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OPTIMIZATION OF ULTRASOUND-ASSISTED EXTRACTION OF (POLY)PHENOLIC COMPOUNDS FROM BLUEBERRY (*Vaccinium myrtillus*) LEAVES

Article Highlights

- The degree of correlation (R^2) for all Responses is extremely high
- High temperatures are most effective in extracting anthocyanins
- The extraction of flavonoids is better at medium solid-to-solvent ratios
- The extraction is most effective with a medium amount of ethanol in the solvent

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Abstract

The present paper aims to discover the optimal conditions for ultrasound-assisted extraction (UAE) of (poly)phenolic chemicals from blueberry (Vaccinium myrtillus) leaves. UAE was performed under the following process conditions: temperature: 25–65 °C, ethanol concentration in the extraction solvent: 30–90 vol.%, and solid-to-solvent ratio: 1:15-1:45 w/v. Statistical analysis was performed using Design-Expert software, using the Box-Behnken design. The study's responses were the content of total (poly)phenols, flavonoids, and anthocyanins in the derived extracts. The results indicated that the corresponding response surface models were highly statistically significant ($p < 0.0001$) and sufficient to describe and predict the content of total (poly)phenols, the content of flavonoids, and the content of anthocyanins with R^2 of 0.965, 0.980 and 0.972, respectively. The optimal conditions for the extraction are for total (poly)phenols 48.4 °C, 51.3 vol.% ethanol, and 1:43.8 w/v solid-to-solvent ratio; flavonoids 58.5 °C, 48.0 vol.% ethanol, and 1:29.8 w/v ratio; and anthocyanins 64.2 °C, 73.5 vol.% ethanol, and 1:44.7 w/v ratio. The use of UAE enhances extraction yields by increasing the release of bioactive compounds, while the application of the Box-Behnken design allows for precise determination of optimal extraction parameters, thereby achieving maximum yields and efficiency.

Keywords: anthocyanins; blueberry; extraction; flavonoids; optimization; (poly)phenols.

SCIENTIFIC PAPER

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Blueberries (*Vaccinium* spp.) are well-known for their excellent taste and nutritious value around the world [1]. Furthermore, research has shown that blueberry fruits have a variety of bioactive qualities, including antioxidant activity [2], anticancer [3], anti-inflammatory [4], and cardioprotective properties [5]. Anthocyanins, phenolics, and other antioxidants are found in various blueberry species, including *Vaccinium angustifolium*, *Vaccinium ashei Reade*, *Vaccinium corymbosum* L., and *Vaccinium myrtillus* L. [6]. The presence of bioactive substances such as anthocyanins, flavonoids, and phenolic acids may be connected to the above-described pharmacological characteristics [7,8]. Anthocyanins, one type of flavonoid, are antioxidants that are crucial in lowering the risk of certain degenerative illnesses in humans

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[9,10]. Additionally, they can prevent cardiovascular disease and improve vision due to their antioxidant and anti-inflammatory properties [11]. The delicious fruit and abundance of anthocyanins have led to a continual increase in blueberry cultivation worldwide. However, in many countries, the leaves are discarded after pruning and represent agrifood waste. Nonetheless, blueberry leaves can be used for preventive effects against anemia, premature aging, and cataracts [8]. Other studies have suggested that blueberry leaf extracts exhibit remarkable biological activities, including hypolipidemic activity [12], anti-leukemic activity [13], suppression of hepatitis C virus [14], antioxidant activity [15], and antimicrobial activity [16]. Some research on the chemical composition of blueberry leaves (*V. angustifolium*) has indicated richness in chlorogenic acids and quercetin glycosides [17]. In leaves of rabbiteye blueberry (*V. ashei*), flavan-3-ols and proanthocyanidins have been identified as major phenolic components alongside chlorogenic acids and flavonol glycosides [18]. Therefore, the application of phenolic compounds from discarded blueberry leaves is environmentally friendly and contributes to the utilization of beneficial health-promoting compounds. Utilizing blueberry leaves not only reduces waste but also supports the circular economy by valorizing agrifood wastes. With increasing interest in maximizing blueberry plant utilization, more scientists are exploring the extraction potential of (poly)phenolic compounds from blueberry leaves.

Supercritical fluid extraction, ultrasonic-assisted extraction (UAE), enzyme-assisted extraction, and solvent extraction are the main techniques that can be used to extract (poly)phenols from plants [6]. Among these, UAE is an effective, economical, and environmentally friendly approach. The mechanism of UAE is as follows. Phases of compression and rarefaction follow one another when the solvent molecules move longitudinally across an elastic media caused by the ultrasonic wave. The solvent molecules will collide with the surrounding molecules during the compression phase. Negative pressure is applied during the rarefaction phase, which causes the molecules to separate and causes cavitation bubbles to form in the liquid. The dissolved gas will enter the bubble and cause the cavitation bubbles to expand. Hotspots would form when the bubbles collapse, and in an ultrasonic bath at normal temperature, the temperature and pressure might reach up to 5000 K and 5.06×10^5 kPa respectively. The plant matrix's cell walls would be destroyed by the hotspots, releasing chemical compounds into the solvent [19]. For numerous reasons, such as simplicity, low acquisition cost, no specific maintenance requirements, and availability in most laboratories, UAE has been widely applied in the extraction of bioactive compounds, not only from blueberry fruits [20], but also from blueberry byproducts as pomace [21,22], or leaves [12,23].

Response surface methodology (RSM) has been successfully used recently to examine process optimization [24,25]. Finding the optimal conditions for the process is the primary goal of the RSM. Using statistical design techniques can reduce variation, the amount of time needed for adjustment, and total cost by increasing efficiency and bringing output outcomes closer to nominal values (goals) [26]. The Box-Behnken design (BBD) is a type of rotatory design that focuses on the midpoints of the edges and center points within a cubic region. This strategy helps to avoid extreme experimental conditions and reduces the likelihood of obtaining inaccurate results [27]. BBD is often used for the UAE process due to its efficiency, especially when dealing with three or more variables. It allows for the evaluation of the independent effects or interactions of these variables on the response variable [28].

