EXTRACTION OF BIOACTIVE COMPOUNDS FROM BLACKBERRIES POMACE

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The aim of this work is to propose a valorization scheme of blackberries pomace through extraction using aqueous/organic solvents. Polyphenolics and anthocyanins were extracted using ethanol-water mixtures at different temperatures from dried blackberries skin and were optimized using a 2^3 factorial design. The statistical analysis has revealed that solid/liquid ratio and temperature are the most important influencing factors of extraction efficiency. The best conditions for anthocyanins extraction were: solid/liquid ratio 1g/50 mL, extraction temperature 60 °C and time 1 h. For polyphenolics extraction the best conditions are quite similar as for anthocyanins extraction: solid/liquid ratio 1g/50 mL, extraction temperature 60°C. The only difference is that the extraction time must be 2 h.

Moreover, this study suggested that blackberries pomace could serve as a valuable source for bioactive compounds.

Keywords: blackberries pomace, anthocyanins extraction, polyphenolics extraction, factorial experiment

1. Introduction

From the large family of berries, blackberries (BB) are among the most known fruits. Blackberry (*Rubus fruticosus*) is a half-shrub of the *Rosaceae* family, which is native across to several continents being cultivated or occupying wild area, in Europe, Asia, Australia, and North and South America. The highest blackberries producer in Europe is Serbia which accounted for 69% of the blackberry area in Europe in 2005 [1]. Romania has also very good conditions for blackberries cultivation, but in 2005 only 100 ha were cultivated with these fruits [1]. Even if, there are not recent reports about blackberries cultures in Romania,

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many agricultural news mentioned that a lot of small farmers decided to extend their cultures for different berries including also blackberries, in the last years.

Berry fruits, and especially blackberries, are considered to have high levels of phenolic compounds including: flavonoids, phenolic acids, tannins and hydrolysable tannins and anthocyanins. In addition, blackberries also contain carbohydrates and several essential vitamins, and minerals [3]. The blackberries seeds are also a source of polyunsaturated fatty acids, mainly linoleic acid and linolenic acid, of sterols (β-sitosterol), and tocopherols (γ-tocopherol) [4].

Their benefits for human health are well known and are based on their high nutritional content. They are mainly used in food industry to produce: juices, jams, alcoholic drinks, compotes, syrups, cakes, ice-cream, yogurts and many others, but could be also fresh consumed.

Blackberry pomace is a valuable by-product from blackberry juice processing which is rich in many bioactive compounds, such as anthocyanins, polyphenolics and oil (especially in seeds) and it is also a valuable source of fibers [2-6]. It could be mentioned that the pomace could represents 20-30% from the initial fruits mass and most of it is considered as unused waste, being a source of environmental pollution [2]. The bioactive compounds derived from blackberries pomace could be used to obtain functional food in order to increase the health attributes of food [7, 8]. In a recent study were analyzed the bioactive components for two spontaneous blackberries varieties from Romania in the two fractions which resulted from blackberries processing: juice and pomace [8]. The conclusions of aforementioned study are that two blackberries' fractions (juice and pomace) are rich in bioactive compounds and have high antioxidant properties, and also convective drying is an efficient method to preserve BB pomace [8].

Starting from this finding, now it is a great interest to develop different techniques to recovery these bioactive substances and to use them in functional food and for other applications. Among these techniques extraction is one of the most used for bioactive compounds separation, due to the fact that it is a robust technique and extraction equipment exists in a great variety.

The main goal of this paper is to investigate the possibility of extraction of anthocyanins and phenolic acids from dried blackberries pomace without seeds. The extraction process is influenced by many variables among them being: the used solvent, the solid-liquid ratio, the temperature and the extraction time. Because it is important to optimize the work conditions, in order to consume less solvents and time, the factorial experiments are a good choice [9]. In order to see the influences of the above-mentioned variables a factorial experiment 2^3 was performed, the response variable being extraction yield for total anthocyanins and phenolic acids.

