independent of extraction time. On the other hand, regardless of the extraction time, lower total flavonoid content and FRAP antioxidant capacity of the extracts were achieved using 50 % aqueous methanol compared to 50 % aqueous ethanol (Table 2). Since 75 and 100 % aqueous methanolic extracts showed higher total flavonoid content and higher antioxidant capacities compared to 75 and 96 % aqueous ethanolic extracts, and since 75 % aqueous methanolic extracts prepared with 90 min extraction had the highest flavonoid contents of all the extracts, aqueous methanol was chosen for further optimization.

T a b l e 2

Total flavonoid content (TF) and antioxidant capacity (FRAP and DPPH) of stinging nettle leaf extracts

$c_{Alc.}$[c] / %	t / min	TF, mg CTE/g D.W. ± S.E.[b]	FRAP, μmol Fe^{2+}/g D.W. ± S.E.	IC_{50}[a] (DPPH), mg/ml ± S.E.
50 % methanol	30	3.27 ± 0.03***	52.7 ± 2.9***	0.338 ± 0.028
50 % methanol	60	3.99 ± 0.04**	61.5 ± 0.9***	0.309 ± 0.024
50 % methanol	90	3.64 ± 0.06	59.4 ± 0.7*	0.664 ± 0.017
75 % methanol	30	4.65 ± 0.32***	61.7 ± 2.2***	0.640 ± 0.047
75 % methanol	60	4.61 ± 0.15***	61.6 ± 2.8**	0.359 ± 0.030
75 % methanol	90	4.68 ± 0.19***	63.7 ± 1.5	0.421 ± 0.021
100 % methanol	30	1.82 ± 0.15***	11.2 ± 0.8*	1.550 ± 0.329***
100 % methanol	60	1.82 ± 0.15***	15.8 ± 1.1**	4.290 ± 0.529***
100 % methanol	90	1.74 ± 0.14***	19.1 ± 0.3**	2.440 ± 0.215***
50 % ethanol	30	4.19 ± 0.04	67.9 ± 2.5	0.527 ± 0.027
50 % ethanol	60	4.32 ± 0.08	75.5 ± 7.1	0.291 ± 0.048
50 % ethanol	90	3.85 ± 0.04	66.4 ± 1.1	0.452 ± 0.041
75 % ethanol	30	3.18 ± 0.13	45.6 ± 1.7	0.699 ± 0.028
75 % ethanol	60	3.49 ± 0.09	51.8 ± 1.4	0.524 ± 0.020
75 % ethanol	90	3.95 ± 0.06	63.9 ± 0.5	0.529 ± 0.023
96 % ethanol	30	0.58 ± 0.01	3.6 ± 0.1	14.435 ± 0.999
96 % ethanol	60	0.61 ± 0.02	5.3 ± 0.1	9.777 ± 0.543
96 % ethanol	90	0.86 ± 0.03	9.5 ± 0.2	8.976 ± 0.368

[a] IC50 – the concentration of extract required to scavenge 50 % of DPPH radicals; statistical significance: ** $p < 0.01$ and *** $p < 0.001$ (50, 75 and 100 % methanol *vs.* 50, 75 and 96 % ethanol, respectively);
[b] S.E. – standard error; [c] $c_{Alc.}$ – alcohol concentration (methanol or ethanol)

3.2. *Response surface models and optimized values of extraction parameters*

Response surface plots, which visualized the effects of extraction time and methanol concentration on total flavonoid content and antioxidant capacity of the extracts (FRAP and DPPH) are presented in Figures 1, 2 and 3, respectively. Regression coefficients, obtained by the least squares method, are presented in Table 3. Predicted values for total flavonoid content and antioxidant capacity are presented in Table 4.

