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Hereby, we submit an article entitled "Optimation of Condition for Anthocyanins Extraction from Indonesian Varieties Black Rice using Response Surface Model" for possible publication in the Food Research. All authors have read and approved the manuscript and take full responsibility for its content. All authors do not have a conflict of interest regarding this research or its funding.

This article is the product of a research grant funded by the Ministry of Education, Culture, Research, and Technology of the Republic of Indonesia in 2021. This article discusses an effective and economical method of extracting black rice functional compounds. Results were very optimal (109.64 mg/ 100 g anthocyanins, 25.35 mg QE/ 100 g flavonoids, 295.56 mg GAE/100 g total phenolics, and antioxidant activity 61.69% inhibition).

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Optimation of Condition for Anthocyanins Extraction from Indonesian Varieties Black Rice using Response Surface Model

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Abstract

This study aims to extract anthocyanin, flavonoid, and total phenol compounds and antioxidant activity of black rice using the heat-assisted maceration method. Extraction parameters were optimized using response surface methodology by studying the concentration of ethanol (50-60%), citric acid (3-5%), and temperature (45-55 °C) factor. The regression model of all variables was very significant ($p < 0.001$). The best conditions for the extraction process for all variables included ethanol concentration of 56.11%, citric acid concentration of 4.42%, and extraction temperature of 49.29 °C. Under the given solution, the maximum extraction of black rice contained 109.64 mg/100 g of anthocyanins, 25.35 mg QE/100 g of flavonoids, 295.56 mg GAE/100 g total phenols, and antioxidant activity reached 61.69% of inhibition. The overall results show the positive impact of the extraction process of black rice functional compounds using the heat-assisted maceration method.

Keywords: Black rice, Indonesian varieties, extraction, anthocyanins, response surface methode

1. Introduction

Pigmented rice cultivars such as black rice are found and consumed mainly by people in Asian countries. Anthocyanins are the main components of black rice (Nakagawa & Maeda, 2017), and cyanidin-3-glucoside compounds are the largest anthocyanin compounds in black rice which reach 88% (Abdel-Aal et al., 2006). Anthocyanins are responsible for the black color in black rice (Lee, 2010). Other anthocyanin compounds such as cyanidin-3-rutinoside, cyanidin 3,5-glucoside, malvidin 3 glucoside, and peonidin 3 glucoside are also found in black rice and have been confirmed to have several health benefits for the human body (Chen et al., 2012; Hou et al. al., 2013). As a secondary metabolite compound, anthocyanins have a high solubility. This makes the extraction process the ideal method to obtain them.

Generally, the anthocyanin extraction process uses methanol, acetone, and ethanol as solvents. Although its effectiveness is not as good as methanol and acetone, ethanol is considered safer because

it is less toxic than other solvents. Ethanol concentration is the most important factor in the anthocyanin extraction process, in relation to its molecular solubility (Khazaei et al., 2016). Ethanol acidified with HCl has also been reported to increase the hydrolyzed anthocyanin content of black rice (Bae et al., 2017). However, the use of HCl as an acidification medium remains a concern, given its very high toxicity.

Regarding acidified ethanol solvents, citric acid has been widely reported as one of the best ethanol acidification media for anthocyanin extraction, such as anthocyanin extraction in barberry, eggplant, and red cabbage (Hosseini et al., 2016), anthocyanin extraction in blueberries (Xu et al., 2016), purple sweet potato (Ekaputra & Pramitasari, 2020), *Carissa carandas* fruit (Le et al., 2019), black carrot (Espinosa-Acosta et al., 2018), red cabbage (Shiyan et al., 2018), and black rice both in the form of whole-grain and by-products (Halee et al., 2018; Pedro et al., 2016). Citric acid plays a role in lowering the pH value of the solvent, this is related to the stability of the anthocyanin compounds at pH < 6 (Liu et al., 2018). The stability of anthocyanin compounds will increase when the solvent has a pH value of 1-3. Anthocyanins are easier to isolate, characterized by the extract having a red-blue mixed color (do Carmo Brito et al., 2017).

In addition to ethanol concentration and pH, temperature level is also the most widely reported contributing factor in the anthocyanin extraction process (Silva et al., 2017). The combination of ethanol concentration, citric acid, and temperature seems to be the most promising approach to obtain functional extracts from black rice. The previous literature only reported the effectiveness of ethanol-citric acid solvent in the black rice anthocyanin extraction process (Pedro et al., 2016). Studies related to the optimization of the extraction process of black rice functional compounds using a combination of ethanol concentration, citric acid concentration, and temperature have not been reported so far. The extraction process uses a response surface model (RSM) approach, which is used to develop and optimize the process and product conditions so that it can be used to determine the best conditions in the extraction process of black rice functional compounds (Granato et al., 2014).

This study aims to create an optimal model in the extraction process of functional components of Indonesian varieties of black rice using RSM based on the concentration of ethanol, citric acid, and temperature. The result is black rice extract high in anthocyanin, total phenol, flavonoid, and antioxidant activity.

2. Materials and methods

2.1 Materials

Black rice was collected from organic rice farmers in the Karanganyar region, Central Java Province, Indonesia. Other materials include food-grade citric acid from PT Gunacipta Multirasa, distilled water, ethanol reagent, $C_2H_3NaO_2$, KCl, $AlCl_3$, CH_3COOK, $C_{15}H_{10}O_7$ (quercetin), Folin–Ciocalteu Reagent, Na_2CO_3 pro analysis from Merck, 2,2-Diphenyl-1-picrylhydrazyl (DPPH), and $C_7H_6O_5$ (gallic acid) pro analysis from Sigma–Aldrich.

