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Optimization of microwave-assisted extraction of phenolics from blueberry

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Abstract

The influence of microwave-assisted extraction (MAE) on the recovery of phenolic compounds from blueberry was investigated. Response surface methodology (RSM) was applied to optimize the extraction conditions. Microwave power, extraction time and solvent to sample ratio were selected as extraction parameters. Ranges of independent variables were 100-300 W of microwave power, 2-16 min of extraction time and 5:1-50:1 ml/g of solvent to sample ratio. Responses of model were extraction yield and total phenolic content (TPC) and total anthocyanin content (TAC) for extraction of blueberry powder. Optimum conditions of MAE based on maximum levels of responses were 287 W of microwave power, 13 min of extraction time, 40:1 ml/g of solvent to sample ratio. Maximum levels of responses under optimum conditions obtained were an extraction yield of 78.35%, TPC of 30.75 mg GAE/ g blueberry powder and TAC of 8.92 mg Cyn-3-glu/ g blueberry powder.

Key words: Microwave-assisted extraction, blueberry, phenolics, response surface methodology, optimization

1. Introduction

Blueberries (family Ericaceae; genus *Vaccinium*) are considered to be a rich sources of polyphenols, especially flavonoids such as anthocyanins (ELIK & al. [1]). Phenolics, especially anthocyanins, have been identified as having antioxidant properties which present beneficial effects in human health and prevent many degenerative diseases (BOBINAITĖ & al. [2]; NETO [3]). In addition to their antioxidant power, phenolics also have health benefits such as anti-inflammation action (JOSEPH & al. [4]), cancer enzyme inhibition (DUTHIE & al. [5]), antimutagenic activity (NILE & al. [6]), antimicrobial activity (DAGLIA [7]), neuroprotective property (DREISEITEL & al. [8]), nitric oxide production inhibition (DEL RIO & al. [9]) and chemoprotective enzyme inducement (YANG & al. [10]).

Extraction of natural phenolics using conventional methods is an expensive and time consuming process. Therefore, there have been numerous publications exploring that the use of modern extraction techniques such as ultrasound-assisted extraction (UAE), supercritical-fluid extraction (SFE), extraction by superheated water for food materials leads to time and solvent usage reduction, higher efficiency and increase of the extraction yields of valuable components (AZMIR & al. [11], GOGUS & al. [12]). One of the most promising techniques is microwave-assisted extraction (MAE) (CHAN & al. [13]). MAE utilizes microwave energy to heat solvents

that are in contact with solid samples. In contrast with classical heating, the uniform heating by microwave energy allows for the sample to be heated. This allows for the solvent to heat rapidly, resulting in short extraction times. Furthermore, many studies have been reported that MAE can reduce solvent requirements, decrease extraction time and provide better extraction efficiency compared to conventional technique (JOKIĆ & al. [14]; NEMES & al. [15]; VENKATESH & al. [16]).

Studies about microwave assisted extraction from blueberry are limited in the literature (ZHENG & al. [17]). In the present study, phenolic extraction from blueberry using microwave technique has been investigated in detail. Therefore, the following points are considered in this study: (1) investigating the influences of MAE parameters (microwave power, extraction time and solvent to sample ratio) on the extraction yield, total phenolic content (TPC) and total anthocyanins content (TAC) (2) optimizing conditions of the MAE for the yield of extracts, TPC and TAC using response surface methodology.

2. Materials and Methods

Blueberry and reagents

Blueberries (*Vaccinium Corymbosum*, Bluecrop variety) were purchased from an organic farm, Trabzon, Turkey. Blueberries were frozen at -40^{o}C to perform freeze drying process. Whole blueberries were dried in a laboratory freeze-dryer (CHRIST Alpha 1-4 LDplus, Martin Christ, Germany) in 48 h. After drying, blueberries were ground and passed through to a 40-mesh sieve to produce blueberry powder. The moisture content of blueberry powder was under 1%. The freeze-dried blueberry powder was stored in airtight containers in refrigerated conditions until being used.

