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Optimization of Process Parameters for the Extraction of Anthocyanins from Black Rice Bran Using Response Surface Methodology

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This study aimed to optimize different process parameters for the extraction of anthocyanins from black rice bran using Response Surface Methodology (RSM). To determine its degradation profile, the stability of crude anthocyanin extract (CAE) against selected biologically relevant buffers was also evaluated. Two-level full factorial and Box-Behnken designs were employed in the screening and optimization of extraction parameters, respectively. CAE was prepared from black rice bran using conventional and optimized methods, and the resulting extract was determined for their phytochemical content and antioxidant scavenging activities. Stability of the optimized CAE was further evaluated using biologically relevant buffers for 48 h. Results showed that the optimum conditions for the extraction of anthocyanins were 60% ethanol, 0.2% hydrochloric acid, and 215 min of extraction. The CAE obtained from the optimized method was 4 times higher in anthocyanins, 2.3 times higher in 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical scavenging activity, 1.7 times higher in phenolics and ferric reducing antioxidant power (FRAP) values, and 1.5 times higher in flavonoids than that obtained from the conventional method. In terms of its stability, the optimized CAE did not undergo substantial degradation at pH 1, while significant degradation was observed at pH 7.4. Addition of 10% newborn calf serum had no significant effect on the stability of anthocyanin. The half-life of anthocyanins from the optimized CAE ranged from 29.0 to 32.5 h based on first order kinetics. The study suggests that RSM is a practical and effective statistical tool that can be used to optimize the best conditions in extracting anthocyanins from black rice bran.

Key Words: anthocyanin, black rice bran, Box-Behnken design, full factorial design, phytochemical properties, response surface methodology

Abbreviations: BBD – Box-Behnken design, CAE – crude anthocyanin extract, C3G –cyanidin-3-glucoside, DPPH – 2,2-diphenyl-1-picrylhydrazyl, FRAP – ferric reducing antioxidant power, GAE – gallic acid equivalent, NCS – newborn calf serum, RHE – rutin hydrate equivalent, RSM – response surface methodology, TAC – total anthocyanin content, TE – trolox equivalent, TFC – total flavonoid content, TPC – total phenolic content

INTRODUCTION

Anthocyanins are water-soluble secondary metabolites responsible for the bright red, purple, and blue coloration of many flowers, fruits, and vegetables (Rein 2005). The most common sources of anthocyanins are pigmented fruits, mainly grapes, currants, and berries such as blueberry, blackberry, cherry, cranberry, raspberry, and strawberry (Ramos et al. 2014). Anthocyanin-rich vegetables include black carrot, red cabbage, purple eggplant, and purple potato (Khoo et al. 2017). In rice, anthocyanins are mostly located in the bran layer, and their contents are higher in black rice than in red rice varieties (Lokuldilok et al. 2011; Bulatao et al. 2016).

Anthocyanins exist in four structural forms, namely, the purple flavylium cation (pH 1–3), blue anhydrous quinoidal base (pH 6–8), colorless carbinol base (pH 4–5), and the pale yellow chalcone (pH 8–9). They are conventionally extracted through the successive solvent extraction system, which uses mostly acidified alcohols such as methanol and ethanol (Pereira-Caro et al. 2013;

Phetpornpaisan et al. 2014; Shao et al. 2014). Anthocyanin extracts are relatively unstable to heat, pH, light, solvent, enzymes, and the presence of oxidants, which consequently lower the extraction recovery due to degradation (Giusti and Wrolstad 2003; Rein 2005; Scalzo et al. 2008; Castañeda-Ovando et al. 2009). Among these factors, pH and heat significantly affected the degradation of anthocyanins (Sadilova et al. 2007). Hence, it is necessary to consider various factors that could affect the quality and yield of anthocyanins such as the nature of extraction process, solvent system, and time of extraction for the development of natural food supplement and possible synthesis of potent drugs (Bordiga et al. 2014).

Clinical studies suggest that anthocyanins could inhibit the growth of HT-29 colon cancer cell and reduce the Lipid A-induced Interleukin-12 release from murine dendritic cells (Dai et al. 2007). The isolated cyanidin-3-glucoside from blackberry extract was found to protect the $CaCO_2$ cells from peroxyl radical-induced apoptosis (Elisia and Kitts 2008). Several studies have also reported different mechanisms of anthocyanins related to the prevention and management of various lifestyle diseases such as cancer, obesity, diabetes, cardiovascular, and neurological diseases (Konczak and Zhang 2004; Ramos et al. 2014).