2.2 Maceration extraction procedure of anthocyanins

Extraction of black rice anthocyanins refers to the study of Pedro et al. (2016) with modifications. Black rice was milled to flour. 100 g of black rice flour was added with 50-60% concentration of ethanol solvent in water, with a ratio of 1:10 (w/v). Then, 3-5% (w/v) of the total solvent was added with citric acid. The extraction process was carried out in a thermostatic water bath at a temperature range of 45-55 °C for 120 minutes with constant stirring at 500 rpm. The

solution was then filtered using 400 mesh filter paper. The ethanol in the extract was then evaporated using a rotary vacuum evaporator at 60 °C. The viscous extract was stored in a dark glass bottle at -20 °C until analyzed.

2.3 Experimental design

The Box-Behnken design was used to evaluate the effect from a combination of three independent variables (ethanol concentration, citric acid concentration, and temperature) in the extraction of bioactive compounds from Indonesian black rice varieties. Values for ethanol concentrations of 50, 55, and 60%, citric acid concentrations of 3, 4, and 5%, and extraction temperatures of 45, 50, and 55 °C were studied. The dependent variables are anthocyanins, flavonoids, total phenol, and antioxidant activity. The experimental design is presented in fifteen combinations (Table 1), including three center point replicates to confirm errors and assess the incompatibility of the proposed model. All experiments were carried out by design and triplicated.

2.4 Analyses of anthocyanins

Analysis of the anthocyanin content of the extract was done using the differential pH method (Yamuangmorn et al., 2018). 1 mL of the extract was put into two dark test tubes. The first test tube was added with 1 mL of potassium chloride buffer (pH 1.0), and the second test tube was added with 1 mL of sodium acetate buffer (pH 4.5). Each solution was incubated for 15 minutes at room temperature (25 ± 1 °C) impermeable to light. The absorbance was measured using a UV-Vis spectrophotometer with a wavelength of 520 nm and 700 nm. The absorbance value was obtained by subtracting the difference in absorbance at a wavelength of 520 nm and 700 nm at pH 1 with the difference in absorbance at pH 4.5. Anthocyanin levels were obtained by multiplying the absorbance value by the molecular weight of cyanidin-3-glucoside (448.8 g/mol) and the amount of dilution, then divided by the coefficient of molar absorptivity of cyanidin-3-glucoside (26900 l/mol cm) and the width of the cuvette (1 cm). The anthocyanin content of black rice extract was expressed in mg/100g.

2.5 Analyses of flavonoids

Determination of the flavonoid content was done with the modified method of Cai et al. (2016). A 0.5 ml extract sample was prepared in a dark tube, to which 1.5 ml of ethanol, 0.1 ml of 10% $AlCl_3$, 0.1 ml of 1 M CH_3COOK, and 2.8 ml of distilled water were added. The solution was homogenized and incubated at room temperature (25 ± 1 °C) for 30 minutes. The absorbance of the sample was measured using a UV-Vis spectrophotometer with a wavelength of 415 nm. Distilled water are used as blank solution. The standard curve uses a solution of quercertin in distilled water with a concentration range of 20-100 ppm. The flavonoid content of black rice extract was expressed as mg QE/100 g.

2.6 Analyses of total phenolics

The Folin-Ciocalteu method (Pedro et al., 2016), with slight modifications, was used in the analysis of total phenol. 0.5 ml of black rice extract was prepared in a dark tube then 5 ml of Folin Ciolcaleu 10% (v/v) reagent was added. The solution was homogenized for 5 minutes and added 4 ml of 7.5% Na_2CO_3 (w/v). The mixture was incubated for 60 minutes at room temperature (25 ± 1 °C). Ethanol are used as blank solution. Gallic acid in ethanol with a concentration of 100-500 ppm

are used as standard solution. Next, the absorbance was measured using a UV-Vis spectrophotometer with a wavelength of 765 nm. The total phenol content of black rice extract was expressed as mg GAE/100g.

2.7 Determination of free-radical scavenging activity

Antioxidant activity was determined using Pedro et al. (2016) method, with a few modifications. 1.5 ml of 0.2 mM DPPH ethanol was mixed with 0.2 ml of the sample into a test tube, and ethanol was added to a final volume of 3.5 ml. The tubes were tightly closed, homogenized, and incubated at room temperature (25 ± 1 °C) for 60 minutes. The absorbance was measured at a wavelength of 517 nm. The ability of the extract to scavenge DPPH was obtained by subtracting the absorbance of the blank with the sample. The result was then compared with the absorbance of the blank and expressed in % of inhibition.

2.8 Statistical analyses

Design-Expert (version 11.1.2.0) was used in determining the experimental design and data analysis. Prediction model using statistical analysis and analysis of variance (ANOVA). The suitability of the polynomial model equation is expressed by the coefficient of determination (R^2).

3. Results and discussion

3.1 Optimization and modeling of the extraction process from black rice

Optimization of the extraction process refers to the components of anthocyanins, flavonoids, total phenol, and antioxidant activity of black rice extract. The experimental design used the Box-Behnken design to identify different independent variables. In general, the ANOVA test (Table 2) from fifteen trials produced significant linear and quadratic effects ($p < 0.05$). The R^2 values of the levels of anthocyanins, flavonoids, total phenol, and antioxidant activity of the extracts were 0.921, 0.987, 0.978, and 0.962, respectively. Thus, the model is very significant and suitable as experimental data. The regression coefficient data can be used to obtain predictions of polynomial equations. The three-dimensional surface plot refers to the obtained model and is used to assess the relationship between the dependent and independent factors.