2. Materials and methods

2.1. Vegetal material

Blackberries wild variety was collected in Prahova County (Romania) in July-August 2020, being used as vegetal material. They were manually cleaned, washed and then stored at 4±1 °C until their processing. BB juice was extracted from the berries by cold pressing using a Kuvings-Slow juicer (NUC Electronics Co., Ltd., Korea), obtaining a pomace consisting of seeds, peels and pulp fraction as a by-product. The pomace was dried at 50 °C in a food dehydrator (Tribest Sedona Express SDE-P6280, USA). After drying, the seeds were manually separated from dried pomace and then stored at 4±1 °C in sealed plastic bags before oil extraction. In this study was used the dried blackberries pomace (BBP) without seeds.

2.2. Extraction procedure

The initial anthocyanins and polyphenols content in BB dried pomace was determined using a laboratory Soxhlet extractor and water-ethanol (50% v/v) of analytical reagent grade as a solvent. About 5 g of grinded pomace was weighed and extracted with 150 mL water-ethanol solution for 8 h. Moisture content of the pomace was (5.4±0.02%) and was measured using a thermo-balance OHAUS MB23. For factorial experiments, batch extractions in a thermostatic shaker were performed for two hours in different conditions according to the experimental design matrix. In addition to anthocyanins, other favonoids (e.g. flavonols) can be extracted, but they don’t interfere with the anthocyanins analysis. The obtained extracts could be concentrated by vacuum evaporation and could be also encapsulated in order to be protected against degradation when they are used in different cosmetic and pharmaceutical applications.

2.3. Analysis of bioactive compounds

Total phenols in the pomace extract were analysed using the Folin-Ciocalteu method with the phenols content expressed as gallic acid equivalents (GAE) per gram of pomace on dry basis. Monomeric anthocyanins were determined by the pH differential method and the anthocyanin concentrations were expressed as mg cyanidin-3-O-glucoside equivalent (Cy3GlE)/g on dry basis. The extract obtained was diluted in two buffer solutions: potassium chloride pH 1.0 (0.025 M) and sodium acetate pH 4.5 (0.40 M), both adjusted with concentrated hydrochloric acid. The absorbance of the solutions was measured at 510 and 700 nm in a spectrophotometer (UV/VIS Cintra 6 spectrophotometer, GBS Australia), using both buffer solutions as blank. TAC (total anthocyanins concentration) was calculated in terms of cyanidin-3-O-glucoside using Eq. (1):

$$TAC = \frac{A \cdot MW \cdot DF \cdot 1000 \cdot V}{\varepsilon \cdot L \cdot M_{DP}} \quad (1)$$

Where: TAC= total anthocyanins concentration expressed as mg cyanidin-3-O-glucoside/g dm; MW=449.2 g/mol-molar mass of cyaniding-3-O-glucoside; DF=dilution factor; E=26,900 L/cm.mol-molar absorptivity of cyaniding-3-O-glucoside; V=total volume (L), L=cell path length (1 cm) M_{DP}=mass of dry residue. Absorbance (A) was calculated as:

$$A = \left(Abs_{510} - Abs_{700}\right)_{pH=1} - \left(Abs_{510} - Abs_{700}\right)_{pH=4.5} \quad (2)$$

2.4. Factorial experimental design

From preliminary experiments the most important factors influencing the extraction process were: solid/liquid ratio, temperature and the operating time. Taking into account this an experimental study was carried out based on a 2^3 factorial design [10-12]. Table 1 presents the variation levels of the independent variables. The response variable was the yield of TFC (total phenolic content) or TAC calculated using Eq. (3), where M_c is the mass of the extracted compound (mg), M_{dp} is the mass of the dried pomace without seeds (g).

$$Y = \frac{M_C}{M_{dp}} \quad (3)$$

A first-degree model was used to express the extraction efficiency as function of the three independent variables.

$$Y = \beta_0 + \beta_1 x_1 + \beta_2 x_2 + \beta_3 x_3 + \beta_{12} x_2 x_2 + \beta_{13} x_1 x_3 + \beta_{23} x_2 x_3 + \beta_{123} x_1 x_2 x_3 \quad (4)$$

Where: β_0 is the average theoretical value of the response, β_1, β_2 and β_3 are the factor effects of x_1, x_2 and x_3. The coefficients β_{12}, β_{13}, β_{23}, β_{123} represents interaction effects of the other model terms. The STATISTICA 10 statistical package software (Stat Soft Inc., Tulsa, USA) was used for experimental design analysis and data processing. The efficiency of the model, generated after the regression analysis of the response, was tested by ANOVA and Fisher's F-test. The operating variables and their levels are presented in Table 1.