This study will investigate the influence of various process parameters (temperature, ethanol concentration in the extraction solvent, and the solid-to-solvent ratio) on the ultrasound-assisted extraction (UAE) of (poly)phenols from blueberry leaves (*V. myrtillus*). Using the BBD with the MINITAB 21 software, the paper aims to determine the efficiency of the extraction process based on these parameters.

EXPERIMENTAL

Plant materials and reagents

Dried blueberry leaves, obtained from a local market, were used for extraction (Figure 1). They are known for their darker green to brownish color, with a more brittle texture. Ethanol was used for sample extraction, while extract characterization was performed using the following reagents: Folin-Ciocalteu reagent (Carlo Erba, Germany), sodium carbonate (Lach:ner, Czech Republic), gallic acid (Sigma Aldrich, USA), aluminum chloride (Lach:ner, Czech Republic), sodium hydroxide (Lach:ner, Czech Republic), sodium nitrite (Zorka Šabac, Serbia), catechin hydrate (Sigma Aldrich, USA), acetate buffer pH=4.5 (Lach:ner, Czech Republic) and potassium chloride buffer pH=1.0 (Lach:ner, Czech Republic).

Methods

Determination of total (poly) phenol content is based on oxidation-reduction reactions involving hydroxyl groups of phenol and the Folin-Ciocalteu reagent, as well as polymer complex ions of molybdenum and tungsten. The reaction requires

Figure 1. Dried leaves used of V. myrtillus.

a basic environment, which is created by adding sodium carbonate to the reaction mixture. In a test tube, 1.5 ml of working Folin-Ciocalteu solution, 0.2 ml of the sample being tested, and 1.5 ml of sodium carbonate were added. The mixture was left to stand for 30 minutes in the dark at room temperature, and then the absorbance was measured in a 10 mm cuvette at 765 nm, with gallic acid utilized as the standard [29]. A Shimadzu 1800 spectrophotometer (Cole-Parmer, USA) was utilized for spectrophotometric determination, with the calibration curve ranging from 50 to 500 mg/l of gallic acid. The results are given in milligrams of gallic acid equivalent per gram of plant material (mg GAE/g).

The flavonoid content of the sample is determined using the colorimetric technique with aluminum chloride. In an acidic solution, aluminum chloride forms stable complexes with the C-4 keto group or the C-3 and C-5 hydroxyl groups of the present flavones and flavonols, and unstable complexes with ortho-dihydroxyl groups in the A or B ring of flavonoids. In a test tube, 1 ml of the sample being tested and 0.5 ml of 5% sodium nitrite solution were added and left to stand for 5 minutes. Then, 0.5 ml of 10% aluminum chloride was added, and after 6 minutes, 2 ml of 1M NaOH solution was added. The absorbance was measured at 450 nm. The results were expressed as mg of catechin equivalent per milliliter of extract solution. This modified method is described in [30]. For the determination of flavonoids, the calibration curve was in the range of 20 to 200 mg/l of catechin hydrate. The results are given in milligrams of catechin hydrate equivalents per gram of plant material (mg CTH/g).

The quantitative determination of total anthocyanins (non-degraded monomers and products of their degradation) is based on the property of anthocyanins to reversibly change their structure when the pH of the environment changes, which also changes the absorption spectrum. The content of total anthocyanins is determined by the 'pH differential' method, as described in reference [31]. The procedure for determining anthocyanins is as follows: two test tubes are prepared for each sample. In each test tube, 0.5 ml of the prepared sample is pipetted. Then, 3.5 ml of pH 1.0 buffer is added to one test tube, and 3.5 ml of pH 4.5 buffer is added to the other. After 20 minutes, the absorbance of the reaction solutions is measured at 520 nm and 700 nm. The total anthocyanins concentration in the sample is determined as cyanidin-3-glucoside equivalent (mg Cy3G/g) using the formula [32]:

$$C_{tot} = (A \cdot M \cdot F \cdot 10^3) / \varepsilon \cdot l \cdot R \quad (1)$$

where are: C_{tot} - total anthocyanins content; A - $(A_{520nm} - A_{700nm})_{pH=1.0} - (A_{520nm} - A_{700nm})_{pH=4.5}$; M - molar mass (for Cy3G it is 449,2 g/mol); F - dilution factor; 10^3- factor for converting grams to milligrams; ε - molar absorption extinction coefficient (for Cy3G it is 26900 $Lmol^{-1}$ cm^{-1}); l - cuvette thickness (1 cm), and R – factor for recalculating the value of anthocyanins per gram of drug.

A Shimadzu 1800 spectrophotometer was used to determine anthocyanins, the same as it was for total (poly)phenols and flavonoids.

Experimental design and statistical analysis

Experimental design and statistical analysis were performed in Design-Expert 13 software (Stat-Ease Inc, USA) using the RSM.

A BBD, as a form of the RSM, was performed to determine the effect of three experimental factors (temperature, solid-to-solvent ratio, and ethanol concentration in solvent) on the output variables (responses) (Table 1). The extraction time was 30 min and an ultrasonic bath was used for mixing.

Table 1. Coded and actual levels of independent variables used in the RSM design for the process of ultrasonic extraction of blueberry leaves.

Symbol	Independent variables	Levels		
		-1	0	1
A	Temperature [°C]	25	45	65
B	Solid-to-solvent ratio [w/v]	1:15	1:30	1:45
C	The ethanol concentration in the solvent [vol. %]	30	60	90

BBD takes mid-level values of experimental factors, avoiding extreme axial points as in central composite design (CCD) [33]. In this paper, considering the existence of three experimental factors that have three levels, there will be 13 points at the middle level. However, two replicates were performed at the

midpoint of the design to allow estimation of pure error and to calculate the repeatability of the method, resulting in a total of 15 extractions to be performed. To achieve objective results, the experiments were randomized.

The Responses in this study were the content of total (poly)phenols, flavonoids, and anthocyanins in the extract.