3.2 Effect of independent variables on anthocyanin contents

The anthocyanin content of the extract was significantly influenced by the linear effect of the concentration of ethanol and citric acid ($p < 0.01$). The effect from the temperature level was not significant ($p > 0.05$), although the trend was positive. This condition is believed to be related to the extraction temperature range being in the optimal range. The same condition was also reported by Ryu & Koh (2018), the extraction temperature of 50 - 60°C had no significant effect on the anthocyanin content of black soybean extract, although there was a tendency to increase. Jha et al. (2017) more specifically reported that the optimal anthocyanin extraction temperature was 49.46 °C, while the optimal extraction temperature in this study was 51.45 °C to produce the best anthocyanins.

Figure 1A-1C is the three-dimensional plot of the three variables on the anthocyanin content of the extract (44.05 – 108.61 mg/100 g). Citric acid plays an important role in triggering the release of anthocyanins from the material, followed by the concentration of ethanol. Citric acid is a weak acid, the addition of an ethanol-water solvent generally results in a solution pH of about 2-3. It is not

possible to adjust the pH of the extract to a lower level even if the concentration is increased. Under these conditions, anthocyanins will maintain the form of flavylium that has good stability (Le et al., 2019; Yang et al., 2010). Citric acid is also easier to diffuse into the plant matrix, which makes anthocyanin hydrolysis process easier (Kurtulbaş et al., 2020).

The role of ethanol in increasing the anthocyanin extract was quite significant. However, when the ethanol concentration reached 57%, the anthocyanin extract decreased drastically. This is related to the higher solubility of anthocyanin molecules in a moderate ethanol medium (± 55%). In addition, the decrease in anthocyanin levels as the ethanol concentration increases is also caused by the presence of unwanted impurities being isolated from the material, which will affect the quality of anthocyanins (Jha et al., 2017; Le et al., 2019). However, the anthocyanin content at the highest ethanol concentration (60%), was still better than at the lowest ethanol concentration (50%). Based on these data, the prediction of the polynomial equation for the anthocyanin content of black rice extract is:

$$Y\ (anthocyanin) = 106.78 + 10.57X_1 + 16.52X_2 + 3.82X_3 - 5.13X_1X_2 + 5.51X_1X_3 - 2.38X_2X_3 - 18.10X_1^2 - 20.23X_2^2 - 14.35X_3^2$$

3.3 Effect of independent variables on flavonoids

Extraction temperature had the strongest positive linear effect among other variables which makes the flavonoid content of black rice extract increased significantly. Many researchers reported the effect of temperature on flavonoid extraction. Rajha et al. (2014) obtained the optimal temperature for solid-liquid extraction of flavonoids from grape by-products at 93 °C. Meanwhile, the temperature of 94.66 °C was the most optimal to obtain high flavonoid Flos populi extract (Sheng et al., 2013). Increasing the extraction temperature causes a decrease in solvent viscosity, which is followed by an increase in molecular movement that cause optimal release of bioactive compounds from black rice.

A high concentration of citric acid generally causes the pH of the solvent to decrease. Here, the concentration of citric acid is positively correlated with flavonoids, although the increase tends to be stable (Figures 3A and 3B). In previous studies, flavonoids are easier to extract at a high pH. The polarity of bioactive compounds at high pH will result in higher dissociation of -OH groups so that the solubility of bioactive compounds increases. Flavonoids will be optimal when the pH of the solvent is between 4.5 – 6 (Karvela et al., 2009). Recent reports support this study, flavonoids are more easily hydrolyzed at an acidic pH of 3.24 (Soquetta et al., 2019). Mai et al., (2020) also investigated the effect of solvent pH on the recovery of *Euonymus alatus* and suggested using a low pH (2.5 – 3.5). Each type of plant has a different amount and type of flavonoid. The position of the -OH group of each compound also affects the flavonoid content of the extract.

Different results were shown by the ethanol factor. Flavonoids increased until the ethanol concentration reached 55%, then began to slope and decrease rapidly when the ethanol concentration was increased further. Ethanol is known to be very efficient in the extraction of flavonoids and their glycosides (Sheng et al., 2013). The presence of water in the ethanol solvent has a positive impact on the extraction process, water will facilitate mass transfer between solids and liquids by increasing the permeability of the plant matrix so that the extraction efficiency is better. The type of flavonoid also affects the extraction process. Less polar flavonoids such as flavonols, aglycones, and flavanones generally will optimally decompose in chloroform, acetone, ethyl acetate,

and hexane solvents (Chaves et al., 2020). After applying the response surface regression method to black rice extract flavonoids, the polynomial equations were as follows:

$$Y\ (flavonoids) = 24.23 + 0.28X_1 + 0.29X_2 + 2.23X_3 - 0.04X_1X_2 + 0.20X_1X_3 - 0.25X_2X_3 - 1.63X_1^2 - 0.26X_2^2 + 0.76X_3^2$$

3.4 Effect of independent variables on total phenolic compounds

The three independent variables had a very significant positive linear effect ($p < 0.01$) on the total phenol extract. Temperature is the main factor that affects the total phenol extract. Increasing the temperature increased the total phenol extract by 37 mg GAE/ 100g (13.69%). Wang et al. (2016) also reported that increasing the extraction temperature from 50 – 70 °C, succeeded in increasing the total phenolic extract of blueberries by 14.48% (43 mg GAE/ 100 g), and red pear peels by 23.33% (42 mg GAE/ 100 g). Higher temperature can separate the phenolic components into smaller structures which cause the viscosity and surface tension to decrease, thereby increasing the solubility in solvents. This condition does not apply when the extraction time is increased. Long extraction time in high temperatures cause degradation of phenolic compounds (Yilmaz & Toledo, 2006).