3. Results and discussion

3.1. Preliminary experiments

Preliminary experiments were done in order to establish the best solvent for TAC and TFC determination. The extractions were performed in a batch system at room temperature and using a solid/liquid ratio of 1/30. The results are presented in Table 2.

Table 1

Matrix of experimental results using 2^3 factorial design

Operating parameters	Level		
	-1	0	1
X_1-solid/liquid ratio (g/mL)	1/30	1/40	1/50
X_2-temperature (°C)	20	40	60
X_3-extraction time (min)	60	90	120

Table 2

Preliminary experiments results using different solvent for anthocyanins and polyphenols extraction from BB pomace

Exp.	Solvent	TAC, (mg/g dm)	TPC, (mg/g dm)
1.	Ethanol (50% v/v)	4.677	14.753
2.	Ethanol (70% v/v)	3.889	12.219
3.	Citric acid solution (4%)	2.667	10.451
4.	Ethanol (50% v/v) with citric acid 4%	2.886	11.594
5.	Ethanol (70% v/v) with citric acid 4%	3.482	10.127
6.	Ethanol (96%)	2.129	5.509

Due to the fact that the best results were obtained using a solution ethanol-water (50% v/v) the experiments of the factorial design were done using this solvent combination.

3.2. Statistical analysis

A total of 11 experiments were performed according to the factorial design 2^3 both for anthocyanins and polyphenols batch extraction from blackberries pomace without seeds.

a) Batch TAC extraction

Table 3 describes the experimental design matrix for anthocyanins and polyphenols extraction from BBP without seeds. Regression coefficients significance was evaluated by Fisher's F-test. The significant terms in the model, generated after the regression analysis of the response, were found using Pareto analysis of variance (ANOVA) for the responses.

Table 3

The 2^3factorial design and the experimental responses

Exp.	Solid/liquid ratio (g/mL)	Temperature (°C)	Extraction time (min)	Y_{TAC} (mg/g dm)	Y_{TFC} (mg/g dm)
1	-1	1	-1	5.90±0.29	33.87±0.05
2	1	1	-1	6.69±0.20	50.07±0.05
3	1	-1	1	4.57±0.77	36.26±0.02
4	1	1	1	6.50±0.45	53.00±0.09
5	-1	-1	-1	2.35±0.17	26.09±0.05
6	-1	1	1	5.53±0.45	37.86±0.03
7	-1	-1	1	2.75±0.12	29.48±0.02
8	1	-1	-1	3.86±0.04	35.86±0.14
9	0	0	0	4.50±0.36	43.42±0.06
10	0	0	0	4.65±0.17	43.50±0.02
11	0	0	0	4.45±0.36	43.62±0.10

The obtained model for anthocyanins extraction in term of coded variables is given by equation (5).

$$Y_{TAC} = 4.7051 + 0.6371 \cdot X_1 + 1.3871 \cdot X_2 + 0.0696 \cdot X_3 - 0.195 \cdot X_1 X_2 + 0.0621 \cdot X_1 X_3 - 0.207\, X_2 X_3 - 0.15375 \cdot X_1 X_2 X_3 \quad (5)$$

The adequacy of the model was determined by assessing the lack of fit, the coefficient of determination (R^2) and the F test value obtained from the analysis of variance (ANOVA). The obtained values are presented in Table 4. According to the values presented in Table 4 the model is high significant having F-value 57.834 and a probability value p= 0.0033 which is less than 0.05. The goodness of fit of the model was checked using the values of R^2 (0.992) and of Adj R^2 (0.975). Starting from the value of Adj R^2, only 2.5 % of the total variation could not be explained by the proposed model.

From the investigated variables the most important are X_1 (solid liquid ratio) and X_2 (temperature) and also the product between X_1X_2 and X_2X_3. This aspect is also illustrated in Fig. 1a, where is presented Pareto chart for TAC extraction.