Response Surface Methodology (RSM), on the other hand, is a multivariate non-linear model, which is widely used in chemical, biological, and industrial applications to predict the optimal conditions of a given system (Liyana-Pathirana and Shahidi 2005; Cacace and Mazza 2003; Anuar et al. 2013). It is a collection of mathematical and statistical techniques used for the modeling and analysis of problems in which the response of interest is influenced by several variables; the objective is to optimize this response (Montgomery 2005; Bas and Boyaci 2007; Myers et al. 2009). RSM is far better than "one-factor-at-a-time" experimentation due to its ability to simultaneously analyze multiple interactions of process variables at the same time (Elksibi et al. 2014). It employs suitable designs involving several independent variables and uses data from experimentation to produce a model that predicts response based on its significant independent variables (Said and Amin 2015). Moreover, RSM can produce designs with a smaller number of experimental runs without affecting the quality of the result, thereby, saving cost, time, and resources of the analysis (Gu et al. 2012; Kuo et al. 2013).

RSM has been successfully used in the optimization of various conditions for the extraction of polyphenols in apple pomace (Mustafa and Kjersti 2010), black rice (Arup et al. 2017), *Epimedium brevicornum* Maxim (Zhao et al. 2014), and green tea (Lee et al. 2013). It was also employed in the optimization of anthocyanin extraction in purple sweet potato (Gongjian et al. 2008), mulberry (Zou et al. 2011), and purple eggplant (Thirunavukkarasu 2011).

In our study, RSM was used to optimize different process variables for the extraction of anthocyanins in black rice bran. The methods employed were the two-level full factorial design to determine the significance of the independent variables and their interactions in the extraction process, and the Box-Behnken design (BBD) for the optimization of process variables. The CAE from the optimized method (optimized CAE) was analyzed for their total anthocyanin content (TAC), total phenolic content (TPC), total flavonoid content (TFC), 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical scavenging activity, and ferric reducing antioxidant power (FRAP). The optimized CAE was further assessed for its 48-h stability against selected biologically relevant buffers.

MATERIALS AND METHODS

Rice Samples and Processing

Ominio, a traditional black rice variety from Mt. Province, Philippines, was used as sample in the extraction of anthocyanin due to its very high content (Bulatao et al. 2017). About 1 kg of black rice sample was dehulled using a laboratory dehuller (THU-35A Satake, Japan) to obtain the brown rice and was polished using a rice polisher (McGill No. 2, Canada) to collect the bran. The recovered rice bran was then passed through a laboratory sieve (ASTM no. 40) to obtain finer and uniform particles. The bran was placed in a polyethylene plastic bag, sealed, and refrigerated at 4 °C prior to extraction.

Extraction of Anthocyanins using Conventional Method

About 1 g of rice bran was weighed and soaked with 5 mL of 85% acidified ethanol (ethanol and 1N HCl, 85:15 v/v). The mixtures were agitated for 2.5 h at 300 rpm at room temperature and then centrifuged at 3000 rpm for 10 min using refrigerated centrifuge (Allegra x-22R, Beckman Coulter, USA). The supernatants were collected through decantation and transferred to another 15-mL centrifuge tube. The extracts were refrigerated at 4 °C until analysis. The anthocyanin content of the extracts was determined

Table 1. Independent variables and their values for two-level full factorial experimental design.

Variable	Factor	Unit	Level	
			-1	1
Ethanol concentration	A	%	20	80
HCl concentration	B	%	0	0.10
Extraction time	C	min	60	240

Table 2. Independent variables and their values for Box-Behnken design.

Variable	Factor	Unit	Level		
			-1	0	1
Ethanol concentration	A	%	30	55	80
HCl concentration	B	%	0	0.1	0.20
Extraction time	C	min	60	150	240

Table 3. Composition of treatments in the stability study of optimized CAE.

Treatment	Composition	Temperature
T_0	CAE + pH 1.0 HCl/KCl buffer	25 °C
T_1	CAE + pH 7.4 phosphate buffer saline (PBS)	
T_2	CAE + pH 7.4 PBS + 10% new born calf serum	
T_3	CAE + pH 1.0 HCl/KCl buffer	37 °C
T_4	CAE + pH 7.4 PBS + 10% new born calf serum	

CAE – crude anthocyanin extract

using the pH-differential method as described by Anuar et al. (2013).

Optimization of Process Parameters for the Extraction of Anthocyanin Using RSM

Extraction of anthocyanin was optimized based on the conventional method using RSM models. The anthocyanins from black rice bran were extracted at different levels of factors that were varied in every extraction run. The factors employed in this study were solvent concentration, acid concentration, and extraction time. About 1 g of rice bran was weighed and soaked with 5 mL of the given solvent (ethanol) and acid (hydrochloric acid) concentrations. The mixtures were agitated based on the specified time in each run at 300 rpm at room temperature. The anthocyanin content of each extraction run was determined using the pH-differential method as described by Anuar et al. (2013).