T a b l e 3

Regression coefficients (Reg. cf.), standard errors (S.E.), and p-values of the fitted second-order polynomial equation for total flavonoid content (TF) and antioxidant capacity (FRAP and DPPH) of the extracts

Equation's term	TF			FRAP			IC_{50}[d] (DPPH)		
	Reg. cf.	S.E.	p[c]	Reg. cf.	S.E.	p	Reg. cf.	S.E.	p
Intercept									
β_0	4.76***	0.16	<0.001	63.48***	2.13	<0.001	0.90	0.74	0.31
Linear									
β_1 (X_1[a])	–0.92**	0.09	0.002	–21.25***	1.17	< 0.001	1.16	0.41	0.06
β_2 (X_2[b])	0.05	0.09	0.576	2.77	1.17	0.098	0.17	0.41	0.71
Cross-product									
β_{12} ($X_1 X_2$)	–0.11	0.11	0.377	0.28	1.43	0.855	0.14	0.50	0.79
Quadratic									
β_{11} (X_1^2)	–1.93**	0.15	0.001	–25.73**	2.03	0.001	1.13	0.70	0.21
β_{22} (X_2^2)	–0.17	0.15	0.339	–1.70	2.03	0.464	–0.64	0.70	0.43

[a] X_1 – methanol content; [b] X_2 – extraction time; [c] statistical significance: **p < 0.01 and ***p < 0.001;
[d] IC_{50} – the concentration of the extract required to scavenge 50 % of DPPH radicals

T a b l e 4

Experimental (R_{exp}), predicted (R_{pred}), and residual (Res.) values for total flavonoid content (TF) and antioxidant capacity (FRAP and DPPH) of the extracts

Coded values		TF			FRAP			IC_{50}[a] (DPPH)		
X_1[b]	X_2[c]	R_{exp}	R_{pred}	Res.	R_{exp}	R_{pred}	Res.	R_{exp}	R_{pred}	Res.
1	1	1.74	1.68	0.06	19.1	17.9	1.2	2.44	2.85	–0.41
0	–1	4.65	4.53	0.11	61.7	59.0	2.7	0.64	0.09	0.55
–1	1	3.64	3.74	–0.10	59.4	59.8	–0.4	0.66	0.25	0.42
0	0	4.61	4.76	–0.15	61.6	63.5	–1.8	0.36	0.90	–0.54
0	1	4.68	4.64	0.04	63.7	64.6	–0.9	0.42	0.43	–0.00
1	–1	1.82	1.79	0.03	11.2	11.7	–0.6	1.55	2.24	–0.69
–1	0	3.99	3.75	0.24	61.5	59.0	2.5	0.31	0.87	–0.56
1	0	1.82	1.91	–0.09	15.8	16.5	–0.7	4.29	3.19	1.10
–1	–1	3.27	3.41	–0.14	52.7	54.8	–2.1	0.34	0.20	0.14

[a] IC_{50} – the concentration of extract required to scavenge 50 % of DPPH radicals;
[b] X_1 – coded value of methanol content; [c] X_2 – coded value of extraction time

The polynomial equation used to model the influence of the methanol content and the extraction time on total flavonoid content, as well as FRAP antioxidant capacity, produced satisfactory fits to the data (R^2 = 0.99, R^2_{adj} = 0.97, and ADD = 3.3 % for total flavonoid content; R^2 = 0.99, R^2_{adj} = 0.98, and ADD = 3.6 % for FRAP antioxidant capacity), while in the case of DPPH antioxidant capacity, the coefficient of determination was somewhat lower, while the value of AAD was high (R^2 = 0.80, R^2_{adj} = 0.46, and ADD = 67.7 %). The following equations are mathematical models that describe the influence of the methanol content and extraction time on total flavonoid content [Eq. (3)], FRAP [Eq. (4)], and DPPH [Eq. (5)] antioxidant capacity:

$$TF = -1.93\,X_1^2 - 0.92\,X_1 - 0.17\,X_2^2 + 0.05\,X_2 - 0.11\,X_1\,X_2 + 4.76 \quad (3)$$

$$FRAP = -25.73\,X_1^2 - 21.25\,X_1 - 1.70\,X_2^2 + 2.77\,X_2 + 0.28\,X_1\,X_2 + 63.48 \quad (4)$$

$$IC_{50} = 1.13\,X_1^2 + 1.16\,X_1 - 0.64\,X_2^2 + 0.17\,X_2 + 0.14\,X_1\,X_2 + 0.90 \quad (5)$$