The experimental data were fitted to a second-order polynomial model to obtain the regression coefficients. The generalized second-order polynomial model used in the RSM is as follows:

$$Y = a_0 + \sum a_i X_i + \sum a_{ii} X_i^2 + \sum a_{ij} X_i X_j \quad (2)$$

where Y represents the experimental response, a_0 is a constant, a_i, a_{ii}, and a_{ij} are coefficients of linear, quadratic, and interactive regression models, and X_i and X_j are independent variables in coded values.

Lack of fit, coefficient of determination (R^2), and p-value obtained by analysis of variance (ANOVA) were used to assess the adequacy of the developed model. Regression analysis and Surface plots were generated to explain the effects of independent variables on the response.

RESULTS AND DISCUSSION

According to the BBD with three factors, 15 extractions were performed, and the measured and predicted values of response are shown in Table 2. The table also shows the extraction yield, i.e. the measured response value presented as mass percentage (w/w).

Table 2. Yield, measured and predicted values for the response variables.

Std	Run	Process parameters			Responses								
					Total (poly) phenol content			Flavonoid content			Anthocyanin content		
		Temp [°C]	Solid-to-solvent ratio [w/v]	Ethanol concentration in solvent [vol%]	Measured [mg/g]	Predicted [mg/g]	Yield [%; w/w]	Measured [mg/g]	Predicted [mg/g]	Yield [%; w/w]	Measured [mg/g]	Predicted [mg/g]	Yield [%; w/w]
3	1	25	1:45	60	56.42	57.76	5.64	26.04	25.79	2.60	0.21	0.24	0.021
10	2	45	1:45	30	56.79	53.88	53.8	29.27	29.27	2.93	0.24	0.21	0.024
13	3	45	1:30	60	52.20	50.25	5.22	33.57	36.28	3.36	0.29	0.33	0.029
15	4	45	1:30	60	49.50	50.25	4.95	30.13	36.28	3.01	0.42	0.33	0.042
1	5	25	1:15	60	32.52	30.17	3.52	23.85	23.97	2.39	0.20	0.22	0.020
6	6	65	1:30	30	54.70	55.26	5.47	42.16	42.29	4.22	0.32	0.37	0.032
8	7	65	1:30	90	48.84	47.27	4.88	34.24	33.99	3.42	0.54	0.54	0.054
7	8	25	1:30	90	39.38	38.82	3.94	18.29	18.16	1.83	0.31	0.26	0.031
11	9	45	1:15	90	26.68	29.59	2.67	20.97	20.98	2.10	0.19	0.22	0.019
5	10	25	1:30	30	40.34	41.91	4.03	25.78	26.03	2.58	0.06	0.06	0.006
14	11	45	1:30	60	49.04	50.25	4.90	35.87	36.28	3.59	0.34	0.33	0.034
4	12	65	1:45	60	57.47	59.82	5.75	40.34	40.22	4.03	0.70	0.69	0.070
12	13	45	1:45	90	50.66	49.88	5.07	22.27	22.65	2.23	0.45	0.46	0.045
9	14	45	1:15	30	35.87	36.65	3.59	30.91	30.53	3.06	0.11	0.09	0.011
2	15	65	1:15	60	51.24	49.90	5.12	41.38	41.63	4.14	0.39	0.36	0.039

From Table 2, it can be observed that the highest content of total (poly)phenols (57.47 mg/g) was achieved at a higher temperature (65 °C), a higher solid-to-solvent ratio (1:45 w/v), and an ethanol concentration of 60 vol.%, while the lowest content (26.68 mg/g) was achieved at a temperature of 45 °C, a solid-to-solvent ratio of 1:15 w/v, and an ethanol concentration of 90 vol.%. It can be concluded that higher temperatures and higher solid-to-solvent ratios increase the efficiency of (poly)phenol extraction, while lower (poly)phenol content was obtained at medium temperature values and lower solid-to-solvent ratios, suggesting that these conditions are less efficient for the extraction of total (poly)phenols. The highest flavonoid content (42.16 mg/g) was obtained at higher temperatures (65 °C), the lowest ethanol concentration (30 vol.%), and medium solid-to-solvent ratios (1:30 w/v), while lower flavonoid contents (18.29 mg/g) were obtained at lower temperatures (25 °C), higher ethanol concentrations (90%), and a solid-to-solvent ratio of 1:30 w/v. This may indicate that high ethanol concentrations and low temperatures are not suitable for flavonoid extraction. Similar to flavonoids, the highest anthocyanin content (0.70 mg/g) was achieved at a temperature of 65 °C, and the lowest content (0.06 mg/g) at low temperatures (25 °C) and low ethanol concentration (30 vol.%). UAE has shown efficiency in extracting bioactive compounds due to its ability to enhance solvent penetration and allow better diffusion of compounds from plant cells. However, comparing the effect of UAE from this study and microwave-assisted extraction (MAE) from the study [34], it can be observed that MAE achieved higher yields of (poly)phenols in shorter extraction periods.

For detailed determination of the influence of process parameters on ultrasound-assisted extraction, ANOVA analysis and evaluation of the obtained models are used.

The experimental data of each measured variable were fitted into a complete quadratic model. Polynomial

coefficients for the surface response model were calculated by multiple regressions. An *F*-value and a *p*-value were also calculated for each member of the regression model. Choosing a reliability level of 95%, a *p*-value greater than 0.05 was not considered statistically significant. The adjusted R^2 and predicted R^2 were evaluated, to determine whether the given model is adequate after eliminating parameters that do not have a significant impact, i.e., whether the model can accurately predict the responses under different process conditions. ANOVA results for the response surface quadratic model of blueberry leaf extraction are shown in Table 3.

Table 3. ANOVA results for the response surface quadratic model of blueberry leaf extraction.

