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# Optimization of microwave assisted extraction and antioxidant activities of anthocyanins from blackberry using response surface methodology

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**Abstract**

Blackberry contains high amount of anthocyanins, whose extraction method is closely related with anthocyanin content and antioxidant activity. The extraction yield and antioxidant capacity as the comprehensive evaluation indexes, a Box–Behnken design (*BBD*) of response surface methodology (*RSM*) was employed to further optimize microwave-assisted extraction (*MAE*) conditions for blackberry anthocyanins (*BBAC*). A significant correlation was found between the double indexes extraction yield and

antioxidant capacity ($P < 0.01$). The results showed that optimized extraction conditions were microwave power 469 W, solvent concentration 52 %, liquid-solid ratio 25 g/mL, and microwave time 4 min. Under these conditions, the mean experimental value of extraction yield (2.18±0.06 mg/g), *ABTS* assay (32.18±1.54 μMTEAC/g) and *DPPH* assay (27.18±1.33 μMTEAC/g) were achieved, respectively, which corresponds well with the predicted values. Moreover, these mean experimental value increased more than 120 % compared with the ethanol leaching extraction.

**Keywords**

Blackberry anthocyanins

Comprehensive evaluation indexes

Microwave-assisted extraction

Antioxidant activity

Response surface methodology

## 1. Introduction

Blackberry is a specie of fruit belonging to the Rubus genus in the Rosaceae family, native chiefly to northern temperate regions, and it is abundant in Northeastern America and in the Pacific coast[1, 2]. Blackberry has not only been used in food industries to produce juice, ice cream, yoghurt, jams, wines and jellies[3, 4], but also used for the treatment of various diseases as an astringent, antiscorbutic, diuretic, antidiabetic, and in chronic diarrhea and enlargement of the spleen[5-8].

In recent years, further researches on chemical components and pharmacologic

effects of blackberry found that it contains many bioactive constituents, such as carbohydrates, flavonoids, amino acid, vitamin, sugar, organic acid, crude protein etc.[9, 10]. It has been shown that blackberry contains higher amount of anthocyanins and other antioxidants than other fruits[11-13].

Anthocyanins are natural water-soluble pigments responsible for orange, red, purple and blue colors of fruits, vegetables and flowers[14]. They have been regarded as potential replacements for synthetic food colorants, and they play important role in human nutrition[15]. Anthocyanins have been reported to have not only the anti-oxidative, anti-inflammatory, immunizing activity, anti-tumor, anti-diabetic effects, anti-aging properties[16-18], but also beneficial effect on coronary heart disease[19], protection against obesity and hypoglycemia[20], memory enhancement[21], and prevention of cancer[22-24]. Therefore, it is interesting to research blackberry anthocyanins (*BBAC*) owe to the antioxidant activity of *BBAC* contain much higher than other common fruits and vegetables [25].

Anthocyanins are highly unstable and very susceptible to degradation. It has been widely acknowledged that bioactivity of anthocyanins can be affected by many factors including its pH, their own chemical structure, concentration, storage temperature, light, oxygen, and the presence of enzymes, flavonoids, proteins and metal ions[26]. Several extraction technologies have been recently suggested to enable rapid extraction of anthocyanins from berries and to prevent their degradation during processing: microwave-assisted extraction[27], supercritical carbon dioxide extraction[28] and ultrasound-assisted ethanol extraction[29]. Among them, microwave assisted

extraction (*MAE*) has gained particular attention due to improved efficiency, reduced extraction time, low solvent consumption, and high level of automationx[30, 31]. *MAE* utilizes the energy of microwaves to cause molecular movement and rotation of liquids with a permanent dipole, leading to rapid heating of the solvent and the sample. *MAE* has been recently reported as more efficient method for extraction of anthocyanins from red raspberries, sour cherry Marasca and blueberry than conventional solvent extraction[27, 32, 33]. To the best of our knowledge, there are no data on *MAE* for isolation of *BBAC.* Therefore, in this study, microwave-assisted ethanol extraction method was used to prepare anthocyanin-containing extract from blackberry.

Response surface methodology (*RSM*), as an effective statistical method, is widely used for the optimization of complex process, extraction technology, and so on. Since it can depict the complete effects of variables, evaluate the interactions between multiple parameters, reduce the number of experimental trials and shorten process time. Moreover, it is more precise and effective than many approaches[34, 35]. Therefore, in this paper, a three-level, four-variable (microwave power, solvent concentration, liquid-solid ratio and microwave time) Box–Behnken design (*BBD*) of *RSM* was employed to further optimize *MAE* conditions for *BBAC*. Antioxidant properties of isolated microwave extracts were correlated with the content of anthocyanins.

## 2. Materials and methods

### *2.1. Materials and reagents*

Fresh blackberry was purchased from Polar Bear Ecological Agriculture Co., Ltd.

(China), and then was kept at -18 °C. Cyanidin-3-O-glucoside chloride was obtained from Guizhou Di Da Technology Co., Ltd. (China). 1,1-diphenyl-2-picrylhdrazyl (*DPPH*) was gained from Tokyo Kasei Kogyo Co., Ltd. (Japan). 2,2'-Azino-bis(3-ethylbenzthiazoline-6-sulfonic acid) (*ABTS*) was acquired from Beijing Lark Technology Co., Ltd. (China). All other chemicals and solvents used were of analytical grade.

### *2.2. Microwave assisted extraction of BBAC*

The *MAE* procedure that was used in the experiment was developed by Li et al. with some modification[36]. After the frozen blackberry was taken out to thaw 10-12 h in room temperature (25-28 °C), it was homogenized by using a household electrical blender (MJ-220BP01A, Guangdong Beauty Life Electrical Appliance Manufacturing Co., Ltd., China), which was selected as sample. A necessary amount of sample (20 g) was weighted and put into a conical flask. Next, the 200 mL 50 % of ethanol concentration was added into the flask. Then the mixtures were extracted in a microwave extraction reaction workstation (Model EM-202MS1, Hefei Royalstar Sanyo Electrical Co., Ltd., China; working at frequency of 2450 MHz with maximum power level of 1080W) under a designed extraction power (400 W), and extraction time (5 min).After the flask was taken out and immediately cooled to room temperature by cooling water bath, the flask was made up for the loss of weight with the same solvent. Finally, the solution in the flask was centrifuged at 4390 g for 5 min in a low-speed centrifuge (Model TDZ5-WS, Changsha Ordinary Instrument Co., Ltd., China), and the supernatant was used for the determination of the total anthocyanin

content (*TAC*).