The influence of the factors on the response variables was estimated using probability value and the regression coefficients (Table 3). Methanol content had significant linear ($p < 0.01$) and quadratic ($p < 0.01$) effects on flavonoid content in the extracts. With the increase in methanol content, total flavonoid content increased up to a certain point (up to 69 % methanol), after which, with a further increase in methanol content, it started to decrease (Fig. 1, Table 2). The quadratic effect of this factor was negative, which indicates that there was a maximal value of total flavonoid content in the range of methanol content tested. This is in accordance with results of our previous study, where the content of methanol in the solvent influenced total phenolic content in stinging nettle leaf extracts in a similar manner, as well as with the results from several other optimization studies that showed that the increase in the content of alcohol in the solvent, after an initial increase, caused a decrease in the content of total flavonoids.[7,20,21] Since methanol is less polar than water, a reduction in the extractability of total flavonoids with a further increase in methanol content (>69 % methanol) indicates that the decrease in solvent polarity did not favor extraction of total flavonoids from the stinging nettle leaf matrix. The solubility of flavonoids and phenolic compounds is heavily influenced by their physicochemical nature, the degree of polymerization, and interaction with other constituents in the plant matrix, as well as by the formation of insoluble complexes.[22,23] This makes the extraction of these compounds very challenging, since there is no uniform approach to the extraction of all flavonoids or of any specific subclass of phenolic compounds whatsoever.[24] The characterization of phenolic compounds present in stinging nettle leaf extract showed that it is rich in flavonol glycosides, and it is known that glycosylation increases the polarity of flavonoids.[24] This could explain the decrease in extractability of flavonoids with a further increase in methanol content in the solvent. Another possible reason for the higher extractability of flavonoids by solvents with higher water content might be the fact that water causes swelling of plant material.[25] This swelling increases the contact surface area between the plant matrix and the solvent, which consequently increases the diffusion of plant components into the solvent and improves extraction efficiency.[25]