Source	df	Total (poly) phenol content				Flavonoid content				Anthocyanin content			
		Sum of Squares	Mean Square	F-value	p-value	Sum of Squares	Mean Square	F-value	p-value	Sum of Squares	Mean Square	F-value	p-value
Model	9	1229.64	136.63	15.46	0.0038	857.36	95.26	26.69	0.0011	0.3694	0.0410	19.27	0.0023
Temperature (A)	1	237.51	237.51	26.88	0.0035	514.56	514.56	144.15	< 0.001	0.1713	0.1713	80.46	0.0003
Solid-to-solvent ratio (B)	1	703.69	703.69	79.64	0.0003	0.0820	0.0820	0.0230	0.8854	0.0638	0.0638	29.97	0.0028
Ethanol concentration(C)	1	61.27	61.27	6.93	0.0463	130.82	130.82	36.65	0.0018	0.0710	0.0710	33.35	0.0022
AB	1	78.06	78.06	8.83	0.0311	2.61	2.61	0.7307	0.4317	0.0228	0.0228	10.73	0.0221
AC	1	6.00	6.00	0.6793	0.4473	0.0462	0.0462	0.0129	0.9138	0.0003	0.0003	0.1381	0.7254
BC	1	2.34	2.34	0.2649	0.6287	2.16	2.16	0.6054	0.4717	0.0045	0.0045	2.13	0.2043
A^2	1	5.68	5.68	0.6430	0.4590	0.7230	0.7230	0.2025	0.6715	0.0112	0.0112	5.24	0.0707
B^2	1	15.89	15.89	1.80	0.2376	53.88	53.88	15.09	0.0116	0.0003	0.0003	0.1188	0.7443
C^2	1	118.79	118.79	13.44	0.0145	161.08	161.08	45.13	0.0011	0.0216	0.0216	10.14	0.0244
Residual	5	44.18	8.84			17.85	3.57			0.0106	0.0021		
Lack of Fit	3	38.35	12.78	4.39	0.1912	0.6014	0.2005	0.0232	0.9938	0.0084	0.0028	2.44	0.3036
Pure Error	2	5.83	2.91			17.25	8.62			0.0023	0.0011		
Cor Total	14	1273.82				875.21				0.3801			
Fit Statistics		R^2=0.9653 Adjusted R^2=0.9029 Predicted R^2=0.5080 Adeq Precision=12.4569				R^2=0.9796 Adjusted R^2=0.9447 Predicted R^2=0.9429 Adeq Precision=15.6404				R^2=0.9720 Adjusted R^2=0.9216 Predicted R^2=0.6343 Adeq Precision=16.6827			

The R^2 values for the content of total (poly)phenols, flavonoids, and anthocyanins in the extracts are 0.965, 0.980, and 0.975, respectively. This showed that the response variability was well explained in the generated model, as the models were able to explain 96.5% of the variation in the total (poly)phenol content, 98.0% of the variation in the flavonoid value, and 97.5% of the variation in the anthocyanin content in the extracts. The R^2 value for all three cases is close to 1, which reveals that there is a good correlation between the independent variables and the response. The adjusted R^2 is the corrected value for R^2 after eliminating terms in the model that do not have a significant effect on the responses. The values of the content of total (poly)phenols, flavonoids, and anthocyanins in the extracts are 0.903, 0.943, and 0.922, respectively. These values are very close to the R^2 values, which means that the proposed models can very easily explain the different variations even by eliminating members whose p-values are greater than 0.05.

Predicted R^2 is used to determine how well a regression model makes predictions. The values for predicted R^2 for the content of total (poly)phenols, flavonoids, and anthocyanins in the extracts are 0.508, 0.945, and 0.634, respectively. The predicted R^2 for total (poly)phenol content (0.508) and anthocyanin content (0.634) is not close to the adjusted R^2 (for total (poly)phenols it is 0.903 and 0.922 for anthocyanins) as expected; that is, the difference is greater than 0.2. This may indicate that the model fits the original data, but the predictions are not accurate enough. This indicates that the model is complicated and begins to model noise in the data (a condition known as 'overfitting the model') [35]. The difference between adjusted R^2 (0.945) and predicted R^2 (0.943) for the content of flavonoids in the extract is extremely small, which means that the obtained model provides valid predictions for the new observations.

Adeq Precision represents the signal-to-noise ratio. Its values for the content of total (poly)phenols, flavonoids, and anthocyanins in extracts are 12.5, 15.6, and 16.7, respectively. The values for all three responses are over 4, which indicates that the signal is adequate.

Lack of fit can be used to confirm the validity of the model. By ANOVA analysis for lack of fit values of all responses, it was determined that the p-value is significantly higher than 0.05, which indicates that the models are adequately adapted to the experimental data.

Influence of process parameters on the value of total (poly) phenol content in the extract

Table 4 shows coded and uncoded coefficients of the regression equation and p-values for members in the proposed quadratic model for the content of total (poly)phenols in blueberry leaf extracts.

ANOVA analysis revealed that the content of total

Table 4. Regression coefficients and p-values for all Responses.

Variables	Total (poly) phenol content			Flavonoid content			Anthocyanin content		
	Coded Regression coefficients	Actual Regression coefficients	p-value	Coded Regression coefficients	Actual Regression coefficients	*p*-value	Coded Regression coefficients	Actual Regression coefficients	*p*-value
Constant	+50.25	-22.27383	<0.0001	+36.28	-14.51734	<0.0001	+0.3291	+0.008202	<0.0001
Temperature (A)	+5.45	+0.557594	0.0035	+8.02	+0.392937	< 0.0001	+0.1463	-0.011751	0.0003
Solid-to-solvent ratio (B)	+9.38	+1.73910	0.0003	+0.1012	+1.04854	0.8854	+0.0893	-0.007663	0.0028
Ethanol concentration (C)	-2.77	+0.704903	0.0463	-4.04	+0.704938	0.0018	+0.0942	+0.011736	0.0022
AB	-4.42	-0.014725	0.0311	-0.8075	-0.002692	0.4317	+0.0756	+0.000252	0.0221
AC	-1.22	-0.002042	0.4473	-0.1075	-0.000179	0.9138	-0.0086	-0.000014	0.7254
BC	+0.7650	+0.001700	0.6287	+0.7350	+0.001633	0.4717	+0.0337	+0.000075	0.2043
AA	+1.24	+0.003101	0.4590	+0.4425	+0.001106	0.6715	+0.0550	+0.000137	0.0707
BB	-2.07	-0.009220	0.2376	-3.82	-0.016978	0.0116	-0.0083	-0.000037	0.7443
CC	-5.67	-0.006302	0.0145	-6.60	-0.007339	0.0011	-0.0765	-0.000085	0.0244

(poly)phenols in the extract is strongly influenced by the following parameters ($p < 0.05$): temperature (A), solid-to-solvent ratio (B), ethanol concentration in the solvent (C), the interaction of temperature and the solid-to-solvent ratio (AB) and the square of the ethanol concentration in the solvent (CC).