The interaction of ethanol concentration with temperature gave the greatest contribution (F-value 2.24). However, the total phenol decreased when the ethanol concentration reached more than 55%. Ethanol concentration was reported to reduce the boiling point and polarity of the solution, resulting in a decrease in the isolation of phenolic compounds. This makes a balanced ethanol-water mixture to give a positive contribution to the total phenol extract. Ethanol plays a role in increasing the solubility of solutes, and the role of water is very important in the desorption process of the material matrix so that the extraction will be optimal (Mustafa & Turner, 2011).

Total phenol increased when the solvent medium became more acidic, this can be seen in Figures 3A and 3B. The addition of citric acid causes the pH of the solution to become acidic, this condition will support the separation of phenolic bound to protein and carbohydrate polymers. The solubility of hydrophobic phenolic compounds in micelles will increase, due to decreased proton activity in deprotonated phenols and their ionic characteristics. Thus, the amount of phenol extracted increased with the addition of citric acid (El-Abbassi et al., 2014; İlbay et al., 2014). The polynomial equation of the total phenol extract is predicted as follows:

$$Y\ (phenolics) = 287.81 + 1.87X_1 + 1.72X_2 + 12.85X_3 + 0.20X_1X_2 - 1.05X_1X_3 - 0.05X_2X_3 - 6.82X_1^2 + 1.78X_2^2 + 4.03X_3^2$$

3.5 Effect of independent variables on antioxidant activity

The concentration of ethanol and citric acid had a very significant ($p < 0.001$) positive linear effect on the antioxidant activity of the extract, while the extraction temperature factor had a positive linear effect with a weak significance ($p < 0.05$). The main factors affecting the antioxidant activity of the extract were the concentration of citric acid, then the concentration of ethanol, and finally the temperature. The interaction of ethanol with citric acid and citric acid with temperature is negatively correlated, while the interaction of ethanol - temperature produces a positive correlation. Based on a statistical analysis of the data obtained, the polynomial equation of antioxidant activity of the extract is predicted according to the following equation:

$$Y\ (antioxidant\ activity) = 61.90 + 1.19X_1 + 1.63X_2 + 0.41X_3 - 0.90X_1X_2 + 0.90X_1X_3 - 0.13X_2X_3 - 2.64X_1^2 - 2.56X_2^2 - 1.81X_3^2$$

The antioxidant activity of black rice extract ranged from 53.18 – 62.20% inhibition, higher than that reported by Pedro et al. (2016), namely 29.13 – 51.78% inhibition. The main antioxidant in black rice comes from anthocyanin compounds. Zhang et al. (2006) stated that the cyanidin-3-glucoside compound in black rice acts as an antioxidant hydrogen donor (AH_4). Its presence can disrupt the lipid autoxidation chain reaction so that further adipose oxidation can be stopped. The mechanism is by supplying hydrogen atoms four times (AH_4), offering H to (R-) and then returning R- to the original lipid (RH).

3.6 Optimization of the parameters

Table 3 shows the optimum maceration conditions for the extraction process using RSM. Ethanol with a concentration of 56.11% as a solvent, added with 4.42% citric acid as a medium for reducing pH and the maceration process at 49.29 °C can produce a functional extract of black rice which is rich in anthocyanins (109.64 mg/100 g), high in flavonoids (25.35 mg QE/100 g), total phenol (295.56 mg GAE/100 g), and antioxidant activity (61.96% of inhibition). The hypothesis fit the experimental results obtained based on the optimum conditions using RSM model.

4. Conclusion

The extraction process of functional compounds of Indonesian varieties of black rice using the Box-Behnken design revealed a significant effect of ethanol concentration, citric acid concentration, and extraction temperature on the yield. Furthermore, the interaction between treatments showed good optimization results. The results support black rice as an important source of anthocyanin compounds, which can be extracted well using a simple method using a combination of ethanol-water solvents, citric acid with the help of controlled heat. This can provide new opportunities, especially for the beverage industry to develop functional drinks.

Conflict of interest - Disclose any potential conflict of interest appropriately.

The authors declare no conflict of interest.

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420 Table 1. The Box–Behnken design and experiment data

No	Independent variables and coded			Response variables			
	Ethanol (%)	CA* (%)	T** (°C)	Anthocyanin [(a)]	Flavonoids [(b)]	Phenolics [(c)]	AA*** [(d)]
1	50 (-1)	3 (-1)	50 (0)	44.05 ± 1.42	21.60 ± 0.14	277.48 ± 0.85	53.48 ± 0.41
2	50 (-1)	4 (0)	55 (1)	65.57 ± 2.12	25.43 ± 0.19	270.28 ± 0.85	56.16 ± 0.25
3	50 (-1)	4 (0)	45 (-1)	59.06 ± 0.00	21.13 ± 0.24	299.08 ± 0.85	56.10 ± 0.16
4	50 (-1)	5 (1)	50 (0)	74.58 ± 0.71	22.13 ± 0.14	281.28 ± 1.13	57.84 ± 0.16
5	55 (0)	3 (-1)	45 (-1)	48.05 ± 1.42	22.03 ± 0.28	279.88 ± 0.85	55.52 ± 0.16
6	55 (0)	3 (-1)	55 (1)	50.55 ± 0.71	26.73 ± 0.05	304.68 ± 1.41	55.57 ± 0.25
7	55 (0)	5 (1)	45 (-1)	98.60 ± 0.71	23.23 ± 0.00	282.68 ± 1.41	59.76 ± 0.08
8	55 (0)	5 (1)	55 (1)	91.60 ± 0.71	26.93 ± 0.14	307.28 ± 1.13	59.29 ± 0.08
9	60 (1)	3 (-1)	50 (0)	72.58 ± 0.71	22.63 ± 0.09	283.88 ± 0.28	57.38 ± 0.33
10	60 (1)	4 (0)	45 (-1)	72.07 ± 1.42	20.90 ± 0.38	273.08 ± 1.41	56.97 ± 0.08
11	60 (1)	4 (0)	55 (1)	100.60 ± 0.71	26.00 ± 0.05	297.68 ± 1.13	60.63 ± 0.00
12	60 (1)	5 (1)	50 (0)	82.59 ± 0.71	23.00 ± 0.14	288.48 ± 0.57	58.13 ± 0.08
13	55 (0)	4 (0)	50 (0)	105.61 ± 0.71	24.27 ± 0.24	288.08 ± 1.70	61.79 ± 0.33
14	55 (0)	4 (0)	50 (0)	108.61 ± 0.71	24.37 ± 0.09	287.68 ± 3.96	62.20 ± 0.08
15	55 (0)	4 (0)	50 (0)	106.11 ± 1.42	24.07 ± 0.14	287.68 ± 0.00	61.73 ± 0.08