The *TAC* was investigated according to the procedure described in Ivanovic et al. with some modification[37]. Briefly, 1 mL supernatant and 5 mL 1% (v/v) solution of hydrochloric acid in methanol were added to hydrolysis tube, and then were kept in a 100 °C water bath for 10 min. The absorbance of the solution was measure at 530 nm and using Cyanidin-3-O-glucoside chloride (0.2113 mg/mL) as a standard on Microplate Reader (Spectra Max Plus 384, Molecular Devices Co., Ltd., America). The *BBAC* yield (mg/g) was calculated using the formula as follows:

$$The\ BBAC\ Yield\left(mg/g\right)=\frac{C*V*N}{M*1000} \quad \text{Eq. (1)}$$

Where C is the concentration of *BBAC* in standard curve (μg/mL)，V represents the volume of extraction solution (mL), N represents dilution multiple and M is the sample weight (g).

### *2.3. Determination of ABTS radical scavenging activity of BBAC*

The *BBAC* were evaluated for their ability to scavenge $ABTS^{\cdot+}$ radicals. The measurements were carried out using a Microplate Reader in the kinetic mode following procedures described by Re et al. [38]. $ABTS^{\cdot+}$ was produced by the reaction of 7 mM *ABTS* solution with 2.5 mM potassium persulfate for 16 h in the dark at room temperature. The $ABTS^{\cdot+}$ solution was diluted with water to an absorbance of 0.70 (±0.02) at 734 nm and equilibrated at 30 °C. 50 μL of the *BBAC* solution samples or Trolox standards in ethanol was added to 1000 μL of diluted $ABTS^{\cdot+}$ solution, and then added into the ELISA plate. The absorbance values were taken continuously for 20 min at 734 nm at 25 °C. The standard curve was generated based on the percentage

of inhibition of the blank absorbance by Trolox at 20 min versus Trolox concentration (25-800 μmol/L). The total antioxidant capacity of samples was calculated as Trolox equivalent (*TE*) based on the percentage of inhibition of the blank absorbance by samples at 20 min. The scavenging activity (*SA*) and Trolox equivalent antioxidant capacity (*TEAC*) with the $ABTS^{\cdot+}$ radicals of *BBAC* were determined using the following equation:

$$SA_{ABTS^{\cdot+}}(\%) = \frac{A_{control} - A_{sample}}{A_{control}} * 100\% \quad \text{Eq. (2)}$$

$$TEAC_{ABTS^{\cdot+}}(\mu MTEAC / g) = \frac{TEAC_{sample}}{c_{sample}} \quad \text{Eq. (3)}$$

Where $A_{Control}$ is the absorbance of the 1 mL $ABTS^{\cdot+}$ mixed with 50 μL ethanol solution , $A_{Sample}$ represents the absorbance of the 1 mL $ABTS^{\cdot+}$ mixed with 50 μL samples, $TEAC_{sample}$ is the equivalent Trolox concentration of samples in standard curve (μM) and $C_{sample}$ represents the concentration of sample (mg/mL). The determination was carried out three times, and in triplicate.

### *2.4. Determination of DPPH scavenging activity of BBAC*

*DPPH* has been used extensively as free radical to evaluate reducing substances. The *BBAC* were evaluated for their abilities to scavenge $DPPH^{\cdot}$ radicals. The measurements were carried out using a modified protocol based on Yang et al.[39]. Briefly, 0.5 mL different concentrations of the *BBAC* solution or Trolox standards (3.125-100 μmol/L) in ethanol was added to 0.5 mL $DPPH^{\cdot}$ solution (0.2 mM in anhydrous ethanol) and then added into the ELISA plate. The absorbance readings were taken continuously for 40 min at 517 nm at 25 ℃. The standard curve was

generated based on the percentage of inhibition of the blank absorbance by Trolox at 40 min versus Trolox concentration. The total antioxidant capacity of samples was calculated as Trolox equivalent (*TE*) based on the percentage of inhibition of the blank absorbance by samples at 40 min. The scavenging activity (SA) and *TEAC* with the *DPPH·* radicals of the *BBAC* were determined using the following equation:

$$SA_{DPPH.}(\%) = \frac{A_{control} - A_{sample}}{A_{control}} * 100\% \quad \text{Eq. (4)}$$

$$TEAC_{DPPH.}(\mu MTEAC / g) = \frac{TEAC_{sample}}{c_{sample}} \quad \text{Eq. (5)}$$

Where $A_{Control}$ is the absorbance of the 0.5 mL *DPPH·* mixed with 0.5 mL ethanol solution, $A_{Sample}$ represents the absorbance of the 0.5 mL *DPPH·* mixed with 0.5 mL samples, $TEAC_{sample}$ is the equivalent Trolox concentration of samples in standard curve (μM) and $C_{sample}$ represents the concentration of sample (mg/mL). The determination was carried out three times, and in triplicate.

### *2.5. Experimental design*

After determining the yield and the bioactivity of *BBAC*, the single-factor test was used for obtaining the preliminary range of extraction variables, and a three-level-four-factor *BBD* of *RSM* was used to determine the optimal combination of both extraction and antioxidant activities variables. Based on the results of single factor experiments, four independent variables (Table 1) were microwave power ($X_1$, W), solvent concentration ($X_2$, %), liquid-solid ratio ($X_3$, g/mL) and microwave time ($X_4$, min), while the response variables were the extraction yield and antioxidant activities of *BBAC*. Each variable was designated as three levels, coded +1, 0 and −1

for high, intermediate and low value, respectively. The response could be related to the selected variables by the following second-order polynomial model:

$$Y = \sum A_0 + \sum_{i=1}^{k} A_{ij} X_i + \sum_{i=1}^{k} A_{ii} X_i^{\,2} + \sum_{i=1}^{k-1} \sum_{j=i+1}^{k} A_{ij} X_i X_j \quad \text{Eq. (6)}$$

Where $Y$ is the response variable, $A_0$, $A_i$, $A_{ii}$ and $A_{ij}$ are the regression coefficients for intercept, linear, quadratic and interaction terms, respectively. $X_i$ and $X_j$ are the encoded independent variables ($i \neq j$) affecting the response of $Y$.

### *2.6. Statistical analysis*

SPSS 18.0 software (SPSS Inc., Chicago, IL, USA) was used to analyze the bioactivity experimental and single-factor data. The experimental design and the regression analysis of experimental data exploited Design-Expert 8.0.5 (Trial version, Stat-Ease Inc., Minneanopolis, MN, USA).