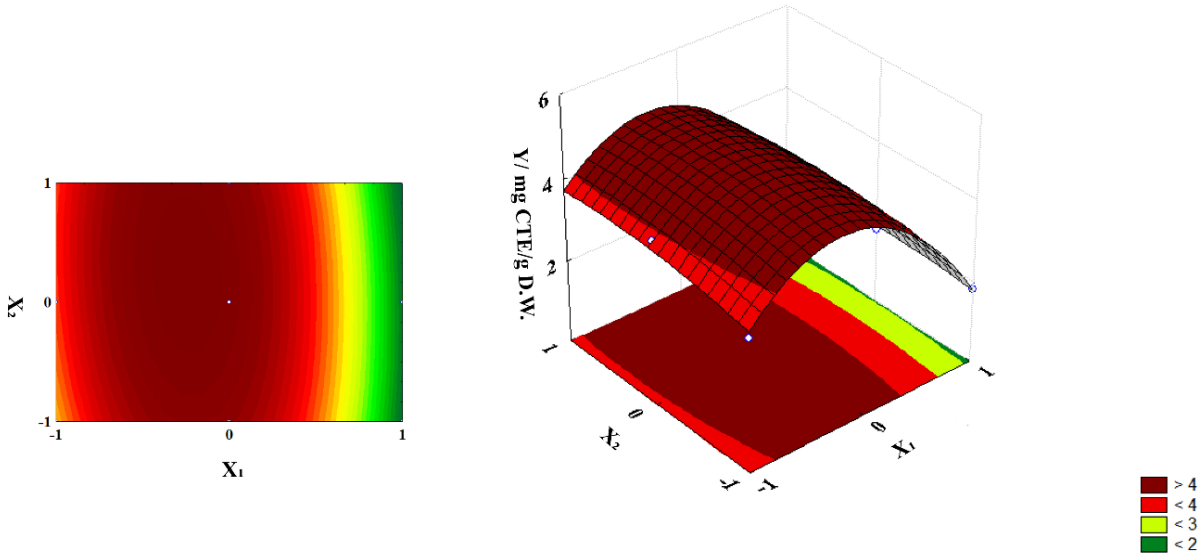

Fig. 1. Response surface plot for total flavonoid content (Y, mg CTE/g D.W.) as a function of methanol content (X_1) and extraction time (X_2) in coded form

Methanol content had significant linear ($p < 0.001$) and quadratic ($p < 0.01$) effects on FRAP antioxidant capacity, which is similar to the effect on total flavonoid content: the increase in methanol content to a certain point (65 % methanol) caused an increase in FRAP antioxidant capacity, but a further increase caused a decrease in this response (Fig. 2). This is to be expected, since the quadratic effect of this factor was also negative for FRAP antioxidant capacity, which implies the existence of a maximum in this response in the range of methanol content tested. It is widely accepted that flavonoids are powerful antioxidants, since there is a great deal of evidence in the literature of their

free radical scavenging and metal-ion reducing abilities.[26] The fact that methanol content influenced FRAP antioxidant capacity in the same manner as it affected total flavonoid content is not surprising, since flavonoids are known for their antioxidant capacity.[1] Furthermore, flavonoids detected in stinging nettle leaf extracts are all flavonols, which have a molecular structure that enhances the ferric-reducing ability; namely, structural specificities of these compounds (hydroxyl groups in an *ortho* position on the B-ring and the 3-hydroxy-4-keto-5-hydroxy structure) are paramount for good ferric-reducing capacity.[7,26]