By discarding members that do not have a large impact, the regression equation for the content of total (poly)phenols in the extract has the following form:

$$Y = -22.27383 + 0.55759 \cdot A + 1.73910 \cdot B + 0.70490 \cdot C - 0.01473 \cdot AB - 0.0063 \cdot CC \quad (3)$$

To assess the influence of input parameters on the content of total (poly)phenols in the extract, surface plots were constructed, as shown in Figure 2.

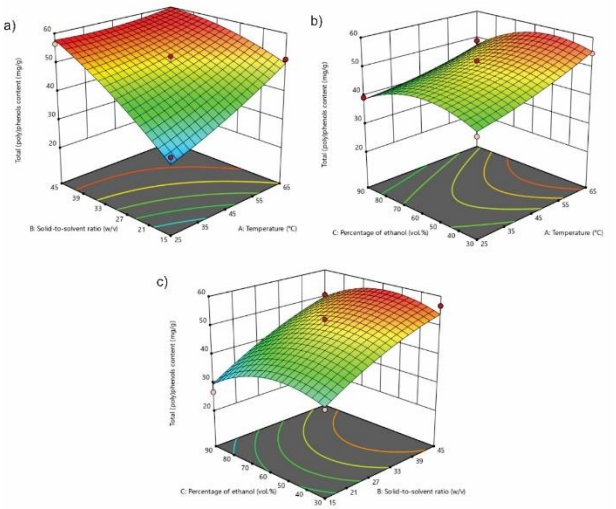

Figure 2. Surface plots for the content of total (poly)phenols in the extracts in the interaction of (a) solid-to-solvent ratio and temperature, (b) ethanol concentration in the solvent and temperature, and (c) ethanol concentration in the solvent and solid-to-solvent ratio.

Figure 2a shows the influence of the solid-to-solvent ratio (B) and temperature (A) on the value of the total (poly)phenols content in the extracts based on the mean level (0) of the ethanol concentration in the solvent (C). It was observed that the value of the response increases linearly with the increase in the solid-to-solvent ratio (B) and temperature (A). The lowest value of the Response (<35 mg GAE/g) is achieved in the range of the solid-to-solvent ratio of 1:15–1:20 w/v and temperature of 25–35 °C, while the highest values of the Response (>55 mg GAE/g) are achieved in over the entire range of the solid-to-solvent ratio of 1:40–1:45 w/v independent of temperature. Bai et al. found that a higher solid-to-solvent ratio improved the extraction yield of phenolic compounds from plant materials using UAE [36]. Similar to our findings, they observed a linear increase in the extraction efficiency with an increase in the solid-to-solvent ratio. Chemat et al. highlighted that the solid-to-solvent ratio is a critical parameter in the UAE, influencing the mass transfer and solubility of phenolic compounds [37]. Their findings support our results, emphasizing the importance of optimizing this ratio to achieve maximum extraction efficiency.

Figure 2b shows the influence of the ethanol concentration in the solvent (C) and temperature (A) on the value of the total (poly)phenols content in the extracts at the mean value of the solid-to-solvent (B) ratio. It is observed that low and high ethanol concentration in the solvent leads to a slightly lower value of the Response, than when ethanol with medium values (45–65 vol.%) is used. Herrero et al. reported that both very low and very high ethanol concentrations can reduce extraction efficiency. Low ethanol content may not sufficiently disrupt cell walls, while high ethanol content can reduce solvent polarity, hindering the extraction of polar phenolic compounds [38]. This aligns with our results showing lower extraction efficiency at low (<35 vol.%) and high (80–90 vol.%) ethanol concentrations. Observing the interaction of parameters A and C, it is observed that Response values of 40–45 mg GAE/g are achieved at lower temperatures (25–40°C) in the entire range of ethanol concentration in the solvent (C). By raising the temperature, there is an increase in the value of the Response (>55 mg GAE/g), where this increase is more pronounced at the ethanol concentration of

30–65 vol.% than at the ethanol concentration of 65–90 vol.%. Chemat *et al.* found that moderate temperatures (around 50–60°C) optimize UAE efficiency by increasing solvent penetration and compound solubility without degrading sensitive phenolic compounds [37]. This is consistent with our findings of optimal extraction at increased temperatures.

From Figure 2c, it can be seen that the high content of ethanol in the solvent (80–90 vol.%) and the low solid-to-solvent ratio (1:15–1:20 w/v) have an extremely unfavorable effect on the extraction of total (poly)phenols from blueberry leaves. Also, at the same solid-to-solvent ratio and ethanol concentration in solvent lower than 35 vol.%, the extraction of total (poly)phenols is unfavorable (<35 mg GAE/g). With an increase in the solid-to-solvent ratio, there is a linear increase in the value of the Response, whereby this increase is more pronounced with the use of ethanol concentration of 30–65 vol.%.

