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# Optimization of ultrasound-assisted extraction (UAE) of (poly)phenolic compounds from blueberry (*Vaccinium myrtillus*) leaves using full-factorial design

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**Abstract.** In this work, the influence of process parameters (temperature: 25 - 65 °C, ethanol content in the extraction solvent: 30 - 90 vol.%, and solid-to-solvent ratio: 1:15 - 1:45 w/v) on the process of ultrasound-assisted extraction (UAE) of (poly)phenols from blueberry leaves (*Vaccinium myrtillus*) was investigated. Statistical analysis was performed using the MINITAB 21 software, with the application of three-level full factorial designs. The Responses in the study are the content of total (poly)phenols, flavonoids and anthocyanins in the obtained extracts. The extraction of blueberry leaf was significantly ($p < 0.05$) impacted by process variables. The $R^2$, Adjusted $R^2$, and Predicted $R^2$ values in the study are high, showing a significant relationship between the independent variables and the Response. The optimal temperature for all three Responses is 65 °C, the optimal solid-to-solvent ratio for total (poly)phenols and anthocyanins is 1:45 w/v and for flavonoids is 28.03 w/v, while the optimal ethanol content in the solvent for total (poly)phenols, flavonoids and anthocyanins is 51.21 vol.%, 50.61 vol.% and 83.33 vol.%, respectively.

***Keywords*:** blueberry; ultrasound-assisted extraction; optimization; (poly)phenolic compounds.

## 1. Introduction

Blueberry is a tree species widespread to the northern hemisphere, which belongs to the *Vaccinium* genus and family *Ericaceae* [1]. On a commercial level, important blueberry species include high-bush blueberry (*Vaccinium corymbs* L.), rabbiteye blueberry (*Vaccinium virgatum Aiton*), low-bush blueberry (*Vaccinium angustifolium Aiton*), and European bilberry (*Vaccinium myrtillus* L.) [2].

Blueberries are the most antioxidant-rich fresh fruit that has been investigated, with a high level of (poly)phenols in both the peel and the pulp. According to research, blueberry leaves are high in polyphenols and procyanidins, such as chlorogenic acid, quercetin glycosides and oligomeric proanthocyanidins [3-6]. As a result, blueberry leaves could be employed as a low-cost raw material with favorable biological activity and a high concentration of phenolic chemicals in medicines, cosmetics, food additives, and functional foods [7].

Blueberries have the highest anthocyanin content of any common fruit [8, 9]. Anthocyanins are the pigments that give ripe berries their red, blue, and purple colors. The anthocyanin concentration of berries increases rapidly throughout ripening, providing a visual indication to distinguish between early and fully ripe fruit [10]. The range of cyanidin-3-glucoside equivalents/g fresh weight of the 215 blueberry genotypes was 0.925 to 2.1 mg [11].

Solvent extraction, enzyme-assisted extraction (EAE), ultrasonic-assisted extraction (UAE), and supercritical fluid extraction are the most used methods for extracting blueberry anthocyanins [1, 12-14]. Among them, the ultrasonic-assisted extraction has primary importance since it is efficient, cost-effective, and environmentally friendly. The use of ultrasound in extraction has significance because it can generate cavitation and expedite plant cell disintegration, making mass transfer more efficient [15].

The one factor at a time (OFAT) method has long been used to identify crucial variables. The optimization of multiple variables or factors that condition the assay in this model is done by analyzing each factor independently while holding all the others constant, which frequently leads to incomplete results because it does not investigate or determine crucial interactions between the elements. Furthermore, it requires a significant usage of time and resources [16].

Design of Experiments (DOE) is a methodical and statistically based on factorial designs, which might be fractional or whole. Because certain interactions are not as important as the main effects, fractional factorial designs are appropriate. A full factorial design, on the other hand, includes all conceivable factor combinations in an assay, making it a more powerful design than a fractional one [16].

In this study, the influence of various process parameters on the ultrasound-assisted extraction (UAE) of (poly)phenols from blueberry leaves (*Vaccinium*

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*myrtillus*) will be investigated. The parameters under investigation include temperature (25 – 65 °C), ethanol content in the extraction solvent (30 - 90 vol.%), and solid-to-solvent ratio (1:15 - 1:45 w/v). Through methodically adjusting these variables and utilizing statistical analysis using three-level full factorial designs with the MINITAB 21 software, the objective of the paper is to offer an understanding of how these factors interact with each other and collectively influence the efficiency of the extraction process. Such an investigation holds significant implications for the development of efficient and sustainable extraction protocols for (poly)phenols, with potential applications in various industries including pharmaceuticals, nutraceuticals, and food processing.