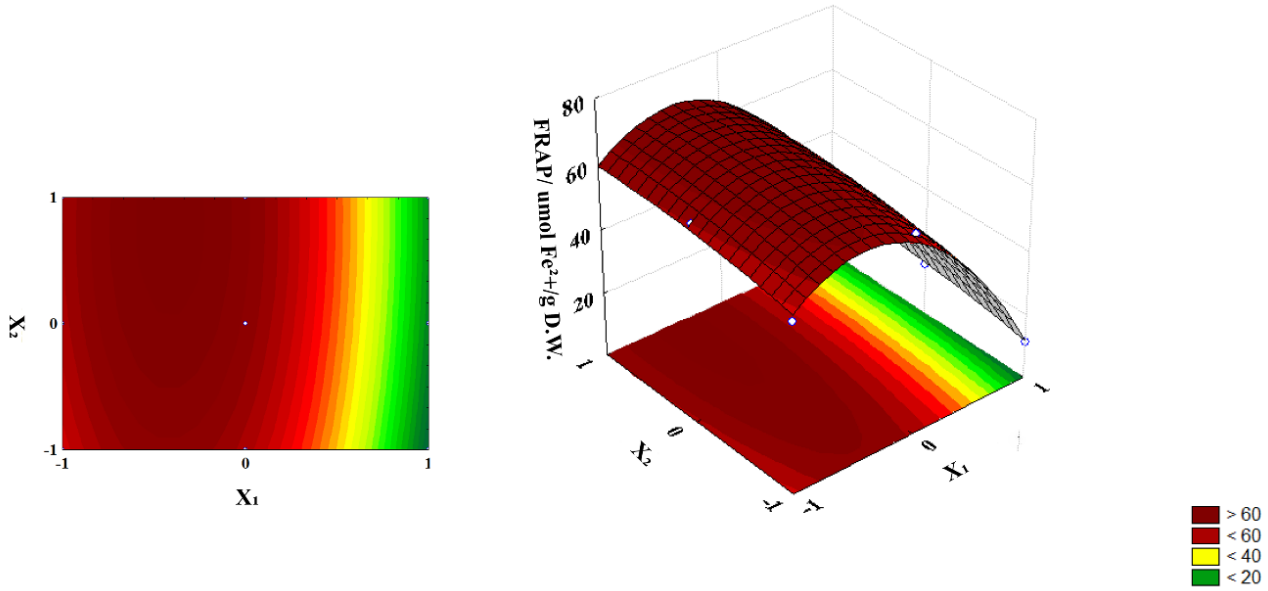

Fig. 2. Response surface plot for FRAP antioxidant capacity (Y, μmol Fe^{2+}/g D.W.) as a function of methanol content (X_1) and extraction time (X_2) in coded form

The results of the regression analysis showed that antioxidant capacity assessed by the DPPH method had no significant terms for the tested factors, either linear or quadratic (Table 3). The fact that the quadratic term of methanol content for this response was positive indicated that there was a minimum in the IC_{50} value. Having in mind that this value is inversely correlated with antioxidant capacity, this means that the increase in methanol content first caused an increase in antioxidant capacity, but after a certain point DPPH antioxidant capacity started to decline. Even though non-significant, the effect of methanol content on DPPH antioxidant capacity is in an agreement with the effects of this factor on total flavonoid content and FRAP antioxidant capacity.

It is expected that the total flavonoid concentration in the bulk solution, as well as the antioxidant capacity of the extracts, increases with an increase in extraction time.[9] Contrary to this, extraction time had no effect on total flavonoid content and FRAP and DPPH antioxidant capacities since neither the linear nor quadratic effect of this factor was significant for any of these responses (Table 3). For the total flavonoid content, this is in accordance with the fact that extraction time did not affect of the total phenolic content in the stinging nettle leaf extracts when aqueous methanol was used as the solvent.[7] This result is also in agreement with another study that also reported that extraction time did not influence the yield of phenolic compounds.[27] This could indicate that the increase in total flavonoid content that would be expected with the increase in extraction time was, at least partially, overpowered by the oxidation and/or degradation of flavonoids in the extracts, as extracted flavonoids are very susceptible to oxidation by other compounds usually present in bulk solution; this could have influenced the total flavonoid content causing a decrease with an increase in extraction time.[28] Furthermore, oxidation and degradation of flavonoids could also be the reason why extraction time did not significantly influence the DPPH and FRAP antioxidant capacity of the extracts.

The nature of the response surface system (maximum, minimum, or saddle point) depends on the signs and magnitudes of the second-order coefficients in the equation used as a model.[11] According to the RSM, maximal total flavonoid content in

stinging nettle leaf extract would be achieved using 69 % aqueous methanol and 67 min extraction to give a projected total flavonoid content of 4.88 mg CTE/g D.W (Fig. 4). Total flavonoid content achieved with these values of extraction parameters was in accordance with this projected value, at 4.79 mg CTE/g D.W. In the literature, 50–80 % aqueous methanol solvents have been reported to be the most effective for the extraction of flavonoids from different plant sources.[29] The wide range of methanol content is probably due to structural differences in the extracted flavonoids, differences in plant material and extraction technique, as well as differences in other extraction factors that influence the extractability and yield of flavonoids.[30] Maximal total flavonoid content was achieved with higher methanol content and longer extraction time (69 % aqueous methanol and 67 min) compared to previously described optimal extraction conditions for maximal total phenolic content (54 % and 38 min).[7] In our previous study, as well as flavonoids, several phenolic acids were identified in stinging nettle leaf extracts, and they accounted for a significant proportion of the quantified phenolic compounds.[7] Flavonoids are less polar than phenolic acids; this could be the reason why a less polar solvent with a greater methanol content is necessary to achieve the maximal total flavonoid content compared to the more polar solvent used for maximal total phenolic content (69 % vs 54 % aqueous methanol, respectively).[30] The longer extraction time necessary for achieving maximal total flavonoid content could be explained by differences in the location of subclasses of phenolic compounds in the plant cell; i.e., flavonoids are usually located in vacuoles, while phenolic acids are mostly bound to cell wall constituents.[31]