Influence of process parameters on the value of flavonoid content in the extract

ANOVA analysis for the content of flavonoids in blueberry leaf extracts (Table 4) revealed that the following parameters have a great influence ($p<0.05$) on the extraction of flavonoids from blueberry leaves: linear terms - temperature (A) and ethanol concentration in the solvent (C), and quadratic terms - the solid-to-solvent ratio (BB) and ethanol concentration in solvent (CC). The abbreviated regression equation for the content of flavonoids in the extract has the following form:

$$Y = -14.51734 + 0.39293 \cdot A + 0.70493 \cdot C - 0.01697 \cdot BB - 0.00733 \cdot CC \quad (4)$$

Figure 3 shows Surface plots for flavonoid content in the extract.

Figure 3a shows the influence of the solid-to-solvent ratio (B) and temperature (A) on the value of the flavonoid content in the extracts based on the mean level (0) of the ethanol concentration in the solvent (C). At lower temperatures (25–30 °C), at very low solid-to-solvent ratios (1:15–1:20 w/v) on the one hand, and very high solid-to-solvent ratios (1:40–1:45 w/v) on the other hand, work unfavorably for the extraction of flavonoids, and under these conditions <25 mg CTH/g of flavonoids is extracted. This aligns with Bai *et al.*, who found that an optimal solid-to-solvent ratio is crucial for maximizing extraction efficiency due to its impact on mass transfer dynamics [36]. From the plot, it can be seen that the parameter of solid-to-solvent ratio (B) has no great influence on the Response, which graphically confirmed the results of the ANOVA analysis; on the other hand, the plot shows a great influence of temperature, i.e. with the increase of that parameter there is a marked increase in the content of flavonoids in the extract. The highest content of flavonoids (>40 mg CTH/g) is achieved at temperatures higher than 55 °C, at any solid-to-solvent ratio. This observation is consistent with Chemat et al., who reported enhanced extraction efficiency at higher temperatures (55–65°C), attributed to improved solvent penetration and enhanced solubility of flavonoids [37].

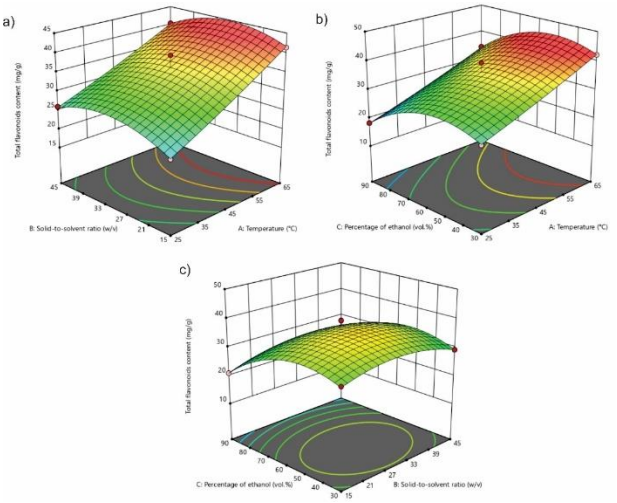

Figure 3. Surface plots for the content of flavonoids in the extracts with mutual interaction: (a) the solid-to-solvent ratio and temperature, (b) ethanol concentration in the solvent and temperature, and (c) ethanol concentration in the solvent and the solid-to-solvent ratio.

Figure 3b shows that both the ethanol concentration in the solvent (C) and the temperature (A) have a significant effect on the ultrasound-assisted extraction of flavonoids from blueberry leaves. The use of a solvent containing 80–90% ethanol is unfavorable for the extraction of flavonoids. This finding mirrors Herrero et al., who noted reduced extraction efficiency at very high ethanol concentrations due to solvent polarity effects and inadequate disruption of cell walls [38]. This influence is particularly clear during extraction at lower temperatures (25–35 °C) because <20 mg CTH/g flavonoids are extracted. Contrary to those process conditions, with ethanol concentration in the solvent in the interval 30–60 vol.% and temperatures 55–65 °C, there is the most intensive extraction of flavonoids (>40 mg CTH/g).

Observing the influence of the ethanol concentration in the solvent (C) and the solid-to-solvent ratio (B), Figure 3c, it is noticed that a plateau is reached at certain values. The maximum value of flavonoids in the extract (>35 mg CTH/g) is achieved when the ethanol concentration in the solvent is in the

range of 35–65% and the solid-to-solvent ratio is in the range of 1:20–1:35 w/v. Moving away from that range, the flavonoid content in the extract decreases, which is particularly clear with an increase in the ethanol concentration up to 90%. This corroborates with findings by various authors emphasizing the critical role of balanced ethanol concentration and solid-to-solvent ratio for maximizing bioactive compound extraction efficiency [37].

Influence of process parameters on anthocyanin content value in the extract

ANOVA analysis (Table 4) revealed that the extraction of anthocyanins from blueberry leaves is influenced by the following factors: temperature (A), solid-to-solvent ratio (B), and ethanol concentration in the solvent (C), square of temperature (AA) and square of ethanol concentration in the solvent (CC). By eliminating factors that have no influence, the regression equation for anthocyanin content in the extract takes the form:

$$Y = 0.0082 - 0.01175 \cdot A - 0.00766 \cdot B + 0.01173 \cdot C + 0.00013 \cdot AA - 0.000085 \cdot CC \quad (5)$$

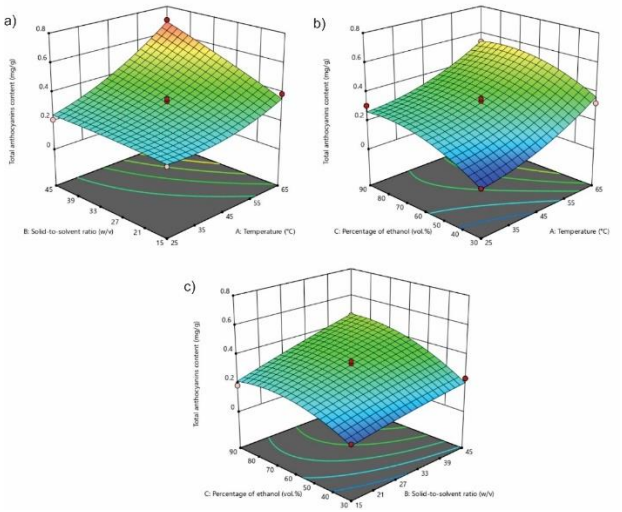

Figure 4. Surface plots for the content of anthocyanins in the extracts with mutual interaction: (a) solid-to-solvent ratio and temperature, (b) ethanol concentration in the solvent and temperature, and (c) ethanol concentration in the solvent and the solid-to-solvent ratio.