## 2. Experimental

### *2.1. Plant materials and methods*

An ethanol solution was used for extraction of dried blueberry leaves. After the extraction was completed, the content of total (poly)phenols, flavonoids and anthocyanins in the obtained extracts was determined with a Shimadzu UV-1800 spectrophotometer (Cole Parmer, USA).

The measurement of total (poly)plenols was at wavelength of 765 nm, and gallic acid (Sigma Aldrich, USA) was utilized as the standard [17]. The following reagents were used for spectrophotometric measurement: Folin-Ciocalteu reagent (Carlo Erba, Germany), as well as sodium carbonate (Lach:ner, Czech Republic). Measurement of flavonoids was at wavelength of 510 nm, with catechin hydrate (Sigma Aldrich, USA) as the standard [18]. Aluminum chloride (Lach:ner, Czech Republic), sodium hydroxide (Lach:ner, Czech Republic), and sodium nitrite (Zorka Šabac, Serbia) were used for color development. The content of anthocyanins was determined by measuring the absorbance of the extract in potassium chloride buffer pH = 1 and in acetate buffer pH = 4.5, at wavelength of 520 nm and 700 nm [19, 20]. The content of total (poly)phenols, flavonoids and anthocyanins in extract was expressed as gallic acid equivalent (mg GAE/g), catechin hydrate equivalents (mg CTH/g) and cyanidin-3-glucoside equivalent (mg Cy3G/g), respectively.

### *2.2. Experimental design*

The Response Surface Methodology (RSM) was applied as the experimental design to investigate the influence of process parameters (temperature, solid-to-solvent ratio and ethanol content in the solvent) on the efficiency of (poly)phenols extraction from blueberry leaves. Response Surface Methodology is a statistical technique that allows for the exploration of complex interactions among different factors, resulting in the development of a mathematical model describing the relationship between input variables (process parameters) and system response (extraction efficiency) [16].

Experimental design and statistical analysis were performed in MINITAB 21 - Trial Version (Minitab, LLC, USA) using Three-level full factorial designs. Also, two replicates were performed at the center point of the design to allow estimation of the pure error and to calculate the repeatability of the method, resulting in a total of 27+2=29 extractions to be performed. Each extraction lasted 30 min, with ultrasound-assisted mixing.

Table 1 shows Coded and actual levels of independent variables used for extraction of phenolic compounds from blueberry leaves.

**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 | | |
|---|---|---|---|---|
| | | 0 | 1 | 2 |
| A | Temperature [°C] | 25 | 45 | 65 |
| B | Solid-to-solvent ratio [w/v] | 1:15 | 1:30 | 1:45 |
| C | Ethanol content in solvent [vol. %] | 30 | 60 | 90 |

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 response surface method is as follows:

$$Y = a_0 + \sum a_i X_i + \sum a_{ii} X_i^2 + \sum a_{ij} X_i X_j \qquad (1)$$

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 contour plots were generated to explain the effects of independent variables on Response.

## 3. Results and discussion

The results of the measurement of the Responses are shown in Table 2. In order to determine the influence of process parameters on extraction, ANOVA analysis and evaluation of the obtained models are used.

**Table 2.** Measured values for the Response variables.

| Run | Process parameters | | | Responses | | |
|---|---|---|---|---|---|---|
| | Temp [°C] | Solid-to-solvent ratio [w/v] | Ethanol content in solvent [vol%] | Total (poly)phenols content [mg/g] | Flavonoids content [mg/g] | Anthocyanins content [mg/g] |
| 1 | 25 | 15 | 30 | 26.95 | 20.66 | 0.0561 |
| 2 | 25 | 15 | 60 | 32.52 | 23.85 | 0.2024 |
| 3 | 25 | 15 | 90 | 22.85 | 11.94 | 0.1808 |
| 4 | 25 | 30 | 30 | 40.34 | 25.78 | 0.0611 |