The values of extraction parameters for achieving maximal antioxidant capacity of the extracts differed substantially depending on the assessment method used. Firstly, the FRAP method predicted a maximal antioxidant capacity of 68.9 μmol Fe^{2+}/g D.W. with 65 % aqueous methanol and 83 min extraction time (Fig. 4). The experimental value of antioxidant capacity achieved with these parameters was 66.2 μmol Fe^{2+}/g D.W. It is evident that the extraction parameters to achieve maximal FRAP antioxidant capacity are similar to those for maximal total flavonoid content. As previously mentioned, extraction time and methanol content did not affect DPPH antioxidant capacity to a significant extent. Also, the effects of these factors, even though not significant, were fundamentally different; namely, the mixed signs of second-order coefficients for methanol content and extraction time (Eq. 5) indicate that there was a saddle point in the response surface plot for DPPH antioxidant capacity (Fig. 3). This means that in the chosen range of factors the maximum or minimum values of this response were not found. These differences in the effects of extraction parameters on antioxidant capacities could be explained by the differences in the reaction mechanisms of FRAP and DPPH assays.[32]

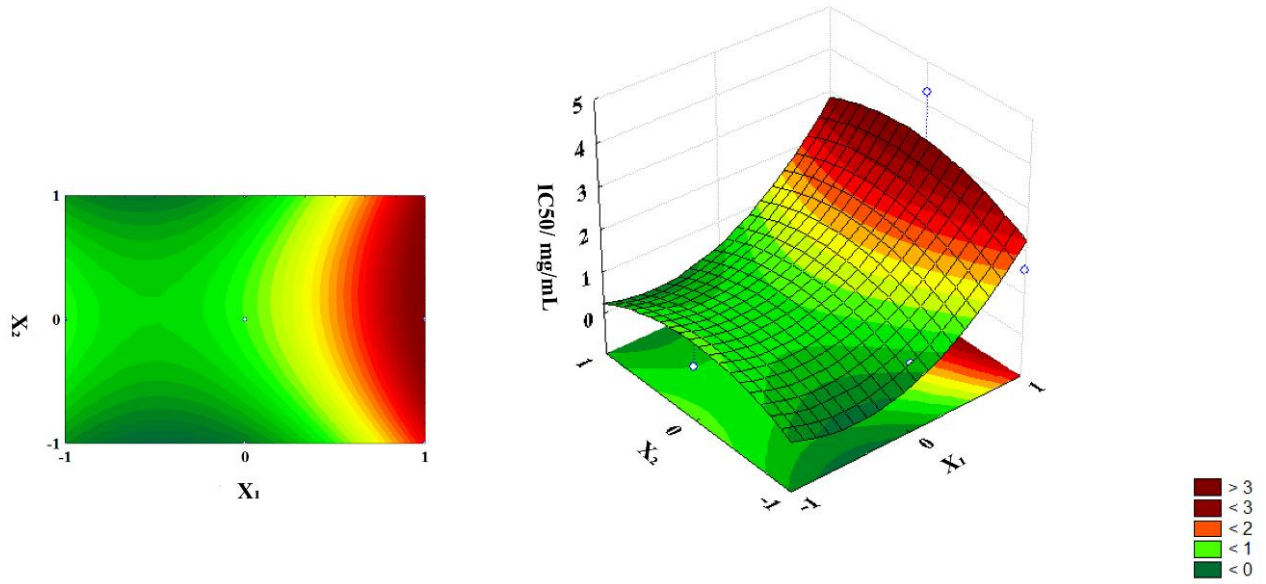

Fig. 3. Response surface plot for DPPH antioxidant capacity (Y, mg/ml) as a function of methanol content (X_1) and extraction time (X_2) in coded form