Experimental Design and Analysis

RSM was employed to determine the optimum conditions for the extraction of anthocyanin from black rice bran. Two-level full factorial design was used to determine the significance of the independent variables and their interactions (Table 1). Meanwhile, BBD (Box et al. 1997) was employed in the optimization of process variables with three different factors at three varying levels -1, 0, and 1 representing low, middle, and high levels, respectively (Table 2). The experimental runs were randomly performed and made in triplicates to ensure reproducibility of the results.

Response Y was partitioned into linear, quadratic, and interactive components. The experimental data were fitted to the second order regression equation for four variables as follows:

$$y = \beta_0 + \Sigma^4_{i=1}\beta_i X_i + \Sigma^4_{i=1}\ \Sigma^4_{j=1}\beta_i X_i\ \beta_j X_j + \Sigma^4_{i=1}\beta_{ij} X_i^2$$

where y is the predicted response quantified by extraction recovery, X_i is the independent variable, β_0 is the model constant, β_i is the linear coefficient, β_{ii} is the quadratic coefficient, and β_{ij} is the cross-product coefficient.

The experimental design and statistical analysis were carried out using Design-Expert software version 9.0 (Stat-Ease Inc., Minneapolis, USA). Analysis of variance (ANOVA) was used to determine the

Table 4. Arrangement and responses of the two-level full factorial design.

Run No.*	Ethanol (%)	HCl (%)	Extraction Time (min)	Total Anthocyanin Content (mg/g)
1	20	0.00	60	32.1
2	20	0.00	60	34.7
3	20	0.00	60	40.7
4	80	0.00	60	43.5
5	80	0.00	60	42.5
6	80	0.00	60	42.6
7	20	0.10	60	34.7
8	20	0.10	60	29.2
9	20	0.10	60	32.1
10	80	0.10	60	54.6
11	80	0.10	60	57.7
12	80	0.10	60	57.8
13	20	0.00	240	47.5
14	20	0.00	240	44.5
15	20	0.00	240	51.3
16	80	0.00	240	65.1
17	80	0.00	240	55.7
18	80	0.00	240	59.7
19	20	0.10	240	39.9
20	20	0.10	240	38.8
21	20	0.10	240	41.2
22	80	0.10	240	77.6
23	80	0.10	240	81.3
24	80	0.10	240	80.1
25	50	0.05	150	83.4
26	50	0.05	150	90.2
27	50	0.05	150	83.3
28	50	0.05	150	90.1
29	50	0.05	150	81.3

*Runs are composed of 8 treatments (in triplicates) and 5 center points.

significance of the model at 5% level of significance ($p \leq 0.05$). The experimental validity of the model was determined by comparing the experimental values with the predicted values.

Phytochemical Analyses and Antioxidant Assays

Total Anthocyanin Content (TAC)

The TAC of the sample was measured using the pH-differential method of Anuar et al. (2013). Ethanolic extracts of anthocyanins were diluted separately using 0.025 M potassium chloride buffer (pH 1.0) and 0.4 M sodium acetate buffer (pH 4.5). The wavelength at maximum absorbance of the extract was determined using the wavelength scan mode of the UV-Vis spectrophotometer (DU 730, Beckman Coulter, USA). The absorbance of the diluted extracts was measured at maximum wavelength (λmax) and 700 nm using UV-Vis Spectrophotometer (DU 730, Beckman Coulter, USA). The final absorbance of the extract was calculated as

Table 5. ANOVA results of the two-level full factorial design.

Source	Sum of Squares	DF	Mean Square	F-value	p-value*
Model	5186.4	7	740.9	79.2	<0.0001
Ethanol (A)	2694.7	1	2694.7	288.1	<0.0001
HCl (B)	160.0	1	160.0	17.1	0.0005
Extraction time (C)	1402.6	1	1402.6	150.0	<0.0001
AB	731.0	1	731.0	78.2	<0.0001
AC	169.1	1	169.1	18.1	0.0004
BC	0.1	1	0.1	0.1	0.9030
ABC	28.9	1	28.9	3.1	0.0942
Curvature	5406.1	1	5406.1	578.0	<0.0001
Pure error	187.1	20	9.35		
Cor Total	10779.5	28			

follows:

$$A = [(A_{\lambda max} - A_{700nm})_{pH1} - (A_{\lambda max} - A_{700nm})_{pH4.5}]$$

The TAC was expressed as cyanidin-3-glucoside (C3G) equivalent and was calculated as follows:

$$TAC\ (mg.g^{-1}) = [(A \times MW \times DF \times V)/ (\varepsilon \times L \times m)]$$

where A is the absorbance of the solution, V is the solvent volume (mL), MW is the molecular weight of C3G (449.2 $g.mol^{-1}$), ε is the molar absorptivity (25965 $L.cm^{-1}\ mol^{-1}$), L is the cell path length (1 cm), DF is the dilution factor, and m is the mass of the sample (g).