Table 4

ANOVA test for response function Y_{TAC} (extraction yield) for anthocyanins extracted from BBP without seeds

Term	Sum of squares	DF	Mean square	*F*-value	*p*-value
Model	19.36297	7	2.766138	57.83441	0.003390[a]
X_1	3.24743	1	3.24743	299.762	0.003319[a]
X_2	15.39293	1	15.39293	1420.885	0.000703[a]
X_3	0.03878	1	0.03878	3.580	0.199023
X_1X_2	0.30537	1	0.30537	28.188	0.033693[a]
X_1X_3	0.03088	1	0.03088	2.850	0.233425
X_2X_3	0.34570	1	0.34570	31.910	0.029938[a]
$X_1X_2X_3$	0.00189	1	0.00189	0.175	0.716670
Lack of Fit	0.12182	1	0.12182	11.245	0.078589
Pure Error	0.02167	2	0.01083		
Total SS	19.50645	10			
R^2=0.992	Adj R^2=0.975				
[a)] *The values are significant at p<0.05.*					

The simplified polynomial model obtained by neglecting the non-significant terms (p-values>0.05) in Eq. (5) is described by Eq. (6).

$$Y_{TAC} = 4.7051 + 0.6371 \cdot X_1 + 1.3871 \cdot X_2 - 0.195 \cdot X_1 X_2 - 0.207 X_2 X_3 \qquad (6)$$

The effects of extraction parameters on the TA extraction yield were investigated using the response surface plots. These plots were obtained by depicting two variables within the experimental range and keeping the third variable constant at zero level. Fig. 2 a,b,c, shows the effect of solid-liquid ratio, temperature, and extraction time on the anthocyanins extraction yield. The highest extraction yield was observed at the highest solid-liquid ratio and temperature.

Extraction efficiency for BBP was optimised by using the desirability function available in STATISTICA 10. From the desirability profiles the optimum could be attended at X_1=1, X_2=1 and X_3=-1, that means, solid liquid ratio=1/50, temperature=60 °C and extraction time 1 h. For these values the extraction yield is 6.65±0.10 (obtained experimentally) and 6.69 indicated by STATISTICA (Fig. 3a).

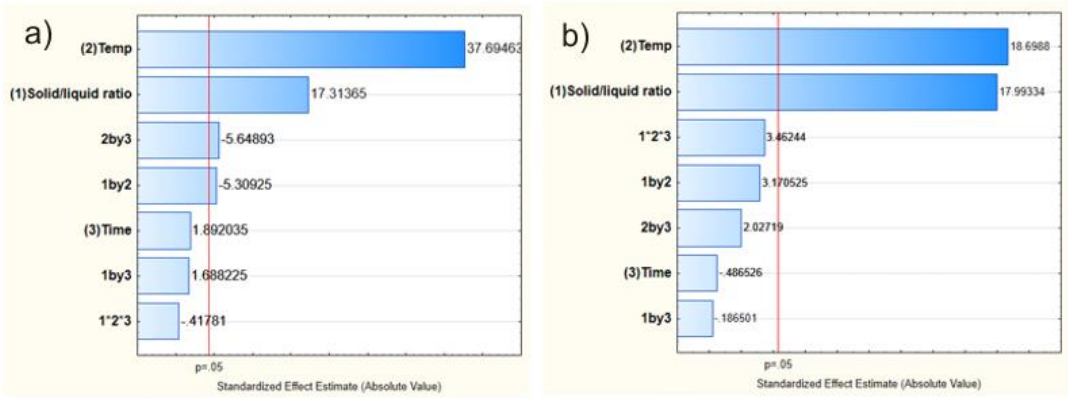

Fig.1. Pareto charts for: a) TFA extraction and b) TFC extraction.