The regression coefficients were performed in the form of analysis of variance (*ANOVA*). Student's t-test was employed for evaluating the statistical significance of the regression coefficient, and Fischer's *F*-test at a probability (*P*) of 0.001, 0.01 or 0.05 was used to determine the second-order model equation and its fitness was expressed by the regression coefficients $R^2$. The adequacy and significance of the model were tested by *F*-value, determination coefficient ($R^2$) and lack of fit secured from *ANOVA*.

## 3. Results and discussion

### *3.1. Effect of microwave power on yield and antioxidant capacity of BBAC*

It is very important for anthocyanins extraction to keep microwave at an optimal working power[40]. In the present study, the effect of various microwave power points

196 within 100-700 W used on extraction and antioxidant capacity of *BBAC* was
197 investigated, while keeping the solvent concentration, liquid-solid radio and
198 microwave time at 50 %, 10 g/mL and 5 min, respectively. As shown in Fig. 1a,
199 *BBAC* content in extract, *ABTS* assay and *DPPH* assay increased with the increase of
200 microwave power from 100 to 400 W, and peaked at around 400 W. Further
201 enhancing of power, however, resulted in decreasing *BBAC* content in extract. Thus,
202 the power chosen for *BBAC* extraction was 400 W. This was identical with the result
203 reported by Zou et al. [41].

204 ***3.2. Effect of solvent concentration on yield and antioxidant capacity of BBAC***

205 Solvent concentration played prominent roles in getting high extraction
206 efficiency of anthocyanins during *MAE*[42]. As shown in Fig. 1b, the effect of solvent
207 concentration on extraction yield was investigated in this study. Increasing in the
208 tested ratio of ethanol to raw material (from 0 % to 100 %) could improve *BBAC*
209 extraction, and the increase leveled off at ratio of 40 %. However, as to the
210 antioxidant capacity of *BBAC*, the increase leveled off at ratio of 60 %. As the results
211 of statistical analysis showed that significant differences were for the solvent
212 concentration tested ($P < 0.05$). Considering the solvent cost problem，therefore an
213 optimal ratio of 40 % was favorable for anthocyanins production. Zheng et al.
214 reported the similar results[33].

215 ***3.3. Effect of liquid-solid ratio on yield and antioxidant capacity of BBAC***

216 Liquid-solid ratio played outstanding roles in getting high extraction efficiency
217 of anthocyanins during *MAE*[33]. To investigate the effect of liquid-solid ratio on

extraction yield and antioxidant capacity of *BBAC*, a liquid-solid ratio range from 5 to 25 g/mL was tested while the microwave power, solvent concentration and microwave time were kept at 400 W, 40 % and 5 min, respectively. An increase of *BBAC* content and *ABTS*assay was observed with the increase of liquid-solid ratio from 5 to 25 g/mL, but further increase of liquid-solid ratio resulted in increasing *BBAC* content not significantly, and *DPPH* assay reach maximum at 20 g/mL (Fig. 1c). Therefore, liquid-solid ratio 20 g/mL was chosen as the optimal one in the present experiment. Similar result was obtained in Yang's research[43].

### *3.4. Effect of microwave time on yield and antioxidant capacity of BBAC*

Microwave time played significant roles in getting high extraction efficiency of anthocyanins during *MAE*[40]. It will have an effect on the final yield and antioxidant capacity of *BBAC* in the recovery, the energy cost and the efficiency of extraction. In this study, an increase of *BBAC* extraction was observed with the elevation of microwave time from 1 to 9 min, reaching the highest at 3 min, but further increase of microwave time resulted in decreasing *BBAC* content, *ABTS* assay and *DPPH* assay (Fig. 1d). Thus, the microwave time 3 min was chosen as the optimal one based on the study. This conclusion agreed with the opinion of Zou et al.[41].

### *3.5. Optimization of extraction conditions of BBAC*

#### *3.5.1. Correlation analysis for -extraction yield and antioxidant capacity*

The aim of the correlation analysis was to seek possible statistical correlation between anthocyanins and antioxidant activity and to develop an extraction method for blackberry to produce anthocyanins extracts with high antioxidant activity. To

240 study the relationships between anthocyanins and antioxidant activities of blackberry,
241 the data of extraction yield, *ABTS* assay and *DPPH* assay of BBAC in Table 2 were
242 analyzed using bivariate correlation analysis. It can be seen from Table 3 that the
243 correlation between the double indexes extraction yield and antioxidant capacity were
244 very significant with *P* values ($P < 0.01$). Therefore, the selection of one index can
245 reasonably evaluate the result of optimize the approach of *MAE* conditions for *BBAC*.
246 So the extraction yield index was chose to evaluate the result of RSM.

247 ***3.5.2. Statistical analysis and the model fitting***

248 The *BBD* of *RSM* in the experimental design involves four independent variables,
249 three levels and five replicates at the center point (Table 1), which was carried out to
250 measure the inherent variability and process stability. The experimental conditions
251 and the fit statistics of extraction yield of 29 runs with *BBD* design were shown in
252 Table 2, and all tests were performed in triplicate. As shown in Table 2, the extraction
253 yield of *BBAC* values (mg/g) varied from 1.85 to 2.18 mg/g， ABTS assay and
254 *DPPH* assay of *BBAC* (μMTEAC/g) varied from 20.01 to 30.39 and 19.21 to 28.13
255 μMTEAC/g , respectively.

256 The results of extraction yield affected by microwave power, solvent
257 concentration, liquid-solid ratio and microwave time were fitted to a second-order
258 polynomial equation, and the values of regression coefficients were calculated.

259 The effects of four variables were highly significant on extraction yield of *BBAC*
260 (Table 4). The predicted model of the extraction yield value was obtained by the
261 following second-order polynomial equations:

$Y_1=2.14+0.048X_1+0.034X_2+0.057X_3-0.021X_1X_2+0.042X_1X_3+0.016X_2X_3+0.078X_3X_4-0.066X_1^2-0.011X_2^2-0.068X_3^2-0.082X_4^2$ Eq. (7)

The predicted values of extraction yield based on the above quadratic predictive model were shown in Table 2.

The statistical significance of regression equation was evaluated by *F*-test, *T*-test and *AVNOA* for response surface quadratic polynomial model were presented in Table 4. The results of high model *F*-value (22.014) and low *P*-value ($P< 0.0001$) turned out that the models were highly significant. The determination coefficient ($R^2$) for model (0.958) was close to 1.0, which represented the satisfactory correlation between actual and predicted values. The value of adjusted determination coefficient $R^2$ (Adj. $R^2$) value was 0.916, which means most variation (> 91.6 %) of the extraction yield could be predicted by the model, and less than 8.4 % variations could not be explained by the model.