421 (*) Citric Acid; (**) Temperature; (***) Antioxidant Activities; [(a)] mg/100 g; [(b)] mg QE/100 g; [(c)] mg GAE/100 g; [(d)] % of Inhibition

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423 Table 2. Significance level of ANOVA and regression coefficient value of quadratic model

Coefficients		Estimated coeficients			
		Anthocyanins	Flavonoids	Phenolics	AA
Intercept		106.78	24.23	287.81	61.90
Linear	X_1 ethanol (%)	10.57**	0.28**	1.87**	1.19**
	X_2 Citric acid (%)	16.52**	0.29**	1.72**	1.63**
	X_3 Temperature (°C)	3.82	2.23**	12.85**	0.41*
Interactions	$X_1 X_2$	-5.13	-0.04	0.20	-0.90**
	X_1X_3	5.51	0.20*	-1.05	0.90**
	X_2X_3	-2.38	-0.25*	-0.05	-0.13
Quadratic	X_1^2	-18.10**	-1.63**	-6.82**	-2.64**
	X_2^2	-20.23**	-0.26*	1.78*	-2.56**
	X_3^2	-14.35**	0.76**	4.03**	-1.81**
R^2		0.921	0.987	0.978	0.962
F-value		24.78**	165.07**	92.60**	53.33**

(model)		

*significant at 0.05 level; **significant at 0.01 level.

Table 3. Optimum conditions of extraction for black rice

Factor		**Optimal Condition**
Ethanol (%)		56.11 (%)
Citric Acid (%)		4.42 (%)
Temperature (°C)		49.29 (°C)
Respons	**Prediction value**	**Experimental value**
Anthocyanins (mg/100 g)	109.64	112.10
Flavonoids (mgQE/100 g)	25.35	25.43
Phenolics (mgGAE/100 g)	295.56	296.95
Antioxidant Activity (% of Inhibition)	61.96	61.24

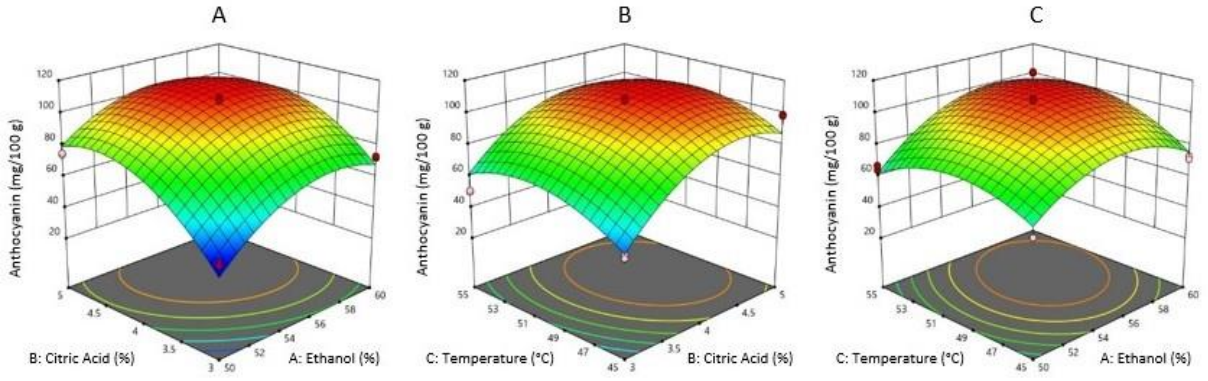

Figure 1. Response surface graphs and contour plots for the effects of ethanol concentration, citric acid concentration, and temperature on anthocyanin content of black rice extract