| Run | Process parameters | | | Responses | | |
|---|---|---|---|---|---|---|
| | Temp [°C] | Solid-to-solvent ratio [w/v] | Ethanol content in solvent [vol%] | Total (poly)phenols content [mg/g] | Flavonoids content [mg/g] | Anthocyanins content [mg/g] |
| 5 | 25 | 30 | 60 | 44.14 | 28.67 | 0.2034 |
| 6 | 25 | 30 | 90 | 39.38 | 18.29 | 0.3076 |
| 7 | 25 | 45 | 30 | 59.38 | 27.26 | 0.0962 |
| 8 | 25 | 45 | 60 | 56.42 | 26.04 | 0.2104 |
| 9 | 25 | 45 | 90 | 49.15 | 16.46 | 0.2705 |
| 10 | 45 | 15 | 30 | 35.87 | 30.91 | 0.1052 |
| 11 | 45 | 15 | 60 | 37.09 | 27.90 | 0.2049 |
| 12 | 45 | 15 | 90 | 26.68 | 20.97 | 0.1854 |
| 13 | 45 | 30 | 30 | 48.40 | 32.56 | 0.1473 |
| 14* | 45 | 30 | 60 | 52.20 | 33.57 | 0.2916 |
| 15 | 45 | 30 | 90 | 39.80 | 21.65 | 0.3787 |
| 16 | 45 | 45 | 30 | 56.79 | 29.27 | 0.2360 |
| 17 | 45 | 45 | 60 | 61.18 | 30.74 | 0.3577 |
| 18 | 45 | 45 | 90 | 50.66 | 22.27 | 0.4509 |
| 19 | 65 | 15 | 30 | 49.87 | 40.04 | 0.1677 |
| 20 | 65 | 15 | 60 | 51.24 | 41.38 | 0.2901 |
| 21 | 65 | 15 | 90 | 36.01 | 26.73 | 0.2279 |
| 22 | 65 | 30 | 30 | 54.70 | 42.16 | 0.3248 |
| 23 | 65 | 30 | 60 | 57.70 | 43.16 | 0.6012 |
| 24 | 65 | 30 | 90 | 48.84 | 34.24 | 0.5370 |
| 25 | 65 | 45 | 30 | 61.96 | 45.49 | 0.4223 |
| 26 | 65 | 45 | 60 | 57.47 | 40.34 | 0.7004 |
| 27 | 65 | 45 | 90 | 55.30 | 32.48 | 0.7905 |
| 28* | 45 | 30 | 60 | 49.04 | 35.87 | 0.3387 |
| 29* | 45 | 30 | 60 | 49.50 | 39.40 | 0.3571 |

*In order to determine Lack-of-Fit and Pure Error, the extraction was repeated at the central point (14th, 28th and 29th extractions)

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, in order to determine whether the given model is adequate after eliminating parameters that do not have a significant impact, whether the model can accurately predict the Responses under different process conditions. Table 3 shows ANOVA results for responses to ultrasound-assisted extraction (UAE) of blueberry leaves.

**Table 3.** ANOVA results for the response surface quadratic model for all Responses of ultrasound-assisted blueberry leaf extraction.

| Source | df | Total (poly)phenols content | | | | Flavonoids content | | | | Anthocyanins 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 | 3199.32 | 355.481 | 60.71 | 0.000 | 2013.87 | 223.764 | 45.73 | 0.000 | 0.878 | 0.098 | 56.44 | 0.000 |
| Temperature (A) | 1 | 16.56 | 16.561 | 2.83 | 0.109 | 0.79 | 0.792 | 0.16 | 0.692 | 0.021 | 0.021 | 11.99 | 0.003 |
| Solid-to-solvent ratio (B) | 1 | 166.77 | 166.766 | 28.48 | 0.000 | 67.51 | 67.510 | 13.80 | 0.001 | 0.001 | 0.001 | 0.36 | 0.554 |
| Ethanol content in solvent (C) | 1 | 111.63 | 111.627 | 19.07 | 0.000 | 118.03 | 118.029 | 24.12 | 0.000 | 0.023 | 0.023 | 13.09 | 0.002 |
| $A^2$ | 1 | 16.64 | 16.639 | 2.84 | 0.108 | 21.93 | 21.931 | 4.48 | 0.048 | 0.016 | 0.016 | 9.14 | 0.007 |
| $B^2$ | 1 | 9.97 | 9.966 | 1.70 | 0.208 | 69.09 | 69.093 | 14.12 | 0.001 | 0.008 | 0.008 | 4.39 | 0.050 |
| $C^2$ | 1 | 186.85 | 186.852 | 31.91 | 0.000 | 225.11 | 225.108 | 46.01 | 0.000 | 0.031 | 0.031 | 18.12 | 0.000 |
| AB | 1 | 168.99 | 168.991 | 28.86 | 0.000 | 0.83 | 0.831 | 0.17 | 0.685 | 0.099 | 0.099 | 57.25 | 0.000 |
| AC | 1 | 10.25 | 10.245 | 1.75 | 0.202 | 4.35 | 4.352 | 0.89 | 0.357 | 0.001 | 0.001 | 0.44 | 0.517 |
| BC | 1 | 1.43 | 1.428 | 0.24 | 0.627 | 0.11 | 0.111 | 0.02 | 0.882 | 0.020 | 0.020 | 11.69 | 0.003 |
| Error | 19 | 111.24 | 5.855 | | | 92.97 | 4.893 | | | 0.033 | 0.002 | | |
| Lack of Fit | 17 | 105.44 | 6.203 | 2.14 | 0.366 | 75.71 | 4.454 | 0.52 | 0.825 | 0.031 | 0.002 | 1.57 | 0.458 |
| Pure Error | 2 | 5.80 | 2.900 | | | 17.25 | 8.626 | | | 0.002 | 0.001 | | |
| Total | 28 | 3310.57 | | | | 2106.84 | | | | 0.911 | | | |
| Fit Statistics | | $R^2 = 0.9664$<br>Adjusted $R^2 = 0.9505$<br>Predicted $R^2 = 0.9084$ | | | | $R^2 = 0.9559$<br>Adjusted $R^2 = 0.9350$<br>Predicted $R^2 = 0.8937$ | | | | $R^2 = 0.9639$<br>Adjusted $R^2 = 0.9469$<br>Predicted $R^2 = 0.9049$ | | | |