The lack-of-fit used to measure the failure of the model to represent the data in the experimental domain at points which were not included in the regression. The *F*-value of 0.247 and P-value of 0.967 for extraction yield implied that the lack of fit was not significant relative to the pure error due to noise. Adequate precision compared the range of the predicted values at the design points to the average prediction error. The ratio greater than 4 indicated adequate model discrimination. In this research, the values were well above 4.

The *P*-values were used as a tool to check the significance of each coefficient, which in turn may indicate the pattern of the interactions between the variables. The

smaller the value of $P$ was, the more significant the corresponding coefficient was. It can be seen from Table 4 that linear coefficients ($X_1$, $X_2$, $X_3$), quadratic term coefficient ($X_1^2$, $X_3^2$, $X_4^2$) and cross product coefficients ($X_1X_3$, $X_3X_4$) were very significant with $P$ values ($P < 0.01$). The other term coefficients were significant ($P > 0.05$).

### *3.5.3. Analysis of response surfaces*

The 3D response surface and 2D contour plots were the graphical representations of regression equation. They provided a method to visualize the relationship between responses and experimental levels of each variable and the type of interactions between two test variables. The shapes of the contour plots, circular or elliptical, indicate whether the mutual interactions between the variables are significant or not. Circular contour plot indicates that the interactions between the corresponding variables are negligible, while elliptical contour plot indicates that the interactions between the corresponding variables are significant. In this study, the results of extraction yield of *BBAC* affected by microwave power, solvent concentration, liquid-solid ratio and microwave time is presented in Figs. 2, 3.

Fig. 2a and Fig. 3a, which give the extraction yield of *BBAC* as a function of microwave power and solvent concentration at fixed liquid-solid ratio (20 g/mL) and microwave time (3 min), indicated that the extraction yield increased rapidly with increase in microwave power from 250 to 470 W and decrease slowly with increase of microwave power from 500 to 550 W. The extraction yield of *BBAC* increased with the increase of solvent concentration from 20 % to 60%.It can be seen that the

maximum extraction yield of *BBAC* can be achieved when microwave power and solvent concentration are around 470 W and 60 %, respectively. A similar result was also reported previously by Zou et al. [41].

The extraction yield of BBAC affected by different microwave power and liquid-solid ratio was shown in Figs. 2b and 3b, when the solvent concentration and microwave time were fixed at 40 % and 3 min respectively, indicated that the extraction yield increased rapidly with increase in microwave power from 250 to 410 W, and increased with the increase of liquid-solid ratio from 15 to 21. However, the extraction yield decreased rapidly with the microwave power increasing from 410 to 550 W and liquid-solid ratio from 21 to 25 g/mL. These results were in agreement with a study done by Zou et al.[41].

The Figs. 2c and 3c showed the 3D response surface plot and the contour plot at varying microwave power and microwave time at fixed solvent concentration (40 %) and liquid-solid ratio (20 g/mL). It indicated that the maximum extraction yield of *BBAC* can be achieved when microwave power and microwave time at the threshold level of around 430 W and 2.8 min, respectively. The accordant result was also reported previously by Liazidet al. [41].

The Figs. 2d and 3d illustrated the 3D response surface plot and the contour plot at varying solvent concentration and liquid-solid ratio at fixed microwave power (400 W) and microwave time (3 min), indicated that the extraction yield of *BBAC* increased with the increase of solvent concentration from 20 % to 48%, extraction yield of *BBAC* reached the plateau region where the yield was maximized and did not

328 further increase the yield. The extraction yield increased rapidly with increase in
329 liquid-solid ratio from 15 to 21 g/mL and decrease slowly with increase of microwave
330 power from 22 to 25 g/mL. It can be seen that the maximum extraction yield of *BBAC*
331 can be achieved when solvent concentration and liquid-solid ratio are around 48% and
332 21 g/mL, respectively. This was accordant with the result reported by Zheng et al. [33].

333 In Figs. 2e and 3e, when the 3D response surface plot and the contour plot were
334 developed for the extraction yield of *BBAC* with varying solvent concentration and
335 microwave time at fixed microwave power (400 W) and liquid-solid ratio (20 g/mL).
336 The maximum extraction yield of *BBAC* achieved when solvent concentration and
337 microwave time at the threshold level of around 60 % and 2.7 min, respectively. Zou
338 et al. reported the unanimous results [41].

339 The 3D response surface plot and the contour plot based on the independent
340 variable liquid-solid ratio and microwave time were shown in Figs. 2f and 3f, while
341 the other two independent variables, microwave power and solvent concentration
342 were kept at 400 W and 40 %, respectively. An increase in the extraction yield of
343 *BBAC* could be significantly achieved with the increasing of liquid-solid ratio. It was
344 observed that the extraction yield of *BBAC* increased with the microwave time from 1
345 to 3.8 min, and reached the maximum value at an extraction time around 4.2 min, but
346 beyond this time, extraction yield of *BBAC* decreased. This conclusion conformed to
347 the opinion of Elez Garofulić et al. [32].

348 ***3.6. Verification of predictive model***

349 Response surface optimization is more advantageous than the traditional single

parameter optimization in that it saves time, space and raw material. In order to validate the adequacy of the model equations, verification experiment was carried out under the optimal conditions: microwave power 469 W, solvent concentration 52 %, liquid-solid ratio 25g/mL and microwave time 4 min. Good agreement exist between the values predicted using model equations and the experimental values at the points of interest. To ensure the predicted result was not biased toward the practical value, experimental rechecking was performed using this deduced optimal condition. This set of conditions was determined to be optimal by the *RSM* optimization approach and was also used to validate experimentally and predict the values of the response using the model equation. The mean value of extraction yield (2.18 ± 0.06 mg/g), *ABTS* assay (32.18±1.54 μMTEAC/g) and *DPPH* assay (27.18±1.33 μMTEAC/g) (n = 5), obtained from real experiments, demonstrated the validation of *RSM* model. The validation result revealed that there was no significant difference between experimental and predicted values, suggesting that the response model was adequate for reflecting the expected optimization (Table 5). This result of analysis indicated that the experimental values were good agreement with the predicted ones, and also suggested that the model of Eq. (7) is satisfactory and accurate.

Furthermore, ethanol leaching extraction were compared with the MAE method, as seen in table 5, a mean value of extraction yield (1.81 ± 0.04 mg/g), *ABTS* assay (20.84±1.49 μMTEAC/g) and *DPPH* assay (17.01±0.19 μMTEAC/g) (n = 5) obtained from the ethanol leaching extraction. Those mean value of microwave treated condition increased 120 % compared with the ethanol leaching treated ones.