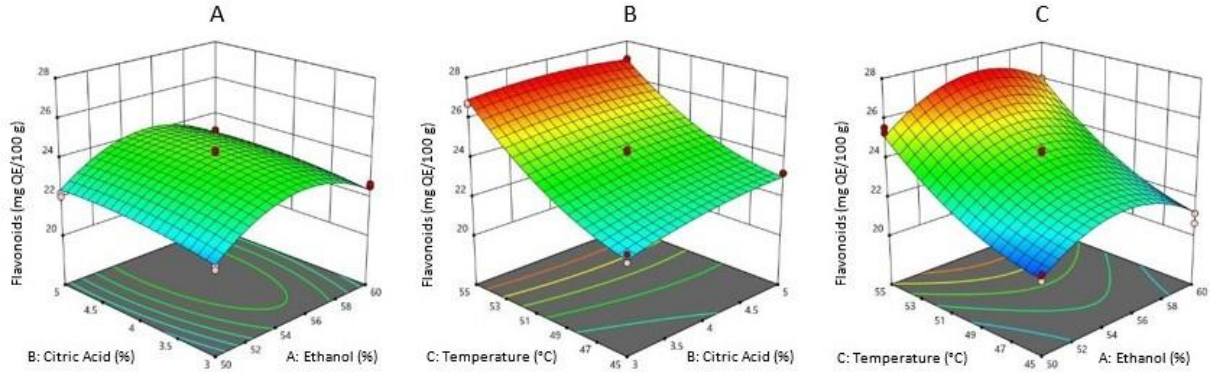

Figure 2. Response surface graphs and contour plots for the effects of ethanol concentration, citric acid concentration, and temperature on flavonoid content of black rice extract

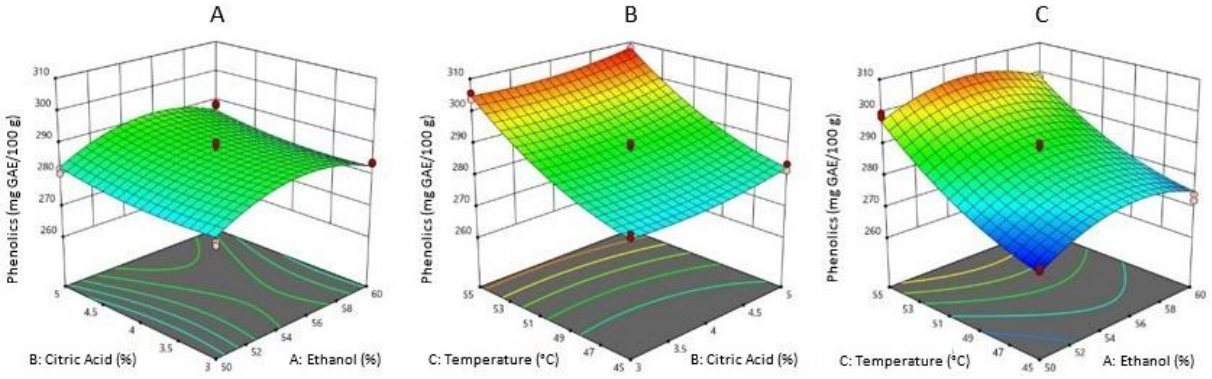

Figure 3. Response surface graphs and contour plots for the effects of ethanol concentration, citric acid concentration, and temperature on phenolics content of black rice

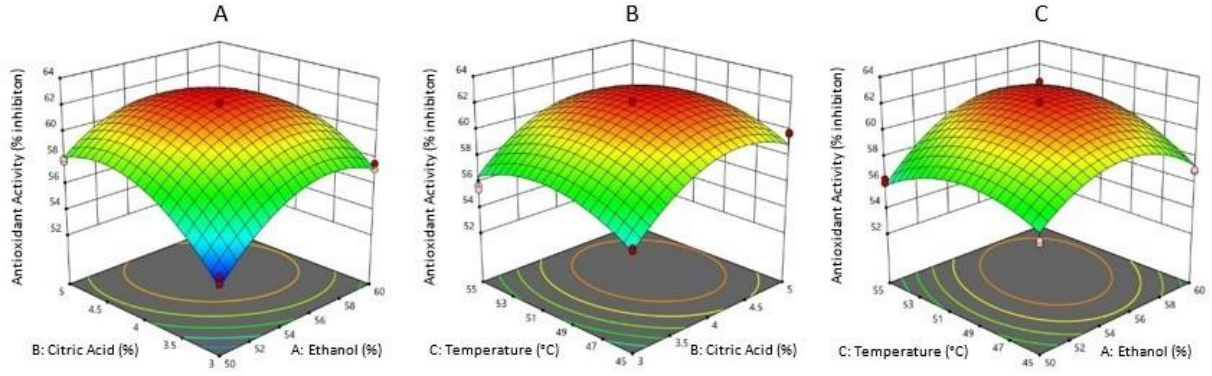

Figure 4. Response surface graphs and contour plots for the effects of ethanol concentration, citric acid concentration, and temperature on antioxidant activity of black rice extract

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Optimazing Extraction of Anthocyanins from Indonesian Varieties Black Rice using Response Surface Model

Abstract

This study aim~~s~~ed to extract anthocyanin, flavonoid, and total phenol compounds and antioxidant activity of black rice using the heat-assisted maceration method. Extraction parameters were optimized using response surface methodology by ~~studying~~ the concentration of ethanol (50-60%), citric acid (3-5%), and temperature (45-55°C) factor. The regression model of all variables was very significant ($p < 0.001$). The best conditions for the extraction process ~~for all variables~~ included ethanol concentration of 56.11%, citric acid concentration of 4.42%, and extraction temperature of 49.29°C. Under the ~~given solution~~best conditions, the maximum extraction of black rice contained 109.64 mg/100 g of anthocyanin~~s~~s, 25.35 mg QE/100 g of flavonoids, 295.56 mg GAE/100 g of total phenols, and antioxidant activity reached 61.69% of inhibition. The overall results show the positive impact of the extraction process of black rice functional compounds using the heat-assisted maceration method.