Folin–Ciocalteu's phenol reagent, ethanol, citric acid, 1,1-diphenyl-2-picrylhydrazyl (DPPH) and trolox were purchased from Sigma-Aldrich. 2,4,6-tripyridyls-triazine (TPTZ) was purchased from Fluka. All other reagents and solvents used were of analytical or chromatographic grade.

MAE procedure

A quantity (quantity was varied as a function of solvent to sample ratio) of blueberry powder was weighed into the flask and mixed with ethanol–water mixture (60:40, v/v). Total volume of extraction solvent was kept constant at 72 ml. The sample solvent mixture was heated by application of microwave for a fixed amount of time and at a fixed power. MAE was carried out with a focused open-vessel microwave system (CEM Corporation, USA, 3100 Smith Farm Road, Matthews, NC 28105-5044). The microwave power level and the extraction time for which microwave was applied were selected according to the experimental design (Table 1). Upon completion of extraction process, extracts were transferred to centrifuge tubes and centrifuged at 6000 rpm at 25^{o}C for 15 min and liquid portion has been collected. Subsequently, solvent was evaporated using the rotary vacuum evaporator (Model VV 2000, Heidolph, Germany). Residual solvent in the extracts was evaporated using a freeze drier and then dry extracts were stored at -18^{o}C until the analyses were performed.

Determination of moisture content

3-5 g of sample were weighed on aluminum tray and put in a drying oven at 105^{o}C until achieving constant weight. The dried blueberries were weighed and the dried matter that remained was determined. All results were expressed on dry matter (DM) basis.

Table 1. Three-factor, three-level face-centered central composite design and results for three variables studied

Standard Order	Microwave Power (W)	Extraction Time (min)	Solvent to sample Ratio (ml/g)	Extraction Yield[1] (%)	TPC (mg GAE/ g blueberry powder)	TAC (mg Cyn-3-glu/ g blueberry powder)
1	100	2	5:1	65.08	14.58	5.14
2	300	2	5:1	66.25	16.30	6.11
3	100	16	5:1	65.19	15.63	5.29
4	300	16	5:1	68.90	18.96	6.32
5	100	2	50:1	76.53	21.66	7.16
6	300	2	50:1	73.76	24.98	7.51
7	100	16	50:1	77.48	29.59	8.80
8	300	16	50:1	77.78	29.12	8.73
9	100	9	27.5:1	74.46	26.07	7.18
10	300	9	27.5:1	77.13	27.88	8.05
11	200	2	27.5:1	73.60	25.88	7.63
12	200	16	27.5:1	74.22	28.03	7.88
13	200	9	5:1	65.18	15.95	5.68
14	200	9	50:1	75.11	26.49	7.97
15	200	9	27.5:1	74.81	28.74	7.80
16	200	9	27.5:1	74.63	28.40	7.83
17	200	9	27.5:1	74.99	28.40	8.49
18	200	9	27.5:1	74.42	27.01	7.91
19	200	9	27.5:1	75.55	28.52	7.77
20	200	9	27.5:1	75.68	28.56	7.73

[1] (g dry extract/g blueberry powder)x100

Total phenolic content (TPC) by Folin-Ciocalteu's assay

The Folin-Ciocalteu method was used to determine total phenol levels in samples, which was adapted from SINGLETON & al. ([18]). TPC values were expressed as gallic acid equivalents (GAE) in mg per g of the powder. All measurements were done in triplicate.

Total anthocyanin content (TAC) by pH-differential method

The pH-differential method was used to determine anthocyanins content of extracts (LEE & al. [19]). Total anthocyanins of samples were expressed as the amount of cyanidin-3-glucoside equivalents with unit of mg per g powder. All measurements were done in triplicate.

DPPH-scavenging activity assay

The DPPH radical scavenging activity of samples was measured according to the method of BRAND-WILLIAMS & al. ([20]). The results were expressed as a DPPH free radical scavenging activity EC_{50} value, which reflects 50% depletion of the free radical. DPPH tests were done in triplicate.