Moreover, the mean value of extraction yield is higher than the result reported by Oancea et al.[44]. And the mean value of antioxidant capacities is higher than the result reported by Reátegui et al.[45]. Therefore, this finding corroborates previous reports that with respect to anthocyanin content, microwave has its superiority in improving efficiency, shortening extraction time, reducing solvent consumption. The anthocyanin content and antioxidant capacity reported herein are higher than those previously reported. This may have been partly due to increased extraction efficiency.

## 4. Conclusions

In the present study, microwave assisted extraction method and ethanol leaching extraction method were screened for the extraction treatment of blackberry, and the extracts exhibited different yields, levels of scavenging effects on *DPPH·* free radicals and $ABTS^{\cdot+}$ free radicals. Microwave assisted extraction method was found to be the most effective one for improving yield and antioxidant capacity of *BBAC* among the tested methods. In the case of ethanol as solvent, optimal extraction conditions for microwave assisted extract of *BBAC* are obtained as follows conditions: microwave power 469 W, solvent concentration 52 %, liquid-solid radio 25 g/mL, and microwave time 4 min. Under this condition, the mean experimental value of extraction yield (2.18±0.06 mg/g), *ABTS* assay (32.18±1.54 μMTEAC/g) and *DPPH* assay (27.18±1.33 μMTEAC/g) were achieved, respectively, which corresponds well with the predicted values and increased more than 120 % compared with the ethanol leaching extraction.

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**References**

1. J. Dai, A. Gupte, L. Gates and R. J. Mumper, , 2009, **47**, 837-847.

2. F. Aqil, A. Gupta, R. Munagala, J. Jeyabalan, H. Kausar, R. J. Sharma, I. P. Singh and R. C. Gupta, *Nutr Cancer*, 2012, **64**, 428-438.

3. A. Rommel, R. E. Wrolstad and D. A. Heatherbell, , 1992, **57**, 385-391.

4. J. L. P. Reátegui, A. P. da Fonseca Machado, G. F. Barbero, C. A. Rezende and J. Martínez, , 2014, **94**, 223-233.

5. M. S. Baliga, S. Fernandes, K. R. Thilakchand, P. D'souza and S. Rao, , 2013, **19**, 191-197.

6. A. Chaturvedi, G. Bhawani, P. K. Agarwal, S. Goel, A. Singh and R. K. Goel, , 2009.

7. H. Sagrawat, A. S. Mann and M. D. Kharya, , 2006, **2**, 96.

8. J. M. Veigas, M. S. Narayan, P. M. Laxman and B. Neelwarne, , 2007, **105**, 619-627.

9. T. J. Hager, L. R. Howard, R. Liyanage, J. O. Lay and R. L. Prior, *J Agric Food Chem*, 2008, **56**, 661-669.

10. E. Sariburun, S. Sahin, C. Demir, C. Turkben and V. Uylaser, *J Food Sci*, 2010,

**75**, C328-C335.

11. B. L. Halvorsen, M. H. Carlsen, K. M. Phillips, S. K. Bohn, K. Holte, D. R. Jr Jacobs and R. Blomhoff, *Am J Clin Nutr*, 2006, **84**, 95-135.

12. R. A. Moyer, K. E. Hummer, C. E. Finn, B. Frei and R. E. Wrolstad, *J Agric Food Chem*, 2002, **50**, 519-525.

13. G. E. Pantelidis, M. Vasilakakis, G. A. Manganaris and G. Diamantidis, , 2007, **102**, 777-783.

14. H. Li, Z. Deng, H. Zhu, C. Hu, R. Liu, J. C. Young and R. Tsao, , 2012, **46**, 250-259.

15. C. W. Haminiuk, G. M. Maciel, M. S. Plata Oviedo and R. M. Peralta, , 2012, **47**, 2023-2044.

16. C. A. Rice-Evans, N. J. Miller, P. G. Bolwell, P. M. Bramley and J. B. Pridham, *Free Radic Res*, 1995, **22**, 375-383.

17. N. P. Seeram and M. G. Nair, *J Agric Food Chem*, 2002, **50**, 5308-5312.

18. J. Valls, S. Millán, M. P. Martí, E. Borràs and L. Arola, , 2009, **1216**, 7143-7172.

19. G. J. Mazza, *Ann Ist Super Sanita*, 2007, **43**, 369-374.

20. B. Jayaprakasam, L. K. Olson, R. E. Schutzki, M. H. Tai and M. G. Nair, *J Agric Food Chem*, 2006, **54**, 243-248.

21. C. Andres-Lacueva, B. Shukitt-Hale, R. L. Galli, O. Jauregui, R. M. Lamuela-Raventos and J. A. Joseph, *Nutr Neurosci*, 2005, **8**, 111-120.

22. H. S. Aiyer, C. Srinivasan and R. C. Gupta, *Nutr Cancer*, 2008, **60**, 227-234.

23. G. D. Stoner, L. S. Wang, N. Zikri, T. Chen, S. S. Hecht, C. Huang, C. Sardo and

437 J. F. Lechner, *Semin Cancer Biol*, 2007, **17**, 403-410.

438 24. L. S. Wang and G. D. Stoner, *Cancer Lett*, 2008, **269**, 281-290.

439 25. F. Aqil, R. Munagala, J. Jeyabalan, T. Joshi, R. C. Gupta and I. P. Singh, , 2014,

440 101.

441 26. M. Rein, , 2005.

442 27. H. Teng, W. Y. Lee and Y. H. Choi, , 2013, **36**, 3107-3114.

443 28. T. Vatai, M. Škerget and Ž. Knez, , 2009, **90**, 246-254.

444 29. Ö. Aybastıer, E. Işık, S. Şahin and C. Demir, , 2013, **44**, 558-565.

445 30. P. L. Buldini, L. Ricci and J. L. Sharma, , 2002, **975**, 47-70.

446 31. C. Sparr Eskilsson and E. Björklund, , 2000, **902**, 227-250.

447 32. I. Elez Garofulić, V. Dragović-Uzelac, A. Režek Jambrak and M. Jukić, , 2013,

448 **117**, 437-442.

449 33. X. Zheng, X. Xu, C. Liu, Y. Sun, Z. Lin and H. Liu, , 2013, **104**, 17-25.

450 34. M. Hosseinpour, M. Vossoughi and I. Alemzadeh, *J Environ Health Sci Eng*,

451 2014, **12**, 33.

452 35. S. P. Kumar, *Indian J Exp Biol*, 2013, **51**, 979-983.