Total Phenolic Content (TPC)

The TPC of the sample extract was determined using the method of Singleton et al. (1999) with minor modifications. About 500 µL of the ethanolic extract was mixed with 2.5 mL of freshly prepared Folin-Ciocalteu reagent (1:10 dilution). After 15 min of incubation, 2 mL of 7.5% Na_2CO_3 was added to the solution and allowed to stand for 1 h to develop its maximum color. The absorbance of the solution was measured at 765 nm against a blank (methanol) using UV-Vis Spectrophotometer (DU 730, Beckman Coulter, USA). The TPC was calculated based on the gallic acid external standard curve and expressed as mg gallic acid equivalent (GAE) per gram of sample.

Total Flavonoid Content (TFC)

The colorimetric method of Bao et al. (2005) with some modifications was employed to determine the TFC of the sample extract. About 500 µL of the diluted methanolic extracts was transferred into test tube containing 2 mL of distilled water. It was mixed with 0.15 mL of 5% $NaNO_2$ aqueous solution, vortex mixed, allowed to stand for 5 min, and added with 0.15 mL of 0.15% $AlCl_3 \bullet 6H_20$ solution. The mixtures were then allowed to stand for another 5 min and added with 1 mL of 1 M NaOH. After 15 min, the absorbance of the solution was measured at 415 nm using UV-Vis Spectrophotometer (DU 730, Beckman Coulter, USA). The TFC was calculated based on the rutin hydrate external standard curve and expressed as mg rutin hydrate equivalent (RHE) per gram of sample.

2,2'-diphenyl-1-picrylhydrazyl (DPPH) Radical Scavenging Activity

The procedure developed by Brand-Williams et al. (1995) with some modifications was used to determine the DPPH radical scavenging activity of the sample extract. Briefly, 500 µL of standard Trolox and ethanolic bran extract were mixed with 5 mL of freshly prepared 0.1 mM DPPH solution and was incubated for 1 h under dark conditions. The absorbance of the solution was measured at 517 nm using UV-Vis Spectrophotometer (DU 730, Beckman Coulter, USA). The DPPH radical scavenging activity was calculated based on the trolox standard curve and expressed as mg of trolox equivalent (TE) per gram of sample.

Ferric Reducing Antioxidant Power (FRAP)

The method of Benzie and Strain (1999) was used to evaluate the FRAP of the sample extract. Different working solutions were prepared by mixing 100 mL of 300 mM acetate buffer, 10 mL of 10 mM 2,4,6-tripyridyl-s-triazine, and 10 mL of 20 mM ferric chloride hexahydrate. About 300 µL of the diluted extract and standards were added into test tubes containing 3 mL of FRAP working solutions. The mixtures were then allowed to stand for 30 min at 37 °C in dark condition. The absorbance of the

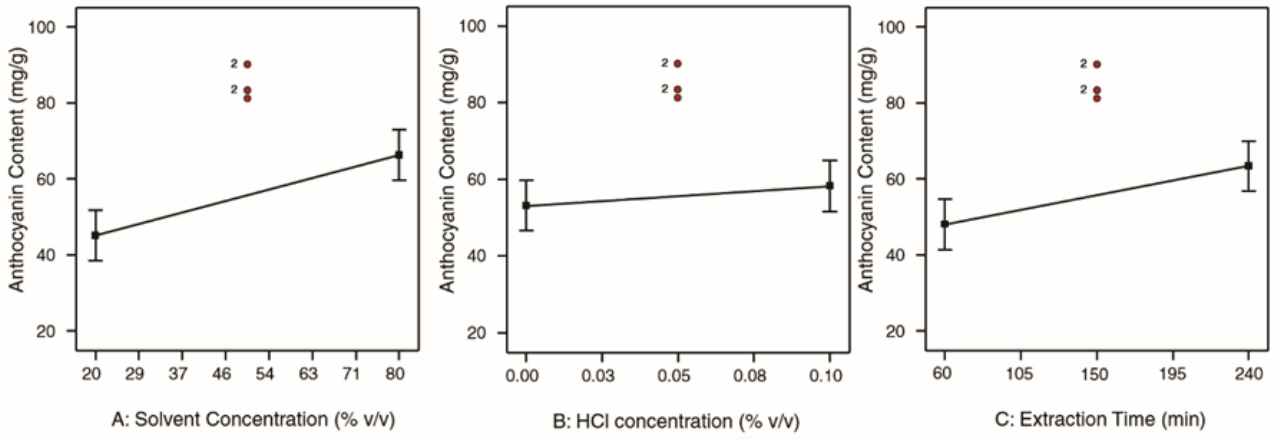

Fig. 1. Second order interaction of the significant factors in the screening process using Full-factorial design (Note: Red dots represent the center points, which indicate the presence of curvature).

solution was measured at 595 nm using UV-Vis Spectrophotometer (DU 730, Beckman Coulter, USA). The FRAP was calculated based on the trolox standard curve and expressed as mg of TE per gram of sample.