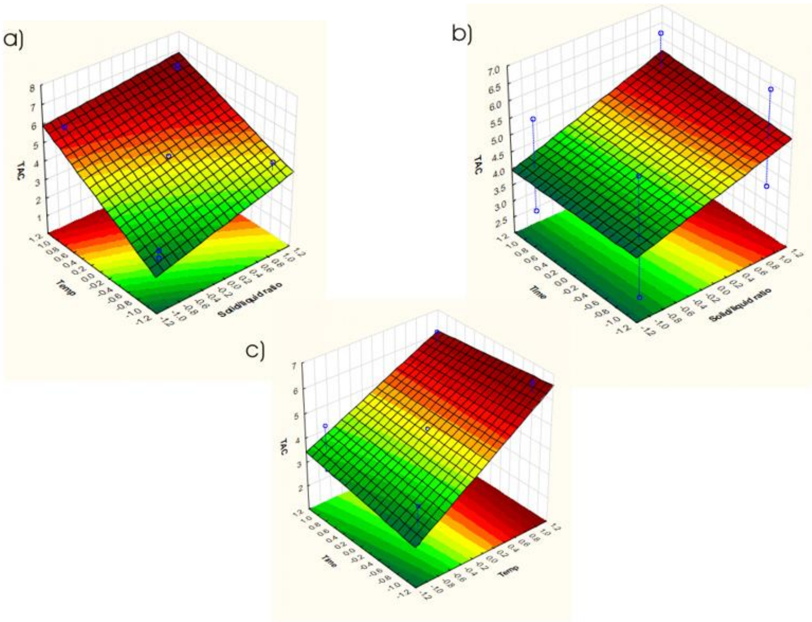

Fig. 2. Response surface plots for the effect of independent variables upon anthocyanins extraction yield: a) solid-liquid ratio and temperature; b) solid-liquid ratio and time and c) time-temperature.

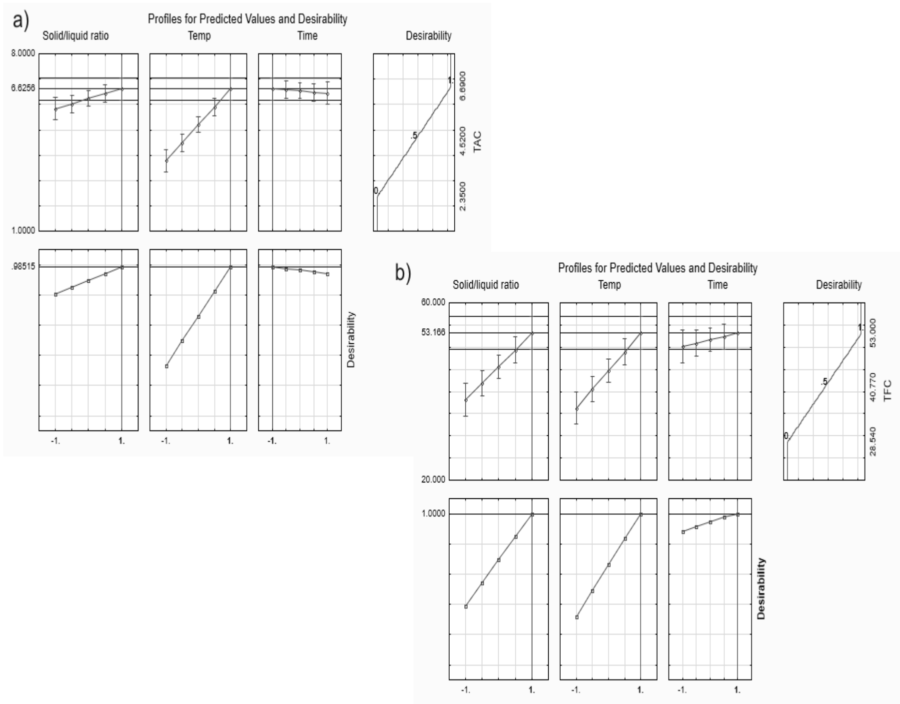

Fig. 3. Desirability profiles for: a) TA extraction and b) TF extraction.

b) Batch TFC extraction from BBP

Data presented in Table 3 were used to calculate the regression coefficients for the polynomial model for TFC extraction. The obtained model for TFC extraction in term of coded variables is given by equation (7).

$$Y_{TFC} = 39.3909 + 5.5475 \cdot X_1 + 5.765 \cdot X_2 - 0.15 \cdot X_3 + 0.9775 \cdot X_1 X_2 - 0.0575 \cdot X_1 X_3 + 0.635 \cdot X_2 X_3 + 1.0675 \cdot X_1 X_2 X_3 \qquad (7)$$

The adequacy of the model was also determined by assessing the lack of fit, the coefficient of determination (R^2) and the F test value obtained from the analysis of variance (ANOVA). The obtained values are depicted in Table 5.