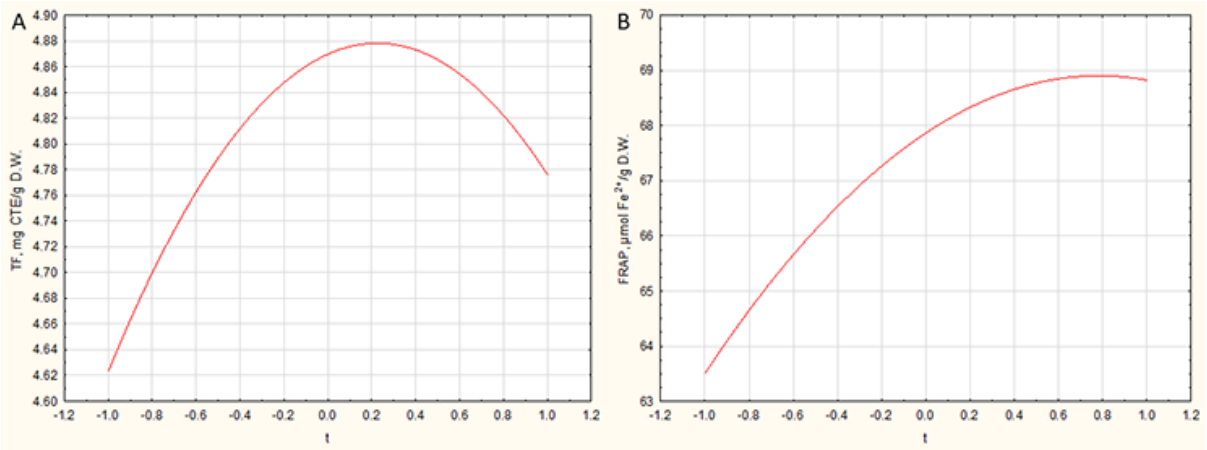

Fig. 4. 2D graphs of the predicted total flavonoids (TF, mg CTE/g D.W.; 69 % MeOH, Fig. 4A) and FRAP antioxidant capacity (µmol Fe^{2+}/g D.W.; 65 % MeOH, Fig. 4B) obtained with the detected optimal extraction solvent (methanol %)
t – time factor in the coded form [–1,1]

4. CONCLUSIONS

This study showed that aqueous methanol was more efficient than aqueous ethanol in the extraction of antioxidants from stinging nettle leaves. RSM proved useful in testing the effects of methanol content and extraction time on total flavonoid content and antioxidant capacity of stinging nettle leaf extracts. The second-order polynomial equation was proven to be appropriate for modeling the influence of methanol content in the solvent and extraction time on total flavonoid content and FRAP antioxidant capacity. We found that the methanol content significantly affected total flavonoid content and FRAP antioxidant capacity, while extraction time had no significant effect on either response. We determined that 69 % aqueous methanol and 67 min extraction time and 65 % aqueous methanol and 83 min extraction time were optimal for achieving maximal total flavonoid content (predicted value 4.88 mg CTE/g D.W.) and maximal FRAP antioxidant capacity (predicted value 68.9 µmol Fe^{2+}/g D.W.), respectively. These results could be the first step in the development of the industrial process of extraction of total flavonoids from the leaves of stinging nettle (a low-cost and low-maintenance phenolic-rich plant), important because dietary flavonoids represent an important source of antioxidants significant to human health.

Acknowledgment: The authors acknowledge their gratitude to the Ministry of Education, Science, and Technological Development of the Republic of Serbia for financial support (contract numbers: 451-03-68/2022-14/200015 and 451-03-68/2022-14/200003).

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