Ferric reducing antioxidant power (FRAP) assay

Antioxidant powers of samples were determined the FRAP assay, which is based on ferric to ferrous reduction in the presence of 2,4,6-tripyridyls-triazine (TPTZ) (BENZIE & al. [21]). Results were expressed as trolox equivalents (TE) in μmoles per g of the blueberry powder. All measurements were done in triplicate.

Experimental Design and Optimization by Response Surface Methodology

Response surface methodology (RSM) was chosen to determine the optimal conditions for MAE from blueberry. The RSM was performed using Design Expert (Stat-Ease, Design-Expert software, version 7). The effect of the independent variables; microwave power (100-300 W), extraction time (2-16 min) and solvent to sample ratio (5:1-50:1 ml/g) was investigated using a three-factor, three-level face-centered central composite design (FCCCD). The complete design consists of 20 runs, including six replications of the centre points for the three independent variables. Table 1 shows the effect of microwave power (100-300 W), the solvent to sample ratio (5:1-50:1 ml/g) and extraction time (2–16 min) on the extract yield, total phenolic content and total anthocyanin content of blueberry extracted by MAE.

The fitness of the model was determined by evaluating the Fisher test value (F-Value), and the coefficient of determination (R^2) as obtained from an analysis of variance (ANOVA). The level of significance for all tests was set at 95% confidence level. FCCCD uses least-squares regression to fit the experimental data to a quadratic model. The quadratic model for the responses is as follows:

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

where Y are the dependent variable, β_0, β_i, β_{ii} and β_{ij} are the regression coefficients for the intercept, linear, quadratic and interaction terms of variables i and j, respectively, and X_i and X_j are the independent variables.

3. Results and Discussion

Preliminary studies

Extraction parameters chosen (microwave power, extraction time and solvent to sample ratio) in this study were based on preliminary experiments and previous studies (ZHENG & al. [17]; MANDAL & al. [22]). Microwave power was operated in range of 100-300 W. Microwave power above 300 W was caused some troubles such as foaming and charring of blueberry. Studied extraction time ranged from 2 to 16 min. Longer extraction time did not cause considerable extraction of phenolics from blueberry. 5:1-50:1 ml/g of solvent to sample ratio was chosen. Higher ratio than 50:1 ml/g of solvent to sample ratio did not show significant recovery of phenolics and also caused waste of solvent.

In general, organic solvents such as methanol, ethanol, acetone and ethyl acetate are used for extraction of phenolic compounds. Organic solvents, however, such as methanol are potentially detrimental to human health. The use of ethanol has many advantages as it has high extraction efficiency and dielectric constant, low toxicity and cost (WU & al. [23]). Therefore, ethanol was selected as extraction solvent for experiments. Aqueous solvent is considered more efficient than the pure solvent in phenolic extraction (BELWAL & al. [24]). Extraction solvent with an ethanol-water ratio of 60:40 (v/v) was kept constant in all experiments due to obtaining highest yield of phenolics in preliminary experiment. Acidified solvents can increase extraction yield (BRIDGERS & al. [25]). Citric acid was used to obtain better yield in extraction.

Model fitting

Response surface models were used for experimental design in MAE. Regression analysis with backward elimination was used to obtain best fitting quadratic models. The backward elimination was employed to eliminate insignificant factors and interactions in the models. The regression models were significant ($p<0.001$) for MAE from blueberry powder with satisfactory coefficient of determination (R^2) that were 0.9826 for the extraction yield, 0.9763 for TPC and 0.9653 for TAC. Large F value and small value of probability of models also confirm that models

are significant (Table 2). Relationship between independent variables and responses could be expressed by the following Eq. (2 - 4).

The extraction yield of blueberry as a function of the independent variables:

$$Y_1 = 74.72 + 0.51*Mw + 0.83*Ti + 5.01*Ra + 0.70*Mw*Ti - 0.92*Mw*Ra + 1.14*Mw^2 - 4.51*Ra^2 \quad (2)$$

TPC of blueberry as a function of the independent variables:

$$Y_2 = 27.75 + 0.97*Mw + 1.79*Ti + 5.04*Ra + 1.05*Ti*Ra - 6.42*Ra^2 \quad (3)$$

TAC of blueberry as a function of the independent variables:

$$Y_3 = 7.83 + 0.31*Mw + 0.35*Ti + 1.16*Ra - 0.21*Mw*Ra + 0.31*Ti*Ra - 0.96*Ra^2 \quad (4)$$

where Y_1, Y_2 and Y_3 are responses of models, Mw is microwave power, Ti is extraction time and Ra is solvent to sample ratio.