The $R^2$ values for the content of total (poly)phenols, flavonoids and anthocyanins in the extracts are 0.9664, 0.9559 and 0.9639, respectively. This showed that the response variability was well explained in the generated

model, as the models were able to explain 96.64% of the variation in the total (poly)phenols content, 95.59% of the variation in the flavonoids value, and 96.39% of the variation in the anthocyanins content in the extracts. The $R^2$ value for all three cases is close to one, which revealed that there is a good correlation between the independent variables and the Responses.

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 total (poly)phenols, flavonoids and anthocyanins content in the extracts are 0.9505, 0.9350 and 0.9469, 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-value is 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 0.9084, 0.8937 and 0.9049, respectively. The difference between Adjusted $R^2$ and Predicted $R^2$ for all three Responses is extremely small, which means that the resulting model provides valid predictions for the new observations.

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.

### *3.1. Influence of process parameters on the value of total (poly)phenols content in the extract*

Table 4 shows the coefficients of the regression equation and p-value, and Figure 1 shows the Pareto diagrams for members in the proposed quadratic model for the content of total (poly)phenols in blueberry leaf extracts.

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

| Variables | Total (poly)phenols content | | | Flavonoids content | | | Anthocyanins content | | |
|---|---|---|---|---|---|---|---|---|---|
| | Actual Regress. Coeff. | SE Coeff | p-Value | Actual Regress. Coeff. | SE Coeff | p-Value | Actual Regress. Coeff. | SE Coeff | p-Value |
| Constant | -14.57 | 7.85 | 0.079 | -7.36 | 7.18 | 0.318 | 0.067 | 0.135 | 0.626 |
| Temperature (A) | 0.395 | 0.235 | 0.109 | 0.086 | 0.215 | 0.692 | -0.01397 | 0.00404 | 0.003 |
| Solid-to-solvent ratio (B) | 1.544 | 0.289 | 0.000 | 0.983 | 0.265 | 0.001 | -0.00300 | 0.00497 | 0.554 |
| Ethanol content in solvent (C) | 0.632 | 0.145 | 0.000 | 0.650 | 0.132 | 0.000 | 0.00899 | 0.00249 | 0.002 |
| AA | 0.00395 | 0.00235 | 0.108 | 0.00454 | 0.00214 | 0.048 | 0.000122 | 0.000040 | 0.007 |
| BB | -0.00544 | 0.00417 | 0.208 | -0.01433 | 0.00381 | 0.001 | -0.000150 | 0.000072 | 0.050 |
| CC | -0.00589 | 0.00104 | 0.000 | -0.006465 | 0.000953 | 0.000 | -0.000076 | 0.000018 | 0.000 |
| AB | -0.01251 | 0.00233 | 0.000 | -0.00088 | 0.00213 | 0.685 | 0.000303 | 0.000040 | 0.000 |
| AC | -0.00154 | 0.00116 | 0.202 | -0.00100 | 0.00106 | 0.357 | 0.000013 | 0.000020 | 0.517 |
| BC | 0.00077 | 0.00155 | 0.627 | 0.00021 | 0.00142 | 0.882 | 0.000091 | 0.000027 | 0.003 |