The simplified fitted polynomial model obtained by neglecting the non-significant terms (*p*-values>0.05) in Eq. (7) is described by Eq. (8).

$$Y_{TFC} = 39.3909 + 5.5475 \cdot X_1 + 5.765 \cdot X_2 \qquad (8)$$

Fig. 4 a,b,c, shows the effect of solid-liquid ratio, temperature, and extraction time on the polyphenols extraction yield. Extraction yield for BBP for UAE was optimised by using the desirability function.

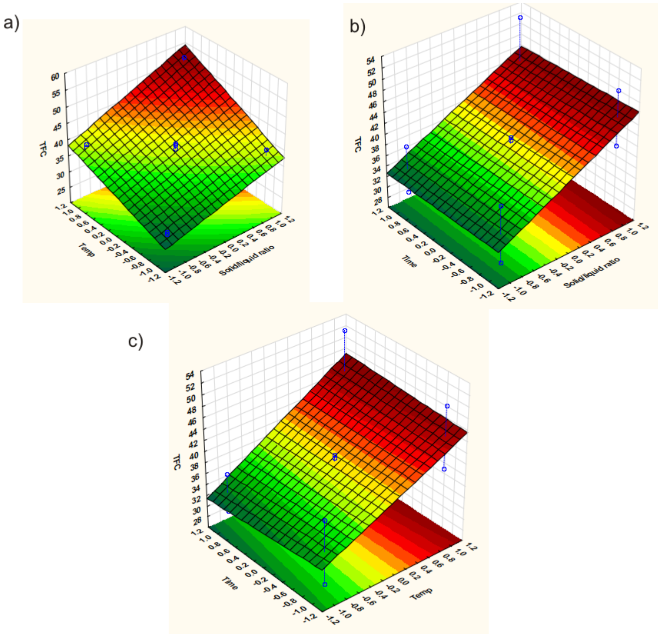

Fig. 4. Response surface plots for the effect of independent variables upon polyphenols extraction yield: a) solid-liquid ratio and temperature; b) solid-liquid ratio and time and c) time-temperature.

Table 5

ANOVA test for response function Y_{TFC} (extraction yield) for polyphenols extraction from BBP without seeds

Term	Sum of squares	DF	Mean square	*F*-value	*p*-value
Model	532.1718	7	76.02454	97.95753	0.001552[a]
X_1	246.1980	1	246.1980	323.7602	0.003074[a]
X_2	265.8818	1	265.8818	349.6451	0.002848[a]
X_3	0.1800	1	0.1800	0.2367	0.674687
X_1X_2	7.6441	1	7.6441	10.0522	0.086734
X_1X_3	0.0265	1	0.0265	0.0348	0.869256
X_2X_3	3.1250	1	3.1250	4.1095	0.179853
$X_1X_2X_3$	9.1165	1	9.1165	11.9885	0.074243
Lack of Fit	0.8074	1	0.8074	1.0618	0.411113
Pure Error	1.5209	2	0.7604		
Total SS	534.5001	10			
R^2=0.995	Adj R^2=0.985				
[a)]*The values are significant at p<0.05*.					

From the desirability profiles the optimum could be attended at X_1=1, X_2=1 and X_3=1, that means, solid liquid ratio=1/50, temperature=60 °C and extraction time 2 h. For these values, the extraction yield is 53±0.19 mg/gdm (obtained experimentally), and 53 mg/g dm indicated by STATISTICA (Fig. 3b).

4. Conclusions

In this study an experimental study regarding TCA and TFC extraction from BBP without seeds was done, using batch extraction. The extraction process was optimized using an experimental statistical design. From the analysis of experimental data, the most important operation parameters are temperature and solid-liquid ratio. The optimum values obtained for extraction yield were: 6.65±0.1 mg/gdm for anthocyanins and 53±0.19 mg/g dm for polyphenols extracted from blackberries dried pomace.

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