3D response surface and contour plots were used to show the effects of process variables on the responses. The response surface and contour plots indicated the effect of two variables on the dependent variable described by the quadratic polynomial equation while third variable was kept constant at middle level, 9 min for extraction time, 200 W for microwave power, 27.5:1 for solvent to sample ratio.

The effect of process variables on the extraction yield

Various factors showed significant effect on the extraction yield. Microwave power was evaluated in terms of the effect on the extraction yield. From Table 2, it was observed that microwave power has significantly ($p < 0.05$) positive linear and quadratic effects on extraction yield. It could be explicated that increase in microwave power results in increased extraction yield. Increase in microwave power results in the rupture of the cell walls and enhance the extraction due to easier penetration of the solvent into the plant matrix (MENDES & al. [26]).

Microwave power had also an interaction effect with other process variables. The interactive effect between microwave power and extraction time was significant ($p < 0.05$) and positive. It means that extraction yield obtained increases significantly as microwave power and extraction time increase simultaneously (Figure 1). In the study carried out by MILUTINOVIC & al. ([27]), a similar trend was found for interaction between microwave power and extraction time. Besides, it was shown that the interactions between microwave power and solvent to sample ratio affected extraction yield significantly (Table 2). As this interaction has negative constant coefficient, the effect of microwave power on extraction yield was negative or positive depending on solvent to sample ratio.

When the effect of extraction time on extraction yield is examined, it is able to understand that time is a more effective independent variable than microwave power due to its greater constant coefficient (Eq. 2). Extraction time displayed quite significant ($p<0.05$) positive effect on the extraction yield. It is concluded that longer extraction time had positive effects on the yield of extraction.

Table 2. Analysis of variance (ANOVA) of responses for MAE experiments

Source	The extraction yield					TPC					TAC				
	SS[1]	DF	MS	F - Value	p-value Prob > F	SS[1]	DF	MS	F - Value	p-value Prob > F	SS[1]	DF	MS	F - Value	p-value Prob > F
Model	348.09	7	49.73	96.70	< 0.0001[2]	510.81	5	101.21	115.42	< 0.0001[2]	21.44	6	3.57	60.3	< 0.0001[2]
Intercept															
Linear															
Microwave Power (Mw)	2.58	1	2.58	5.02	0.0448[2]	9.43	1	10.02	10.63	0.0045[2]	0.99	1	0.99	16.74	0.0013[2]
Extraction Time (Ti)	6.97	1	6.97	13.56	0.0031[2]	32.15	1	31.08	36.37	< 0.0001[2]	1.2	1	1.2	20.32	0.0006[2]
Solvent to sample ratio (Ra)	250.6	1	250.6	487.32	< 0.0001[2]	254.22	1	251.2	287.18	< 0.0001[2]	13.53	1	13.53	228.23	< 0.0001[2]
Interaction															
Mw*Ti	3.93	1	3.93	7.65	0.0171[2]										
Mw*Ra	6.75	1	6.75	13.13	0.0035[2]						0.37	1	0.37	6.24	0.0267[2]
Ti*Ra						8.74	1	8.74	9.87	0.0072[2]	0.78	1	0.78	13.16	0.0030[2]
Quadratic															
Mw^2	4.17	1	4.17	8.11	0.0147[2]										
Ti^2															
Ra^2	65.03	1	65.03	126.47	< 0.0001[2]	206.27	1	206.27	233.1	< 0.0001[2]	4.57	1	4.57	77.11	< 0.0001[2]
Residual	6.17	12	0.51			12.39	14	0.88			0.77	13	0.0059		
Lack of Fit	4.9	7	0.7	2.75	0.1418[3]	10.40	9	1.16	2.91	0.1264[3]	0.36	8	0.046	0.56	0.7775[3]
Pure Error	1.27	5	0.25			1.99	5	0.4			0.41	5	0.081		
Cor Total	354.26	19				523.19	19				22.21	19			
R^2	0.9826					0.9763					0.9653				
Adj R^2	0.9724					0.9679					0.9493				
Pred R^2	0.9462					0.9371					0.9294				