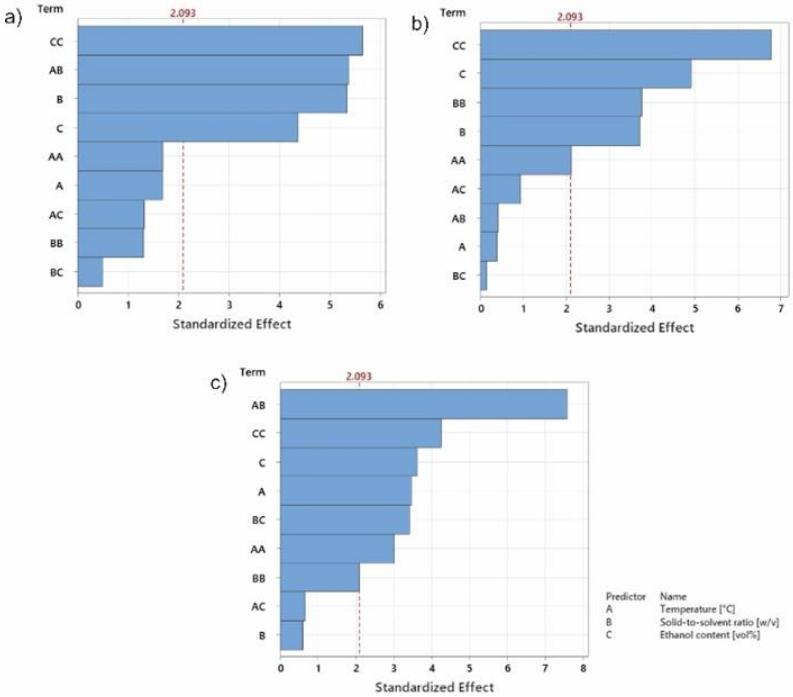


**Figure 1.** Pareto diagrams for the influence of process parameters on a) the content of total (poly)phenols, b) the content of flavonoids and c) the content of anthocyanins in extract.

By analyzing the p-value from Table 4 and the Pareto diagram (Figure 1a), it was determined that the following parameters have an effect on the total (poly)phenols content in the extracts: the square of the

ethanol content in the solvent (CC), the mutual interaction of temperature and the solid-to-solvent ratio (AB), and the linear terms of the solid-to-solvent (B) ratio and the ethanol content in the solvent (C). The temperature term (A) is linear, and will be retained in the abbreviated regression equation even though its p-value is 0.109. Other parameters have a p-value greater than 0.05 and can be excluded. By discarding the elements that are not significant, the regression equation for the total (poly)phenols content in the extract takes the form:

$$Y\ [\text{mg GAE/g}] = -14.57 + 0.395\ A + 1.544\ B + 0.632\ C - 0.00589\ CC - 0.01251\ AB \quad (1)$$

Figure 2 shows contour diagrams for the influence of process parameters on the content of total (poly)phenols in the extract.

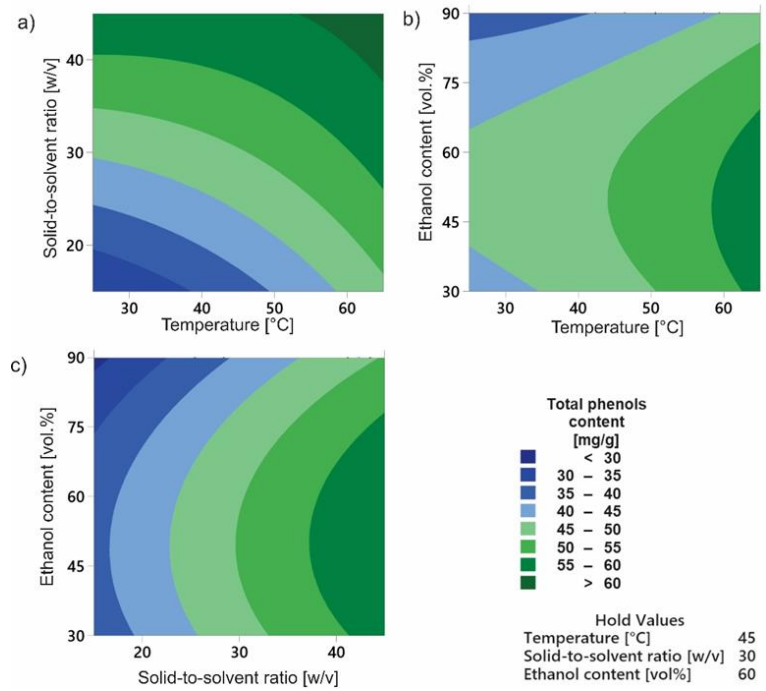


**Figure 2.** Contour plots for the total (poly)phenols content in the extracts in the interaction of: a) the solid-to-solvent ratio and temperature; b) the ethanol content in the solvent and temperature; and c) the ethanol content in the solvent and the solid-to-solvent ratio.