Keywords: Black rice, ~~Indonesian varieties,~~ Extraction, Anthocyanin~~s~~s, Response surface method~~ology~~e

1. Introduction

Pigmented rice cultivars such as black rice are found and consumed mainly by people in Asian countries. Anthocyanins are the main components of black rice (Nakagawa and Maeda, 2017), and cyanidin-3-glucoside compounds are the largest anthocyanin compounds in black rice, around ~~which reach~~ 88% (Abdel-Aal *et al*., 2006). Anthocyanins are responsible for the black color in black rice (Lee, 2010). Other anthocyanin compounds such as cyanidin-3-rutinoside, cyanidin 3,5-glucoside, malvidin 3 glucoside, and peonidin 3 glucoside are also found in black rice and have been confirmed to have several health benefits for the human body (Chen *et al*., 2012; Hou *et al*. ~~.~~, 2013). As a secondary metabolite compound, anthocyanins have a high solubility. This makes the extraction process the ideal method to obtain them.

Generally, the anthocyanin extraction process uses methanol, acetone, and ethanol as solvents. Although its effectiveness is not as good as methanol and acetone, ethanol is considered ~~safer~~ more safe because it is less toxic than other solvents. Ethanol concentration is the most important factor in the anthocyanin extraction process, in relation to its molecular solubility (Khazaei *et al*., 2016). Ethanol acidified with HCl has also been reported to increase the hydrolyzed anthocyanin content of black rice (Bae *et al*., 2017). However, the use of HCl as an acidification medium remains a concern, given its very high toxicity.

Regarding acidified ethanol solvents, citric acid has been widely reported as one of the best ethanol acidification media for anthocyanin extraction, such as anthocyanin extraction in barberry, eggplant, and red cabbage (Hosseini *et al*., 2016), anthocyanin extraction in blueberries (Xu *et al*., 2016), purple sweet potato (Ekaputra and Pramitasari, 2020), *Carissa carandas* fruit (Le *et al*., 2019), black carrot (Espinosa-Acosta *et al*., 2018), red cabbage (Shiyan *et al*., 2018), and black rice both in the form of whole-grain and by-products (Pedro *et al*., 2016; Halee *et al*., 2018;). Citric acid plays a role in lowering the pH value of the

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Suggested title:
The extraction optimization of anthocyanin from Indonesian black rice using response surface methodology

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Cyanidin-3-glucoside and peonidin-3-glucoside are the main anthocyanins in black rice, not other anthocyanin compounds in black rice.
Please refer the recent publication of anthocyanins in black rice.

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Why do anthocyanins as secondary metabolites have high solubility?
High solubility in any solvents?
What are the anthocyanin extraction methods?

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solvent, this is related to the stability of the anthocyanin compounds at pH < 6 (Liu *et al*., 2018). The stability of anthocyanin compounds ~~will~~ increase ~~when the solvent has a~~at pH value of solvent around 1-3~~. Anthocyanins are~~ so they are easier to isolate, characterized by the extract having a red-blue mixed color (do Carmo Brito *et al*., 2017).

In addition to ethanol concentration and pH, temperature level is also the most widely reported contributing factor in the anthocyanin extraction process (Silva *et al*., 2017). The combination of ethanol concentration, citric acid, and temperature seems to be the most promising approach to obtain functional extracts from black rice. The previous literature only reported the effectiveness of ethanol-citric acid solvent in the black rice anthocyanin extraction process (Pedro *et al*., 2016). Studies related to the optimization of the extraction process of black rice functional compounds using a combination of ethanol concentration, citric acid concentration, and temperature have not been reported so far. The extraction process uses a response surface model (RSM) approach, which is used to develop and optimize the process and product conditions so that it can be used to determine the best conditions in the extraction process of black rice functional compounds (Granato *et al*., 2014).

This study aims to create an optimal model in the extraction process of functional components of Indonesian varieties of black rice using RSM based on the concentration of ethanol, citric acid, and temperature. The result is black rice extract high in anthocyanin, total phenol, flavonoid, and antioxidant activity.

2. Materials and methods

2.1 Materials

Black rice was collected from organic rice farmers in the Karanganyar region, Central Java Province, Indonesia. Other materials include food-grade citric acid from PT Gunacipta Multirasa, distilled water, ethanol reagent, $C_2H_3NaO_2$, KCl, $AlCl_3$, CH_3COOK, $C_{15}H_{10}O_7$ (quercetin), Folin–Ciocalteu Reagent, Na_2CO_3 pro analysis from Merck, 2,2-Diphenyl-1-picrylhydrazyl (DPPH), and $C_7H_6O_5$ (gallic acid) pro analysis from Sigma–Aldrich.

2.2 Maceration extraction procedure of anthocyanins

Extraction of black rice anthocyanins refers to the study of Pedro *et al*. (2016) with modifications. Black rice was milled to flour. ~~100 g of b~~Black rice flour (100 g) was added with 50-60% concentration of ethanol solvent in distilled water, with a ratio of 1:10 (w/v). Then, 3-5% (w/v) of the total solvent was added with citric acid. The extraction process was carried out in a thermostatic water bath at a temperature range of 45-55°C for 120 minutes with constant stirring at 500 rpm. The solution was ~~then~~ filtered using 400 mesh filter paper. The ethanol in the extract was then evaporated using a rotary vacuum evaporator at 60°C. The viscous extract was stored in a dark glass bottle at -20°C until analyzed.

2.3 Experimental design

The Box-Behnken design was used to evaluate the effect from a combination of three independent variables (ethanol concentration, citric acid concentration, and temperature) in the extraction of bioactive compounds from Indonesian black rice varieties. Values for ethanol concentrations of 50, 55, and 60%, citric acid concentrations of 3, 4, and 5%, and extraction temperatures of 45, 50, and 55°C were studied. The dependent variables are anthocyanins, flavonoids, total phenol, and antioxidant activity. The experimental design is presented in fifteen

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Please refer to Penjumras et al (2020) entitled RSM for the extraction of bioactive compound from black glutinous rice.