From Figure 4a (influence of the solid-to-solvent ratio (B) and temperature (A)), it can be seen that the extraction of anthocyanins is favored by an extremely narrow range of process parameter values. First of all, it is observed that only at high temperatures (60–65 °C) and high solid-to-solvent ratios (1:40–1:45 w/v) can the maximum yield of anthocyanins in the extract be achieved (>0.6 mg Cy3G/g). In contrast, by comparing Figure 4b and Figure 4c, it can be seen that the extraction of anthocyanins is poorly efficient at the following process conditions: ethanol concentration in the solvent of 30–50 vol.%, temperature of 25–50 °C and the solid-to solvent ratio of 1:15–1:25 w/v. High solid-to-solvent ratios and high temperatures significantly improve extraction efficiency, while moderate ethanol concentrations are less effective. These findings are consistent with established literature, underscoring the importance of precise parameter optimization for maximizing anthocyanin yields using UAE techniques [39,40].

Optimization

Figure 5 shows the optimization plot for the content of total (poly)phenols in the extract. The maximum content of (poly)phenol in the extract is taken as a target, which is why there is only one solution. The optimal process parameters are temperature 48.4 °C, solid-to-solvent ratio 1:43.8 w/v, and ethanol concentration in the solvent 51.3 vol.%, whereby the value of Response is 57.5 mg GAE/g. The composite desirability is equal to one, indicating that the setting provided the most favorable results.

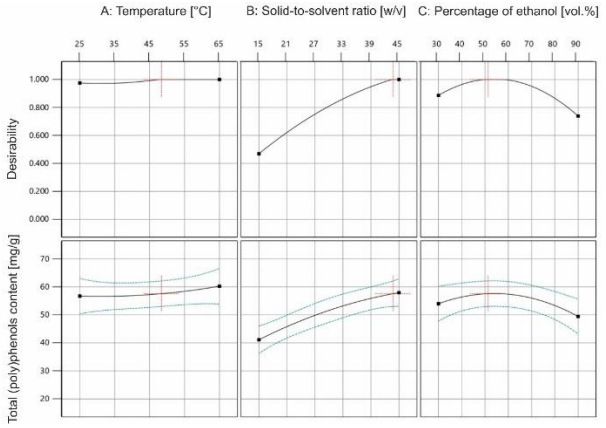

Figure 5. Optimization plot for total (poly)phenols content in the extract.

The optimization plot for the content of flavonoids in the extract is shown in Figure 6. Temperature 58.5 °C, solid-to-solvent ratio of 1:29.8 w/v, and ethanol concentration in the solvent 48.0 vol.% are the optimal process parameters for the extraction of the maximum content of flavonoids (44.38 mg CTH/g). As in the case of optimization of total (poly)phenols, the composite desirability is equal to unity.

Finally, optimization of process conditions for ultrasound-assisted extraction (UAE) of anthocyanins from blueberry leaves was carried out (Figure 7). As in the previous two optimizations, the aim is to maximize Response and composite desirability. The optimal process conditions are temperature 64.2 °C, solid-to-solvent ratio 1:44.7 w/v, and ethanol concentration in

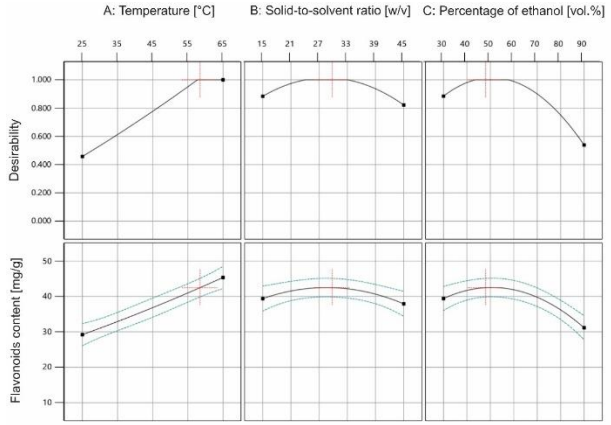

Figure 6. Optimization plot for flavonoid content in extract.

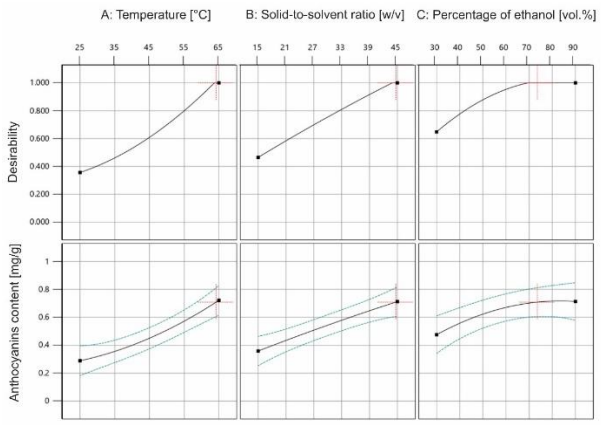

Figure 7. Optimization plot for anthocyanins content in extract.

the solvent 73.5 vol.%. Under these conditions, 0.71 mg Cy3G/g of anthocyanin is extracted.

When the temperature is considered as a process parameter, it is noticeable that temperatures lower than 45 °C are not favorable for extraction. First of all, the viscosity of the solvent at lower temperatures is higher, and the solubility of the dissolved substance and the diffusion coefficient are lower, which adversely affects the extraction process [41]. On the other hand, by analyzing the optimal time for all three Responses, it is noticeable that high temperatures do not have a favorable effect on the extraction of (poly)phenolic compounds. The reason for such a phenomenon lies in the fact that phenolic compounds are thermosensitive, i.e. their thermal decomposition occurs at high temperatures [41,42]. In this work, the optimal temperature for the extraction of total (poly)phenols is lower (48.4 °C) compared to the extraction of anthocyanins (64.2 °C), which means that anthocyanins from blueberry leaves are more resistant to higher temperatures than other phenolic compounds (phenolic acids, stilbenes, tannins, flavonoids, etc.).