Observing the influence of the solid-to-solvent ratio (B) and temperature (A) at the mean value of the ethanol content in the solvent (C), Figure 2a, it is observed that the maximum values of Response (>60 mg GAE/g) are achieved at the highest values of parameter A and parameter B. Such a response value is achieved in the range of solid-to-solvent ratio = 1:40 – 1:45 w/v and temperature 55 - 65 ° C. Also, extremely high response values (50 – 60 mg GAE/g) are achieved at solid-to-solvent ratio = 1:35 – 1:45 w/v in the entire temperature range, as well as at temperatures higher than 50 °C, regardless of the solid-to-solvent ratio. By lowering both parameters, there is a gradual decrease in the value of the Response, so that the minimum content of total (poly)phenols (<30 mg GAE/g) is achieved at a solid-to-solvent ratio = 1:15 - 1:20 w/v and temperatures of 25 - 37 °C.

Observing the interaction of ethanol content in the solvent (C) and temperature (A), Figure 2b, it is observed that the total (poly)phenols content in the extract is the lowest when the extraction is performed at low temperatures using solvents with high or low ethanol content. Also, a small value of the total (poly)phenols content is achieved when using a solvent with 75 - 90% ethanol in almost the entire temperature range. From Figure 2b it is also observed that sufficiently high values of Response are achieved in the range of 37 - 63% ethanol. The highest value of extracted (poly)phenols from blueberry leaves was achieved at temperatures of 60 - 65 °C and the use of 30 - 60% ethanol.

It can be seen from Figure 2c that the extraction of total (poly)phenols from blueberry leaves is not favored by low solid-to-solvent ratios in the entire range of ethanol content in the solvent. By increasing the solid-to-solvent ratio, an increase in the Response is observed, with the maximum value being reached at the solid-to-solvent ratio greater than 1:45 w/v and the use of a solvent with 35 - 65% ethanol.

### *3.2. Influence of process parameters on the value of flavonoid content in the extract*

The p-values were obtained by ANOVA analysis (Table 4) and a Pareto diagram was constructed (Figure 1b), where it was determined that the following parameters have the greatest influence on the content of flavonoids in the extracts: the square of the ethanol content in the solvent (CC), the ethanol content in the solvent (C), the square of the solid-to-solvent ratio (BB), the solid-to-solvent ratio (B) and the square of the temperature (AA). Term A does not have a large effect, but will be retained in the shortened regression equation because it is linear factor. The other members (AC, AB and BC) have a p-value greater than 0.05 and can be excluded. The abbreviated regression equation has the form:

$$Y\ [mg\ CTH/g] = -7.36 + 0.086\ A + 0.983\ B + 0.650\ C + 0.00454\ AA - 0.01433\ BB - 0.006465\ CC \quad (2)$$

Figure 3 shows the contour diagrams for the influence of process conditions on the value of the flavonoid content in the extract.

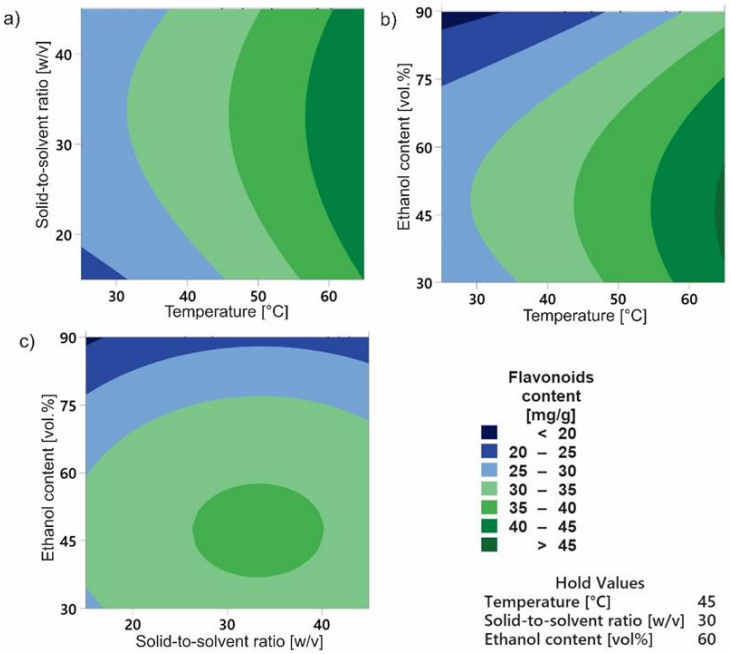


**Figure 3.** Contour plots for the content of flavonoids in the extracts in the interaction of: a) the solid-to-solvent ratio and temperature; b) ethanol content in the solvent and temperature; and c) ethanol content in the solvent and the solid-to-solvent ratio.