453 36. Y. Li, L. Han, R. Ma, X. Xu, C. Zhao, Z. Wang, F. Chen and X. Hu, , 2012, **109**,

454 274-280.

455 37. J. Ivanovic, V. Tadic, S. Dimitrijevic, M. Stamenic, S. Petrovic and I. Zizovic, ,

456 2014, **53**, 274-281.

457 38. R. Re, N. Pellegrini, A. Proteggente, A. Pannala, M. Yang and C. Rice-Evans, ,

458 1999, **26**, 1231-1237.

39. B. Yang, M. Zhao, J. Shi, N. Yang and Y. Jiang, , 2008, **106**, 685-690.

40. Y. Sun, X. Liao, Z. Wang, X. Hu and F. Chen, , 2007, **225**, 511-523.

41. T. Zou, D. Wang, H. Guo, Y. Zhu, X. Luo, F. Liu and W. Ling, , 2012, **77**, C46-C50.

42. A. Liazid, R. F. Guerrero, E. Cantos, M. Palma and C. G. Barroso, , 2011, **124**, 1238-1243.

43. Z. Yang and W. Zhai, , 2010, **11**, 470-476.

44. S. Oancea, C. Grosu, O. Ketney and M. Stoia, *Acta Chim Slov*, 2013, **60**, 383-389.

45. J. L. P. Reátegui, A. P. da Fonseca Machado, G. F. Barbero, C. A. Rezende and J. Martínez, , 2014, **94**, 223-233.

481 **Table 1.**The coded values and corresponding actual values of the optimization parameters

| Solvent | code | Microwave power（W) | Solvent concentration (%) | Liquid-solid rata (g/mL) | Microwave time (min) |
|---|---|---|---|---|---|
| | -1 | 250 | 20 | 15 | 1 |
| ethanol | 0 | 400 | 40 | 20 | 3 |
| | 1 | 550 | 60 | 25 | 5 |

482 **Table 2.**The coded experimental and predicted for RSM design using ethanol as solvent

| Run | X1 | X2 | X3 | X4 | Extraction yield (mg/g) | | ABTS (μMTEAC/g) | | DPPH (μMTEAC/g) | |
|---|---|---|---|---|---|---|---|---|---|---|
| | | | | | Experimental | Predicted | Experimental | Predicted | Experimental | Predicted |
| 1 | 1 | 0 | 1 | 0 | 2.16 | 1.96 | 30.39 | 26.18 | 26.99 | 21.26 |
| 2 | 0 | 0 | 0 | 0 | 2.14 | 2.10 | 28.48 | 23.04 | 24.48 | 22.82 |
| 3 | 1 | -1 | 0 | 0 | 2.09 | 2.08 | 23.06 | 26.82 | 23.55 | 24.69 |
| 4 | 0 | 1 | 1 | 0 | 2.15 | 2.13 | 30.07 | 28.74 | 26.09 | 24.74 |
| 5 | 0 | 0 | 0 | 0 | 2.11 | 2.02 | 27.44 | 22.25 | 25.15 | 20.30 |
| 6 | 0 | 0 | 0 | 0 | 2.10 | 1.98 | 29.85 | 26.54 | 28.00 | 24.83 |
| 7 | 0 | 0 | -1 | -1 | 2.01 | 1.85 | 22.03 | 20.18 | 20.23 | 20.87 |
| 8 | 0 | -1 | -1 | 0 | 2.01 | 2.12 | 19.67 | 31.44 | 19.21 | 27.43 |
| 9 | 0 | -1 | 0 | -1 | 2.04 | 1.95 | 23.57 | 27.34 | 21.35 | 21.39 |
| 10 | 1 | 0 | -1 | 0 | 1.96 | 2.06 | 18.58 | 27.60 | 19.41 | 24.95 |
| 11 | 0 | 1 | 0 | 1 | 2.08 | 1.95 | 28.22 | 29.63 | 25.22 | 25.73 |
| 12 | 0 | 0 | 1 | -1 | 1.98 | 2.03 | 26.30 | 28.14 | 26.02 | 23.78 |
| 13 | 0 | 0 | 0 | 0 | 2.18 | 1.99 | 29.22 | 18.90 | 23.79 | 18.63 |
| 14 | 0 | -1 | 0 | 1 | 1.98 | 2.03 | 24.12 | 19.56 | 21.93 | 21.36 |
| 15 | -1 | 1 | 0 | 0 | 2.10 | 2.07 | 26.38 | 24.17 | 24.57 | 24.23 |
| 16 | 0 | 1 | 0 | -1 | 2.11 | 2.17 | 25.29 | 29.85 | 23.34 | 26.86 |
| 17 | 0 | -1 | 1 | 0 | 2.07 | 1.95 | 24.34 | 23.82 | 23.26 | 21.26 |
| 18 | 1 | 0 | 0 | -1 | 2.05 | 1.96 | 28.58 | 19.82 | 24.64 | 20.32 |
| 19 | 0 | 0 | 1 | 1 | 2.13 | 1.98 | 31.23 | 28.22 | 28.13 | 25.06 |
| 20 | 1 | 1 | 0 | 0 | 2.12 | 2.15 | 28.96 | 30.99 | 25.31 | 27.62 |
| 21 | -1 | 0 | 0 | -1 | 1.93 | 2.03 | 27.71 | 23.88 | 20.66 | 21.37 |
| 22 | 0 | 0 | -1 | 1 | 1.85 | 2.09 | 20.01 | 25.87 | 20.30 | 23.39 |
| 23 | 1 | 0 | 0 | 1 | 2.04 | 2.00 | 28.77 | 24.12 | 24.32 | 22.30 |
| 24 | -1 | 0 | 0 | 1 | 1.95 | 2.09 | 29.65 | 28.47 | 25.84 | 25.63 |
| 25 | 0 | 1 | -1 | 0 | 2.02 | 2.14 | 20.39 | 28.45 | 22.13 | 25.47 |
| 26 | 0 | 0 | 0 | 0 | 2.18 | 2.14 | 27.25 | 28.45 | 25.91 | 25.47 |
| 27 | -1 | -1 | 0 | 0 | 1.97 | 2.14 | 25.53 | 28.45 | 21.31 | 25.47 |
| 28 | -1 | 0 | 1 | 0 | 1.97 | 2.14 | 28.89 | 28.45 | 25.55 | 25.47 |
| 29 | -1 | 0 | -1 | 0 | 1.94 | 2.14 | 23.86 | 28.45 | 21.46 | 25.47 |