48-h Stability Testing of CAE Using Selected Biologically Relevant Buffers

For selection of biologically relevant buffers, the optimized CAE established using RSM was in turn used to determine the stability of anthocyanins. The optimized CAE was freeze-dried (CoolSafe 110, Scanvac, Germany) and stored in a desiccator prior to analysis.

The method developed by Dai et al. (2009) was employed to evaluate the stability of optimized CAE against selected biologically relevant buffers at 25 °C and 37 °C for 48 h. The optimized CAE was diluted to a final concentration of 2 $mg.mL^{-1}$ using a pH 1 buffer (214.6 mM NaCl, 8.7 mM KCl), pH 7.4 phosphate buffered saline (PBS: 30.3 mM Na_2HPO_4, 8.7 mM KH_2PO_4, 0.9% NaCl), and pH 7.4 PBS with 10% Newborn Calf Serum (NCS) (ATCC, Rockville, Maryland). The mixtures were kept at 25 °C. Another set of CAEs were treated with pH 1 buffer and pH 7.4 PBS with 10% NCS and were stored at 37 °C. Table 3 summarizes the composition of each treatment. After mixing with different buffers, aliquot of samples was collected after 0.0, 0.2, 0.5, 2.0, 6.0, 12.0, 24.0, and 48.0 h and were analyzed for its TAC. Degradation of anthocyanins was stopped by adding a drop of 36.5% HCl solution. The stability of the samples was determined by comparing the initial and final concentrations of the optimized CAE.

Statistical Analysis

ANOVA was used in the screening and optimization of process parameters to determine the level of significance of the model at 5% ($p \leq 0.05$) using Design-Expert software version 9.0 (Stat-Ease Inc., Minneapolis, USA). To determine the significant differences between the conventional and optimized CAE in terms of phytochemical contents and antioxidant capacities, t-test ($p \leq 0.5$) was employed using the Statistical Package for Social Sciences (SPSS) version 20 software. All experiments were done in triplicates.

RESULTS AND DISCUSSION

Optimization and Screening of Extraction Variables

Two-Level Full Factorial Design

Two-level full factorial design consisting of 29 runs (5 center points and 8 samples x 3 replicates) was employed to assess the significance of the independent variables on the extraction of anthocyanins from black rice bran (Table 4). It was also used to evaluate the main effects and higher order interactions of the extraction parameters on the yield of anthocyanins. Acidified ethanol was used as solvent because of its efficiency, safety, and acceptability in food applications (Huang et al. 2008; Myjavcova et al. 2010; Celli et al. 2015). Diluted hydrochloric acid was used to acidify the mixtures to prevent degradation of non-acylated compounds during extraction.

Hydrochloric acid is a common part of the human biological system; thus, it is more preferred for use in the extraction of bioactive compounds for food applications. Extraction time was optimized because it plays a crucial role in the recovery of anthocyanins (Anuar et al. 2013).