Observing the interaction of the solid-to-solvent ratio and temperature, it is noticed that, as with the extraction of total (poly)phenols, the lowest values of Response (20 - 25 mg CTH/g) are achieved at low values of solid-to-solvent ratio (15 - 18 w/v) and temperature (25 – 31 °C). Also, low Response values (<30 mg CTH/g) are achieved in the entire range of solid-to-solvent ratios at temperatures lower than 40 °C. By increasing the temperature, there is a constant increase in the value of Response, whereby the highest value of flavonoids in the extract is achieved above 60 °C, independently of the other parameter.

From Figure 3b (influence of ethanol content in the solvent and temperature) it can be seen that the extraction should not be performed with a solvent containing more than 75% ethanol and temperatures of 25 - 30 °C, because the extraction of flavonoids is extremely low (<20 mg CTH/g). Also, the content of extracted flavonoids is low (<30 mg CTH/g) at temperatures lower than 30 °C at any value of ethanol in the solvent, as well as at the concentration of ethanol in the solvent that is greater than 70% in the temperature range 25 - 45 °C. The most intensive extraction of flavonoids takes place at temperatures higher than 58 °C and with the use of 30 - 60% ethanol.

From Figure 3c, it can be seen that the use of a solvent containing more than 65% ethanol is unfavorable for extraction, thus confirming the results from the previous diagram. By lowering the ethanol content in the solvent, independently of the other parameter, the value of Response increases. On the contour diagram, the peak at which the highest value of the Response is realized is clearly visible, and it is found in the ethanol content in the solvent in the amount of 35 - 50% ethanol and the solid-to-solvent ratio of 27 - 40 w/v.

### *3.3. Influence of process parameters on anthocyanin content value in the extract*

Based on the ANOVA analysis for the content of anthocyanins in the extract, it was determined that all process parameters effect on anthocyanins content, except for the interaction of temperature and ethanol content in the solvent (AC) and the solid-to-solvent ratio (B). Given that the solid-to-solvent (B) ratio term is linear, the term AC can only be removed from the regression equation, so the regression equation has the form:

$$Y\ [mg\ Cy3G/g] = 0.067 - 0.01397\ A - 0.00300\ B + 0.00899\ C + 0.000122\ AA - 0.000150\ BB - 0.000076\ CC + 0.000303\ AB + 0.000091\ BC \quad (3)$$

Figure 4 shows contour diagrams for the influence of process parameters on the value of anthocyanins in the extract.

It can be seen from Figure 4a that low temperatures and low solid-to-solvent ratios are unfavorable for anthocyanin extraction. Also, the entire temperature range of 25 - 40 °C is unfavorable for the extraction of anthocyanins at any value of the second parameter, as well as the solid-to-solvent ratio of 15 - 20 w/v in the entire temperature interval, because less than 0.3 mg of Cy3G/g anthocyanins is extracted. By increasing the parameters above those values, a more intensive extraction of anthocyanins is observed, and the highest value of Response (>0.6 mg Cy3G/g of anthocyanins) is achieved at the solid-to-solvent ratio of 1:38 w/v - 1:45 w/v and at a temperature of 60 - 65 °C.

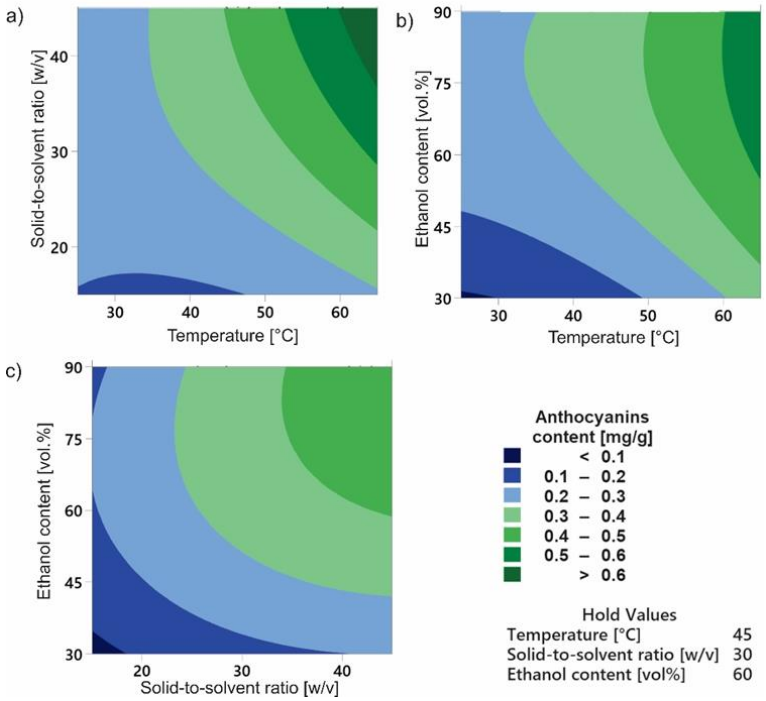


**Figure 4.** Contour plots for the anthocyanins content in the extracts with the interaction of: a) the solid-to-solvent ratio and temperature, b) the ethanol content in the solvent and temperature and c) the ethanol content in the solvent and the solid-to-solvent ratio.