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**Table 3.**The correlation analysis of the extraction yield and antioxidant capacity double indexes

| Index | Extraction yield | | DPPH | |
|---|---|---|---|---|
| | r[a] | P | r[a] | P |
| DPPH | 0.611** | 0.001 | | |
| ABTS | 0.534** | 0.003 | 0.847** | 0.001 |

[a]**Significant at P < 0.01

**Table 4.**ANOVA for the effects of microwave power ($X_1$), solvent concentration ($X_2$), liquid-solid ratio ($X_3$) and microwave time ($X_4$) on extraction yield of BBAC with ethanol as solvent using predicted polynomial models

| Source | Sum of squares | Df | Mean square | F-value | P-Value | Significant[a] |
|---|---|---|---|---|---|---|
| Model | 0.195 | 14 | 0.014 | 22.014 | < 0.0001 | *** |
| $X_1$ | 0.027 | 1 | 0.027 | 42.739 | < 0.0001 | *** |
| $X_2$ | 0.015 | 1 | 0.015 | 23.049 | 0.0003 | *** |
| $X_3$ | 0.039 | 1 | 0.039 | 61.085 | < 0.0001 | *** |
| $X_4$ | 0.001 | 1 | 0.001 | 1.165 | 0.2987 | |
| $X_1X_2$ | 0.002 | 1 | 0.002 | 3.743 | 0.0735 | |
| $X_1X_3$ | 0.007 | 1 | 0.007 | 11.243 | 0.0047 | ** |
| $X_1X_4$ | 0.000 | 1 | 0.000 | 0.537 | 0.4759 | |
| $X_2X_3$ | 0.001 | 1 | 0.001 | 1.717 | 0.2111 | |
| $X_2X_4$ | 0.000 | 1 | 0.000 | 0.376 | 0.5494 | |
| $X_3X_4$ | 0.025 | 1 | 0.025 | 38.996 | < 0.0001 | *** |
| $X_1^2$ | 0.028 | 1 | 0.028 | 43.917 | < 0.0001 | *** |
| $X_2^2$ | 0.001 | 1 | 0.001 | 0.976 | 0.3400 | |
| $X_3^2$ | 0.031 | 1 | 0.031 | 48.870 | < 0.0001 | *** |
| $X_4^2$ | 0.044 | 1 | 0.044 | 70.143 | < 0.0001 | *** |
| Residual | 0.009 | 14 | 0.001 | | | |
| Lack of Fit | 0.003 | 10 | 0.000 | 0.247 | 0.9670 | not significant |
| Pure Error | 0.005 | 4 | 0.001 | | | |
| Cor Total | 0.203 | 28 | | | | |
| $R^2$ | 0.958 | | | | | |
| Adj.$R^2$ | 0.916 | | | | | |
| Pred.$R^2$ | 0.872 | | | | | |
| Adequate Precision | 18.143 | | | | | |

493 **Table 5.**Result of model validation experiments

| NO. | Optimum conditions | | | | Extraction yield (mg/g) | | ABTS (μMTEAC/g) | | DPPH (μMTEAC/g) | |
|---|---|---|---|---|---|---|---|---|---|---|
| | Microwave power | Solvent concentration | Liquid-solid radio | Microwave time | Experimental | Predicted | Experimental | Predicted | Experimental | Predicted |
| | (W) | (%) | (g/ml) | (min) | | | | | | |
| 1 | 469 | 52 | 25 | 4 | 2.22 | 2.19 | 34.13 | 32.94 | 26.23 | 27.78 |
| 2 | 469 | 52 | 25 | 4 | 2.1 | 2.19 | 30.08 | 32.94 | 29.08 | 27.78 |
| 3 | 469 | 52 | 25 | 4 | 2.14 | 2.19 | 33.13 | 32.94 | 25.93 | 27.78 |
| 4 | 469 | 52 | 25 | 4 | 2.17 | 2.19 | 31.54 | 32.94 | 26.65 | 27.78 |
| 5 | 469 | 52 | 25 | 4 | 2.25 | 2.19 | 32.04 | 32.94 | 28.01 | 27.78 |
| Average | | | | | 2.18 | | 32.18 | | 27.18 | |
| Ethanol Leaching Extraction | | | | | | | | | | |
| 6 | 0 | 52 | 25 | 60 | 1.82 | | 21.34 | | 17.14 | |
| 7 | 0 | 52 | 25 | 60 | 1.77 | | 22.02 | | 16.79 | |
| 8 | 0 | 52 | 25 | 60 | 1.84 | | 19.16 | | 17.09 | |
| Average | | | | | 1.81 | | 20.84 | | 17.01 | |

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499 **Figure Captions**

500 **Figure1.**The effect of different microwave power *(a)*, solvent concentration *(b)*,
501 liquid-solid ratio *(c)* and microwave time *(d)* on the extraction yield and antioxidant
502 capacity of *BBAC*.

503 **Figure2.**Response surface (*3D*) showing the effect of microwave power ($X_1$), solvent
504 concentration ($X_2$), liquid-solid ratio ($X_3$) and microwave time ($X_4$) on extraction yield
505 of *BBAC*.

506 **Figure 3.**Contour plots showing the effect of microwave power ($X_1$), solvent
507 concentration ($X_2$), liquid-solid ratio ($X_3$) and microwave power ($X_4$) on extraction
508 yield of *BBAC*.

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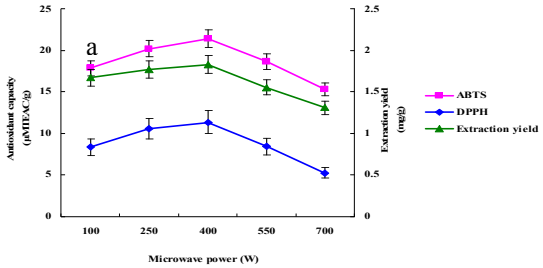


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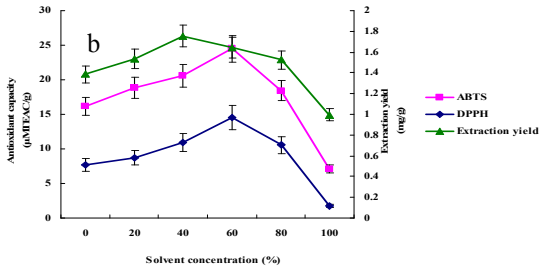


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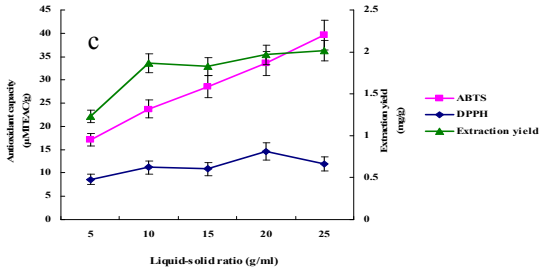