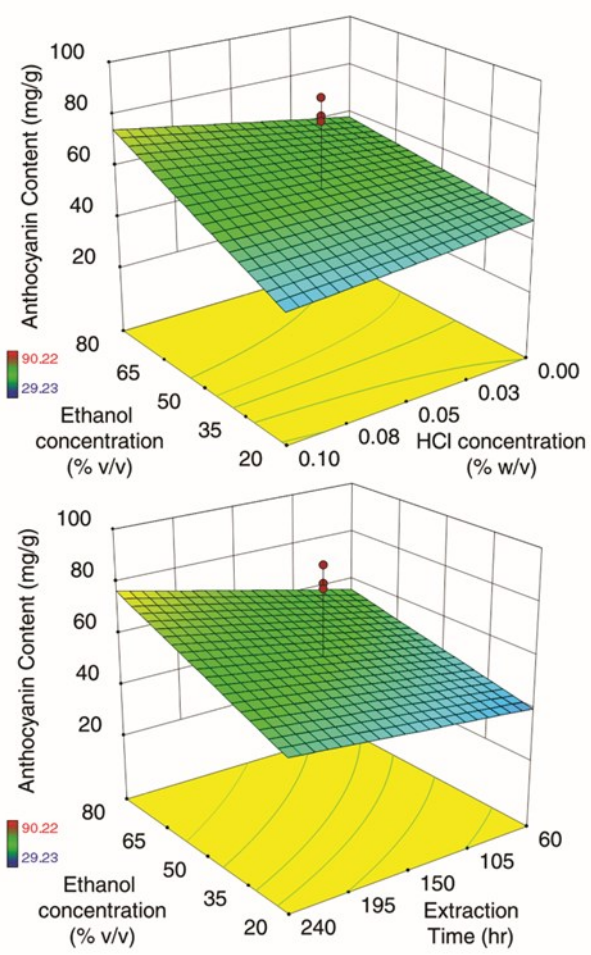

Fig. 2. 3-D representation of the significant interactions between the factors of the model (Note: Red dots represent the center points, which indicate the presence of curvature).

Solvent concentration was also included in the optimization due to its significant contribution in the extraction efficiency of water-soluble anthocyanins (Aybastier et al. 2013; Xu et al. 2016).

Table 5 shows that the developed statistical model was highly significant as indicated in the Fisher's F-Test with high F and low p-value (F = 79.22, p-value < 0.0001). It was also apparent that all independent variables were significant, implying that each factor can significantly affect the extraction yield of anthocyanins in the bran. Similar observation was reported by Celli et al. (2015) on the extraction of anthocyanins from *Haskap* berries.

Results also showed that all independent variables had a positive effect on the response (anthocyanin content). Significantly higher interactions were also observed in the model specifically between solvent (ethanol) and HCl concentrations (AB), and the solvent concentration and extraction time (AC) (Fig. 2). This result implies that increasing or decreasing the value of both variables can significantly alter the response. Similar effect can be obtained if the other variables were left constant. The developed model was found to have a curvature ($p < 0.0001$), which indicates that at least one variable was involved in the higher order interaction. The results also suggested that the optimum region would be near or within the experimental ranges of the independent variables.

Box-Behnken Design (BBD)

The independent variables used in this method were ethanol concentration, HCl concentration, and extraction time. BBD experiment was carried out in 27 runs consisting of 15 center points and 12 runs. Each independent variable was evaluated at three different levels, namely, low, middle, and high (Table 6).

Sequential model sum of squares and model summary statistics were performed to check the adequacy of the model generated from the experimental data. The p -value obtained for quadratic versus 2FI was less than 0.0001, which indicates high degree of significance. The output of the model summary statistics showed that the developed model is quadratic since the value for R-squared and adjusted R-squared were higher than the other models. On the other hand, the cubic model was disregarded because it was aliased, which means that the effect of higher interactions was combined with other factors in the model. Taking into account the exclusion of the cubic model, the BBD matrix was sufficient to interpret the outcome of the present system (Kumar et al. 2008). Table 7 shows the ANOVA for the BBD model. The quadratic regression containing the significant terms demonstrated that the model was highly significant based on the Fischer's F-test with high F value and low P value ($F = 108.71$, $p < 0.0001$). The result also implies that the linear parameters A, B, C, and their interactions such as AB and quadratic parameters A^2 and C^2 were found to be significant in the model ($p < 0.05$). Statistical analysis revealed that the lack of fit was insignificant; therefore, there was no evidence that the model did not adequately explain the variation in the responses. The model having an R-squared of 0.9703 can explain 97% of the variation of TAC leaving approximately 3% that can be attributed to noise. The R-squared for the sample size and the number of terms in the model can be corrected by the value of adjusted R-squared ($R^2adj = 0.9613$), which gives a high correlation between the observed and predicted values. The model had high predicted R-squared and adequate precision values of 0.9383 and 33.76, respectively. The coefficient of variation (CV = 2.72%) could be used as an indicator of the precision and reliability of the model. The