[1] Sum of Squares

[2]Significant at Prob > F less than 0.05 level; [3] not significant at Prob > F higher than 0.05 level

The linear effect of solvent to sample ratio was positive and significant ($p<0.05$) while its quadratic effect was significant and negative, which resulted in a curvilinear increase in extraction yield (Figure 1A and 1B). The extraction yield went up rapidly up to 40:1 ml/g as solvent to sample ratio increases. After that point, however, the further increase of solvent to sample ratio affected the extraction yield negatively. When it was used large volume of solvent, it caused excessive swelling of the materials and extraction efficiency increased (MILUTINOVIĆ & al. [27]). However, in case of further increase in solvent volume, the most of microwave energy was absorbed by extraction solvent and very little microwave energy absorbed directly by the materials (YAN & al. [28]). Therefore, this phenomenon leads to the less efficient extraction.

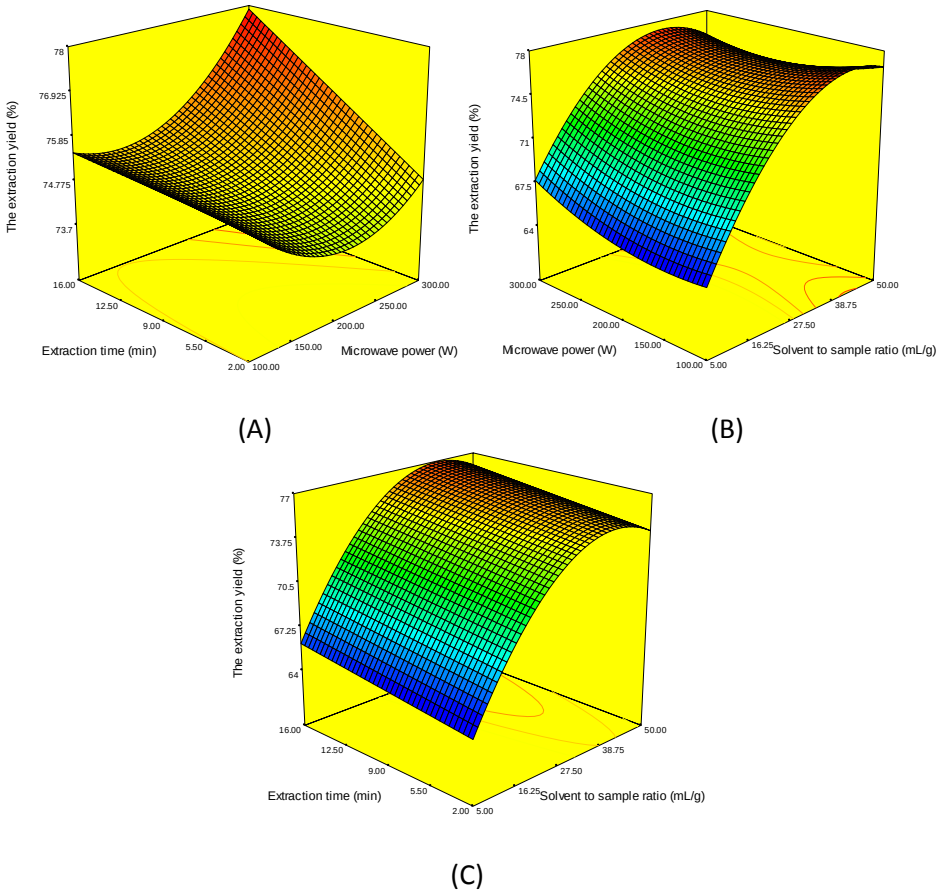

Figure 1. Response surface plots of extraction yield as affected by extraction time, microwave power and solvent to sample ratio in MAE. (A) extraction time (X1) and microwave power (X2); (B) microwave power (X1) and solvent to sample ratio (X2); (C) extraction time (X1) and solvent to sample ratio (X2). The value of the missing independent variable in each plot was kept at the centre point

The effect of process variables on TPC

Microwave power showed a positive linear effect on TPC ($p< 0.05$). It indicates that increase in TPC is noted when microwave power increased from 100 to 300 W (Figure 2A and 2B). However, the effect of microwave power was the least effective independent variable compared to other two variables, namely extraction time and solvent to sample ratio.