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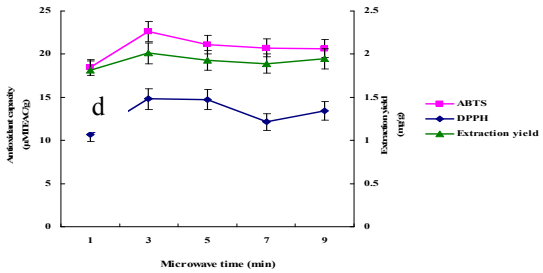


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521 **Figure 1**

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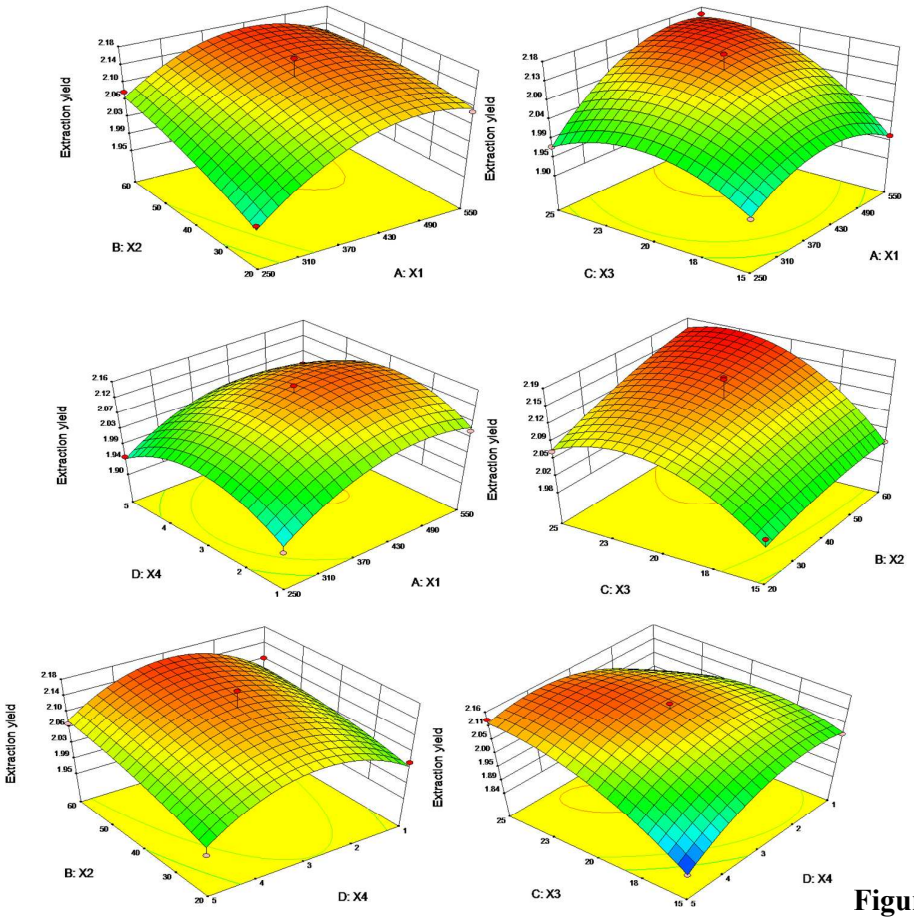


**Figure 2**

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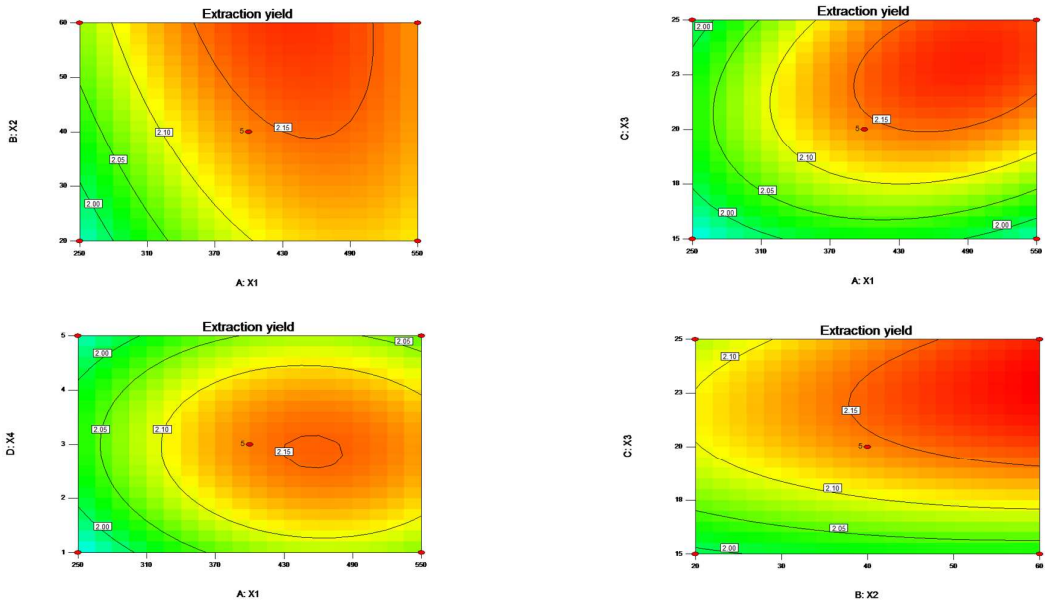

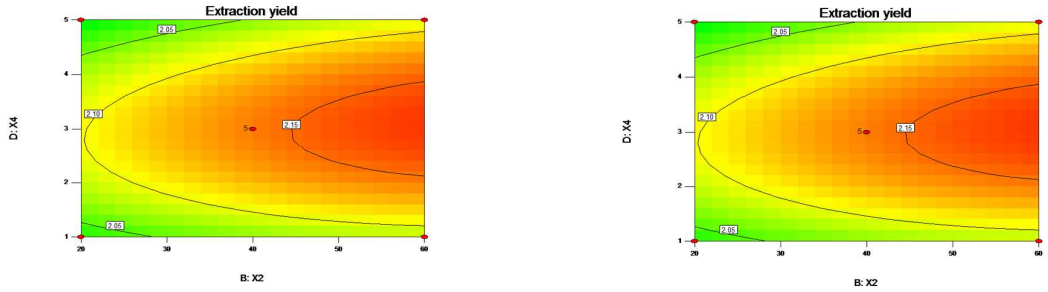


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534 **Figure 3**