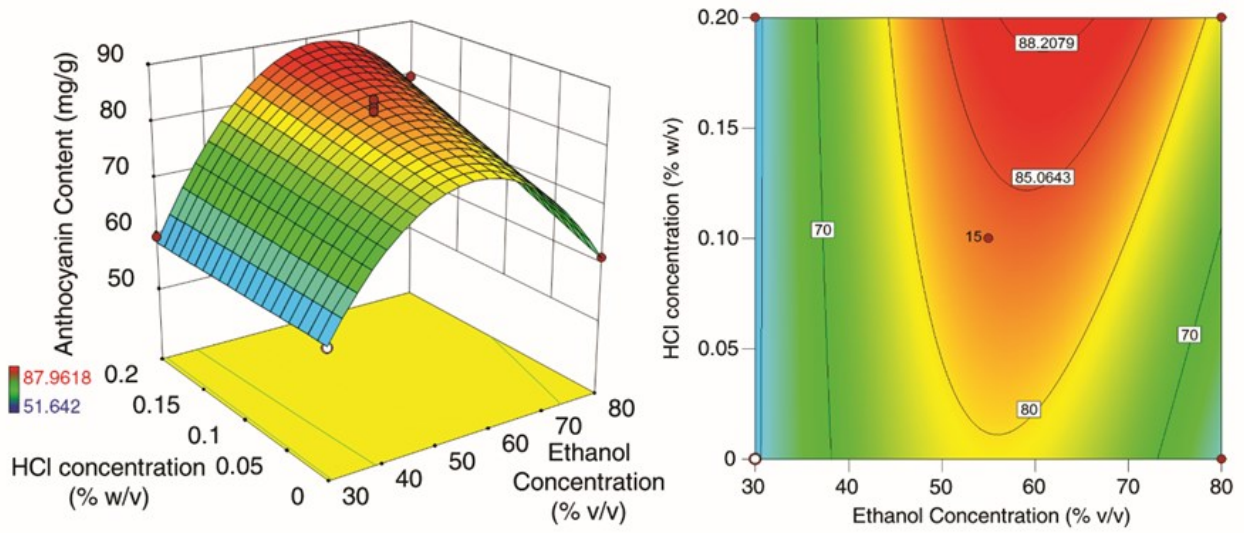

Fig. 3. 3-D and contour plots of the interaction of ethanol and HCl concentration in the extraction of anthocyanins

final equation of the quadratic model in actual variables was as follows:

$$\text{Total Anthocyanin Content (TAC)} = -29.4811 + 3.4675\ A - 55.3945\ B + 0.1284\ C + 1.7592\ AB - 0.03117\ A^2 - 2.9743 \times 10^4\ C^2$$

where A is ethanol concentration, B is HCl concentration, and C is extraction time.

Figure 3 shows the surface graph and the contour plot of the response for the anthocyanin content in relation to the solvent and HCl concentrations. The figures illustrate the interaction effects of these two variables on the anthocyanin content at constant extraction time. Results showed that increasing the acid concentration could result in a higher anthocyanin content, with solvent concentration slightly higher than its center point. The concentration of anthocyanin decreased as the concentration of solvent decreased from the center point (5% solvent concentration) and started to increase at 70% solvent concentration while keeping all variables constant. Similar trend was obtained by Huang et al. (2010) for the optimum extraction of anthocyanin from purple sweet potato roots.

The maximum predictive value of the total anthocyanin content and the predicted value of each variable were determined using the model obtained from RSM (Table 8). The optimized conditions were found to be 60% ethanol concentration, 0.2% HCl concentration, and 215 min of extraction time. The optimized method had a desirability value of 0.982. Under these conditions, the relative error between the predicted value (90.25 $mg.g^{-1}$) and the experimental value (90.20 $mg.g^{-1}$) was about 0.05%, which proves the validity of the model.

Comparison Between the Optimized and Conventional CAEs

Anthocyanin extracts obtained from the optimized (optimized CAE) and conventional (conventional CAE) methods showed significant differences for all the phytochemical analyses tested (Table 9). The CAE obtained from the optimized method was higher in anthocyanins by 4 times, DPPH radical scavenging activity by 2.3 times, phenolics and FRAP values by 1.7 times, and flavonoids by 1.5 times than the CAE obtained from the conventional method. The data suggest that the optimized conditions established using RSM is a promising technique to improve the efficiency and quality of the anthocyanin extracts from black rice bran.

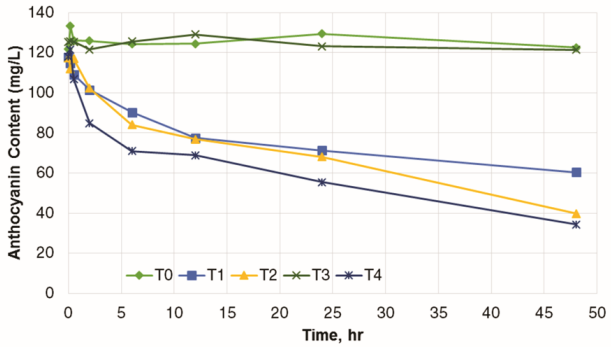

Fig. 4. Stability of the optimized crude anthocyanin content (CAE) in selected biologically relevant buffers.

Table 6. Treatments and responses of the Box-Behnken design.