As in the previous diagram, looking at Figure 4b, it can be seen that at low values of the process conditions, the extraction of anthocyanins is slow. Thus, when using 30 - 45% ethanol and a temperature of 25 - 40 °C, it is possible to extract less than 0.2 mg of Cy3G/g of anthocyanins. Extraction is low enough (<0.3 mg Cy3G/g anthocyanin) at 25 - 40 °C at any value of ethanol content in the solvent, or when using 30-45% ethanol and temperature in the interval 25 - 55 °C. The highest value of the Response is achieved with the use of 60 - 90% ethanol and temperatures above 60 °C. Figure 4c confirms the previous results, that is, with a wide interval of lower values of the process parameters, satisfactory values of the Response are not achieved, and that a sufficiently large value of the Response can only be achieved with higher values of the process parameters.

### *3.4. Optimization*

The optimization plots for all Responses are shown in Figure 5. For all three Responses, the optimal value of temperature is 65 °C, while the values of the solid-to-solvent ratio and the ethanol content in the solvent differ.

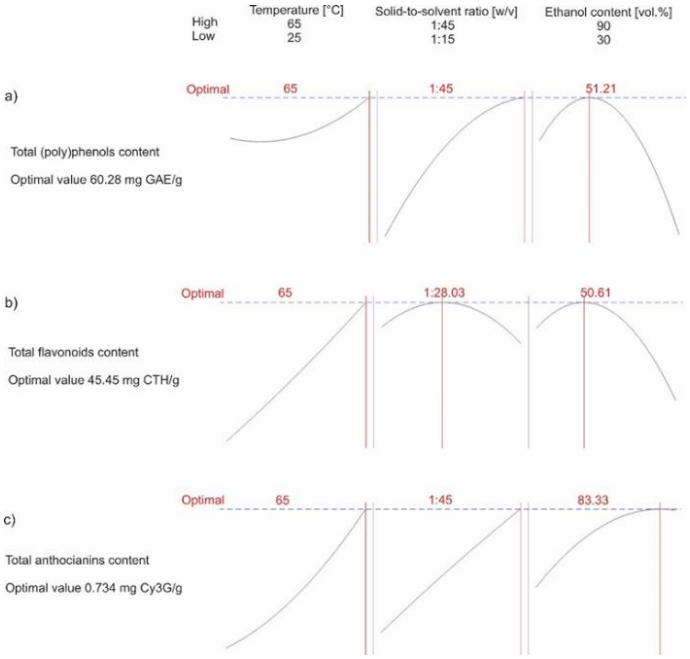


**Figure 5.** Optimization plots for a) the content of total (poly)phenols, b) the content of flavonoids and c) the content of anthocyanins in extract.

By observing the solid-to-solvent ratio, the highest amount of total (poly)phenols and anthocyanins is extracted at a solid-to-solvent ratio of 1:45 w/v, while the maximum flavonoid content is achieved at a solid-to-solvent ratio of 1:28.03 w/v. On the other hand, the most total (poly)phenols and flavonoids are extracted at medium values of ethanol content in the solvent (51.21 vol.% and 50.61 vol.%), while anthocyanins are extracted at higher ethanol content in the solvent (83.33 vol.%).

## 4. Conclusions

In this paper, the effect of temperature, solid-to-solvent ratio, and ethanol content in the solvent on the extraction of (poly)phenolic compounds from blueberry leaf (*Vaccinium myrtillus*) was investigated. The $R^2$, Adjusted $R^2$ and Predicted $R^2$ values in the study are extremely high, indicating that there is a good correlation between the independent variables and the response. Also, by ANOVA analysis, it was determined that the p-value is significantly lower than 0.05, which indicates that the models are adequately adapted to the experimental data. An increase in temperature has a positive effect on the values of all three Responses. Also, it was determined that a higher ethanol content in the solvent enables better extraction only for anthocyanins, but not for total (poly)phenols and flavonoids, where the optimal concentration of ethanol in the range of 50.61 – 51.21 vol.%, most likely because ethanol penetrate more easily in the plant material at such medium ethanol content. Total (poly)phenols and anthocyanins are extracted at larger solid-to-solvent ratios, since their diffusion rate is higher at such high ratios; however, flavonoids are extracted at considerably lower solid-to-solvent ratios, most likely because other compounds limit their diffusion.

## Conflict of interest

The authors declare no conflict of interest.

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