When total (poly)phenols and anthocyanins are examined, it is evident that the highest degree of extraction is obtained at close to the highest solid-to-solvent ratio (1:43.8–1:44.7 w/v). This could be due to the increased contact area between the sample and the solvent, allowing for more effective mass transfer of the (poly)phenolic compounds from the solid matrix to the liquid phase. A higher ratio may result in faster mass transfer, which may result in higher yields due to the amount of solvent available to dissolve the (poly)phenolic compound. Higher solvent content in an extraction system often improves extraction efficiency because more solid material is available for interaction with the solvent [43]. On the other hand, the maximum content of flavonoids is extracted at a solid-to-solvent ratio of 1:29.8 w/v. The most likely explanation for this phenomenon is that a very high solid-to-liquid ratio may cause contaminants to dissolve, reducing the solubility of flavonoids [44].

The extraction of total (poly)phenols and flavonoids has a positive effect on the medium values of the ethanol concentration in the solvent (51.3 vol.% and 48.0 vol.%, respectively), while for the extraction of anthocyanins, the optimal ethanol concentration in the solvent is higher and amounts to 73.5 vol.%. Lower concentrations of ethanol penetrate plant cells more easily, making phenolic extraction easier. Ethanol at greater concentrations can cause protein denaturation, impede phenolic breakdown from the matrix, and diminish the production of (poly)phenolic compounds [45]. The combination of water and ethanol allows efficient (poly)phenol extraction because water acts as a swelling agent and ethanol breaks down the bonds between the solutes and the floral matrix; therefore, high ethanol concentration in solvent yields a smaller yield of (poly)phenolic compounds [46].

In comparison with the full factorial design used in previous research [32], the BBD offers several significant advantages. The BBD is more efficient in investigating quadratic effects and interactions between factors, as it better covers the area of interest without the need for extreme values of the factors. This results in more robust models that can provide more precise estimates of optimal conditions for the processes being studied. Also, The BBD allows for more efficient experimental planning, reducing redundancy and potential errors in conducting experiments. In this way, the obtained results are more reliable and can be better applied in practice.

CONCLUSION

The experimental data fit well into the obtained models, as confirmed by the high degrees of correlation (R^2 and adjusted R^2). The model accurately predicts flavonoid content, but not total (poly)phenols and

anthocyanins. Extraction is adversely affected by low temperatures due to slow diffusion and high temperatures due to the thermosensitivity of phenolic compounds. Anthocyanins can be extracted at slightly higher temperatures owing to their greater heat resistance. Total (poly)phenols and anthocyanins are better extracted at higher solid-to-solvent ratios due to a larger concentration gradient. However, flavonoids are better extracted at lower ratios to avoid components that reduce their solubility. Medium ethanol concentrations are optimal for phenolic compound extraction, as ethanol penetrates plant material effectively, while higher concentrations denature proteins and hinder extraction. The optimization of process parameters using the BBD demonstrated that UAE effectively enhances the release of bioactive compounds, achieving maximum yields under specified conditions as follows: for (poly)phenols, 48.4 °C, 51.3 vol.% ethanol, and 1:43.8 w/v solid-to-solvent ratio; flavonoids, 58.5 °C, 48.0 vol.% ethanol, and 1:29.8 w/v ratio; and anthocyanins, 64.2 °C, 73.5 vol.% ethanol, and 1:44.7 w/v ratio. The bioactive components have potential applications in functional foods, nutraceuticals, pharmaceuticals, and cosmetics, and further research could expand their use.

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NAUČNI RAD

OPTIMIZACIJA ULTRAZVUČNE EKSTRAKCIJE (POLI)FENOLNIH JEDINJENJA IZ LIŠĆA BOROVNICE (*Vaccinium myrtillus*)

Cilj rada je da utvrdi optimalne uslove za ultrazvučnu ekstrakciju (UAE) (poli)fenolnih jedinjenja iz lišća borovnice (Vaccinium mirtillus). UAE je izvedena pod sledećim procesnim uslovima: temperatura od 25–65 °C, koncentracija etanola u rastvaraču za ekstrakciju od 30–90 vol.%, i odnos čvrsta materija prema rastvaraču od 1:15–1:45 v/v. Statistička analiza je izvršena korišćenjem softvera Design-Expert, korišćenjem Boks-Benkenovog dizajna. Odgovori su bili sadržaj ukupnih (poli)fenola, flavonoida i antocijana u dobijenim ekstraktima. Rezultati su pokazali da su odgovarajući modeli površine odgovora visoko statistički značajni ($p < 0,0001$) i dovoljni da opišu i predvide sadržaj ukupnih (poli)fenola, sadržaj flavonoida i sadržaj antocijana sa R^2 od 0,965, 0,980 i 0,972, redom. Optimalni uslovi za ekstrakciju su za ukupne (poli)fenole 48,4 °C, 51,3 vol.% etanola i 1:43,8 v/v odnos čvrsta materija/rastvarač; za flavonoide 58,5 °C, 48,0 vol.% etanola i 1:29,8 v/v odnos čvrsta materija/rastvarač; i za antocijanine 64,2 °C, 73,5 vol.% etanola i 1:44,7 v/v odnos čvrsta materija/rastvarač. Upotreba UAE povećava prinose ekstrakcije povećanjem oslobađanja bioaktivnih jedinjenja, dok primena Boks-Benkenovog dizajna omogućava precizno određivanje optimalnih parametara ekstrakcije, čime se postižu maksimalni prinosi i efikasnost.

Ključne reči: antocijanini; borovnica; ekstrakcija; flavonoidi; optimizacija; (poli)fenoli.