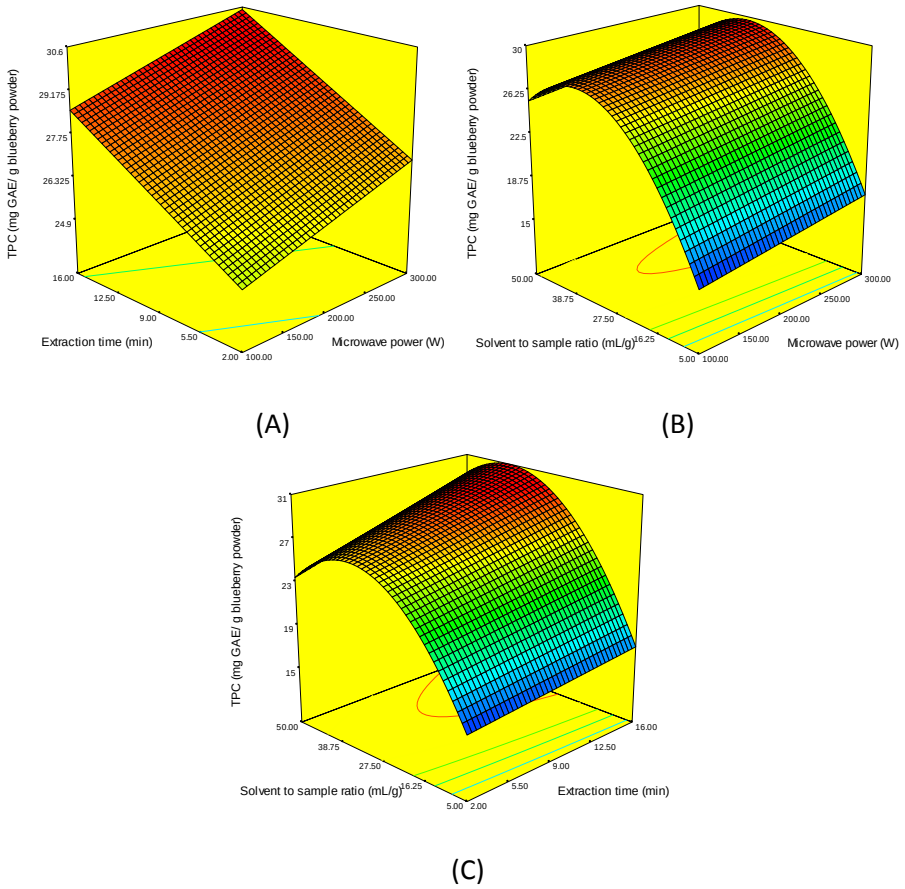

Figure 2. Response surface plots of TPC as affected by extraction time, microwave power and solvent to sample ratio in MAE. (A) extraction time (X1) and microwave power (X2); (B) solvent to sample ratio (X1) and microwave power (X2); (C) solvent to sample ratio (X1) and extraction time (X2). The value of the missing independent variable in each plot was kept at the centre point

ANOVA results showed linear effect of extraction time ($p<0.05$) on TPC. It can be interpreted that as extraction time increases, amount of phenolics extracted from blueberries also increase. Table 2 reveals that the interaction effect between extraction time and solvent to sample ratio is significant ($p<0.05$). As seen in Figure 2B, more yield of phenolics extracted was resulted at higher solvent to sample ratio and microwave power. Solvent to sample ratio was the most important independent variable on TPC. As shown in Table 2, solvent to sample ratio had significant ($p<0.05$) positive linear and negative quadratic effect on TPC. This effect resulted in a curvilinear increase in TPC as in the extraction yield. LI & al. ([29]) reported that solvent to sample ratio was the most effective parameter in MAE of polyphenols from grape seed.