Run	Ethanol (%)	HCl (%)	Extraction Time (min)	Total Anthocyanin Content (mg/g)
1	30	0	150	58.8
2	80	0	150	61.2
3	30	0.2	150	59.5
4	80	0.2	150	79.5
5	30	0.1	60	51.6
6	80	0.1	60	60.4
7	30	0.1	240	60.5
8	80	0.1	240	72.2
9	55	0	60	77.2
10	55	0.2	60	82.7
11	55	0	240	79.5
12	55	0.2	240	88.0
13	55	0.1	150	83.7
14	55	0.1	150	82.7
15	55	0.1	150	81.2
16	55	0.1	150	84.3
17	55	0.1	150	84.8
18	55	0.1	150	86.0
19	55	0.1	150	85.6
20	55	0.1	150	85.9
21	55	0.1	150	84.5
22	55	0.1	150	78.9
23	55	0.1	150	83.9
24	55	0.1	150	84.0
25	55	0.1	150	83.2
26	55	0.1	150	81.0
27	55	0.1	150	82.9

The obtained results concur with the published studies of Celli et al. (2015) for haskap berries extract and Jing et al. (2015) for alfalfa extract.

Stability of Optimized CAE Using Biologically Relevant Buffers

The anthocyanin content of T_0 and T_3 did not undergo substantial degradation (Fig. 4). This result is expected because the most stable form of anthocyanins, which is the flavylium cation, exists at pH 1. The temperature effects in T_0 and T_3 were very minimal indicating that the impact of temperature in acidic solutions was almost negligible. On the other hand, significant reduction of anthocyanins was observed in T_1, T_2, and T_4. This result implies that the anthocyanins degraded faster in alkaline conditions than in acidic medium (Castañeda-Ovando et al. 2009). Moreover, T_2 had faster rate of degradation than T_1, demonstrating that the presence of newborn calf serum permits quicker degradation of anthocyanins. The influence of temperature in T_2 and T_4 showed that the anthocyanins degrade faster at elevated temperature (37°C) than at lower temperature (25°C). The half-life of anthocyanin in optimized CAE ranged from 28.9 to 32.5 h based on the first order kinetics. Similar trends were obtained by Dai et al. (2009) for the degradation rate of anthocyanins from blackberry extracts as affected by pH, temperature, and the presence of 10% NCS.

Table 7. ANOVA for the Box-Behnken model.

Source	Sum of Squares	DF	Mean Square	F-value	p-value*
Model	2866.5	6	477.7	108.7	<0.0001
Solvent (A)	231.0	1	231.0	52.5	<0.0001
HCl (B)	136.9	1	136.9	31.1	<0.0001
Extraction time (C)	99.3	1	99.3	22.6	0.0001
AB	77.4	1	77.4	17.6	0.0004
A^2	1957.3	1	1957.3	445.4	<0.0001
C^2	29.9	1	29.9	6.8	0.0168
Residual	87.9	20	4.4		
Lack of fit	33.7	6	5.6	1.4	0.2638
Pure error	54.2	14	3.9		
Cor Total	2954.4	26			

Pred R-squared : 0.9383 Adeq. Precision : 33.761
R-squared : 0.9703 Adj R-squared : 0.9613

Table 8. Experimental confirmation of predicted value at optimum extraction parameters.

Optimum Parameters*	Predicted Response (mg/g)	DFExperimental Value (mg/g)	Relative Mean Error** (%)
A: 60%			
B: 0.2%	90.25	90.20	0.05
C: 215 min			

*A – Ethanol concentration, B – HCl concentration, C – Extraction time
**Relative Error = [(Exp. Value – Pred. Value)/Exp. Value] x 100%

Table 9. Comparison between optimized and conventional methods of extraction.

Phytochemical Property	Unit	Extraction Method	
		Conventional	Optimized
Total anthocyanin content	mg C3G•g^{-1}	22.4±0.3	90.2±0.7
Total phenolic content	mg GAE•g^{-1}	30.7±1.2	50.1±1.5
Total flavonoid content	mg RHE•g^{-1}	66.2±0.6	92.2±0.6
DPPH radical scavenging activity	mg TE•g^{-1}	33.7±0.3	75.8±0.1
Ferric reducing antioxidant power	mg TE•g^{-1}	70.9±2.3	120.2±0.8

C3G – cyanidin-3-glucoside, DPPH - 2.2-diphenyl-1-picrylhydrazyl, GAE – gallic acid equivalent, RHE – rutin hydrate equivalent, TE – trolox equivalent

CONCLUSION

All independent variables such as ethanol concentration, HCl concentration, extraction time, and some of their interactions were found to be significant in the extraction of anthocyanins. The optimum conditions for the extraction of anthocyanins were found to be 60% ethanol, 0.2% HCl, and 215 min of extraction time. The extract produced from the optimized method had higher TAC, TPC, TFC, DPPH radical scavenging activity, and FRAP value than that obtained from the conventional method. Results further showed that RSM is a useful method in predicting the optimum and efficient conditions of extracting anthocyanin from black rice bran.

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