The effect of process variables on TAC

It was found that microwave power significantly influenced TAC as in TPC. It depicts that the increase in microwave power improves the yield of TAC. Negative interaction between microwave power and solvent to sample ratio was observed for TAC. (Figure 3B).

An ANOVA of extraction time indicated positive linear effect on TAC. Anthocyanins extracted increased linearly with extraction time. There were also significant ($p<0.05$) positive interaction effects of extraction time and solvent to sample ratio, indicating that TAC increases considerably with increase in solvent to sample ratio and extraction time. Solvent to sample ratio

was also observed as the most efficient factor for TAC as it has been observed for two other responses. Table 2 demonstrated positive linear and negative quadratic effects of solvent to sample ratio on TAC. Solvent to sample ratio up to 40:1 ml/g caused increase in TAC however, further increase in solvent to sample ratio led to decline of quantity of extracted anthocyanins from blueberries. A similar observation was also reported in the study of ZHENG & al. ([17]).

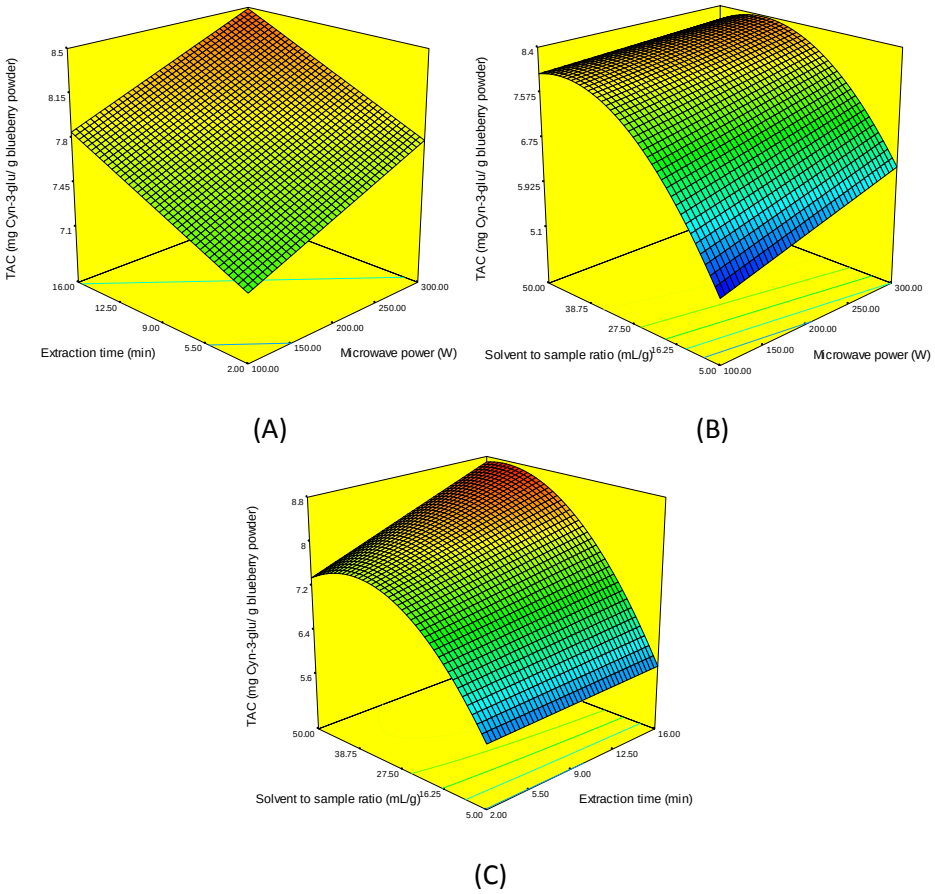

Figure 3. Response surface plots of TAC as affected by extraction time, microwave power and solvent to sample ratio in MAE. (A) extraction time (X1) and microwave power (X2); (B) solvent to sample ratio (X1) and microwave power (X2); (C) solvent to sample ratio (X1) and extraction time (X2). The value of the missing independent variable in each plot was kept at the centre point

Model optimization and verification

FCCCD was used for optimization of extraction parameters. Optimization of the MAE conditions was based on achieving the highest extraction yield, TPC and TAC. The optimized conditions for blueberry powder were 287 W of microwave power, 13 min of extraction time, 40:1 ml/g of solvent to sample ratio with the predicted extraction yield of 78.35%, TPC of 30.75 mg GAE/g blueberry powder and TAC of 8.92 mg Cyn-3-glu/ g blueberry powder.

Predicting the optimum response values is tested to determine the accuracy of the model using the selected optimum conditions. The experiments were carried out in triplicates and the average values were found as 78.47%, 30.38 mg GAE/g blueberry powder and 8.78 mg Cyn-3-glu/ g blueberry powder for extraction yield, TPC and TAC, respectively. The experimental

results were quite close to the predicted ones. Consequently, it can be seen that indicating the RSM model was satisfactory and accurate.

Properties of berry extracts obtained at optimum conditions

Blueberry extracts were obtained at optimum conditions and total phenolic content (TPC), total anthocyanin content (TAC) and antioxidant activity (DPPH and FRAP) of extracts were determined. Table 3 shows TPC, TAC and antioxidant activity of blueberries before and after extraction. It can be understood from those results that 86.9% of phenolics of blueberry could be extracted by MAE. Anthocyanin content of blueberry powder constituted approximately 29% of total phenolics. Anthocyanins can be easily degraded by some factors such as temperature and long processing time (MANDAL & al. [22]; MARTYNENKO & al. [30]). Results from Table 3 have shown that most of anthocyanins content (87.1%) in blueberry could be extracted effectively by MAE without degrading. It might be explained by short processing time of MAE. Besides having rich phenolic content in extracts, their high antioxidant capacities were also very high. One of the most common method to evaluate antioxidant activity of extracts is the DPPH-scavenging activity assay which relies on the decrease of DPPH• absorbance at 517 nm induced by antioxidants. EC_{50} values (the concentration needed to decrease by 50% the initial DPPH concentration) of blueberry powder and extract were found 0.89 and 1.03 mg blueberry powder/ml, respectively. It indicates that blueberry extracts were very strong DPPH• scavengers. FRAP which is another common method to evaluate antioxidant activity was used to determine antioxidant activity of extracts. The FRAP values of blueberry powder and extracts were 277.57 and 242.28 μmoles TE/g blueberry powder, respectively. The FRAP values of blueberry powder and extracts are pretty close to each other. Both results of DPPH and FRAP demonstrated that blueberry extracts show high antioxidant activity. This is due to extracting high amount of phenolics, especially anthocyanins from blueberry powder.

Table 3. Comparison of TPC, TAC, DPPH and FRAP values before and after extraction

	TPC [1]	TAC [2]	DPPH·EC_{50} [3]	FRAP [4]
Before extraction (BE)	35.36	10.24	0.89	277.57
After extraction (AE)	30.75	8.92	1.03	242.28

[1]mg GAE/ g blueberry powder
[2]mg Cyn-3-glu/ g blueberry powder
[3]mg blueberry powder/ ml (EC_{50} = Blueberry concentration needed to decrease by 50% the initial DPPH concentration)
[4]μmoles TE/g blueberry powder

4. Conclusion

Optimization of MAE for TPC, TAC and the yield of extracts was successfully investigated using response surface methodology. The experimental values agreed with the predicted values, thus proving satisfactory and accurate of RSM model. All independent variables (microwave power, extraction time and solvent to sample ratio) demonstrated a significant effect on MAE from blueberry powder. The most effective variable on all responses was solvent to sample ratio, followed by extraction time and the least was microwave power. High amount of phenolics could be extracted from blueberry by using MAE. Besides, it was found that extracts under optimum conditions had high antioxidant capacity. High-quality extracts was obtained in a short time using MAE. Our results showed that MAE was a fast and effective extraction technique and has a great potential to be used in phenolics extraction.

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