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Please state the goal of this research clearly.

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What are the main problems in the extraction of anthocyanins from black rice?
What are the novelties of this research?
What are the contributions of this research?

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combinations (Table 1), including three center point replicates to confirm errors and assess the incompatibility of the proposed model. All experiments were carried out by design and triplicated.

2.4 Analyses of anthocyanins

Analysis of the anthocyanin content of the extract was done using the differential pH method (Yamuangmorn *et al*., 2018). ~~1 mL of the~~ Black rice extract (1 mL) was put into two dark test tubes. The first test tube was added with 1 mL of potassium chloride buffer (pH 1.0), and the second test tube was added with 1 mL of sodium acetate buffer (pH 4.5). Each solution was incubated for 15 minutes at room temperature (25 ± 1°C) impermeable to light. The absorbance was measured using a UV-Vis spectrophotometer with a wavelength of 520 nm and 700 nm. The absorbance value was obtained by subtracting the difference in absorbance at a wavelength of 520 nm and 700 nm at pH 1 with the difference in absorbance at pH 4.5. Anthocyanin levels were obtained by multiplying the absorbance value by the molecular weight of cyanidin-3-glucoside (448.8 g/mol) and the amount of dilution, then divided by the coefficient of molar absorptivity of cyanidin-3-glucoside (26900 l/mol cm) and the width of the cuvette (1 cm). The anthocyanin content of black rice extract was expressed in mg/100g.

2.5 Analyses of flavonoids

Determination of the flavonoid content was done with the modified method of Cai *et al*. (2016). A 0.5 m~~l~~L extract sample was prepared in a dark tube, to which 1.5 m~~l~~L of ethanol, 0.1 m~~l~~L of 10% $AlCl_3$, 0.1 m~~l~~L of 1 M CH_3COOK, and 2.8 m~~l~~L of distilled water were added. The solution was homogenized and incubated at room temperature (25 ± 1°C) for 30 minutes. The absorbance of the sample was measured using a UV-Vis spectrophotometer with a wavelength of 415 nm. Distilled water is used as a blank solution. The standard curve used~~s~~ a solution of quercetin in distilled water with a concentration range of 20-100 ppm. The flavonoid content of black rice extract was expressed as mg QE/100 g.

2.6 Analyses of total phenolics

The Folin-Ciocalteu method (Pedro *et al*., 2016), with slight modifications, was used in the analysis of total phenol. ~~0.5 ml of b~~Black rice extract (0.5 mL) was prepared in a dark tube then 5 m~~l~~L of Folin-Ciocalteu 10% (v/v) reagent was added. The solution was homogenized for 5 minutes and added 4 m~~l~~L of 7.5% Na_2CO_3 (w/v). The mixture was incubated for 60 minutes at room temperature (25 ± 1°C). Ethanol is used as a blank solution. Gallic acid in ethanol with a concentration of 100-500 ppm are used as standard solution. Next, the absorbance was measured using a UV-Vis spectrophotometer with a wavelength of 765 nm. The total phenol content of black rice extract was expressed as mg GAE/100g.

2.7 Determination of free-radical scavenging activity

Antioxidant activity was determined using Pedro *et al*. (2016) method, with a few modifications. A 1.5 m~~l~~L of 0.2 mM DPPH ethanol was mixed with 0.2 m~~l~~L of the sample into a test tube, and ethanol was added to a final volume of 3.5 m~~l~~L. The tubes were tightly closed, homogenized, and incubated at room temperature (25 ± 1°C) for 60 minutes. The absorbance was measured at a wavelength of 517 nm. The ability of the extract to scavenge DPPH was obtained by subtracting the absorbance of

the blank with the sample. The result was then compared with the absorbance of the blank and expressed in % of inhibition.

2.8 Statistical analyses

Design-Expert (version 11.1.2.0) was used in determining the experimental design and data analysis. Prediction model using statistical analysis and analysis of variance (ANOVA). The suitability of the polynomial model equation is expressed by the coefficient of determination (R^2).

3. Results and discussion

3.1 Optimization and modeling of the extraction process from black rice

Optimization of the extraction process refers to the components of anthocyanins, flavonoids, total phenol, and antioxidant activity of black rice extract. The experimental design used the Box-Behnken design to identify different independent variables. In general, the ANOVA test (Table 2) from fifteen trials produced significant linear and quadratic effects ($p < 0.05$). The R^2 values of the levels of anthocyanins, flavonoids, total phenol, and antioxidant activity of the extracts were 0.921, 0.987, 0.978, and 0.962, respectively. Thus, the model is very significant and suitable as experimental data. The regression coefficient data can be used to obtain predictions of polynomial equations. The three-dimensional surface plot refers to the obtained model and is used to assess the relationship between the dependent and independent factors.

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3.2 Effect of independent variables on anthocyanin contents

The anthocyanin content of the extract was significantly influenced by the linear effect of the concentration of ethanol and citric acid ($p < 0.01$). The effect from the temperature level was not significant ($p > 0.05$), although the trend was positive. This condition is believed to be related to the extraction temperature range being in the optimal range. The same condition was also reported by Ryu and Koh (2018), the extraction temperature of 50 - 60°C had no significant effect on the anthocyanin content of black soybean extract, although there was a tendency to increase. Jha *et al*. (2017) more specifically reported that the optimal anthocyanin extraction temperature was 49.46°C, while the optimal extraction temperature in this study was 51.45°C to produce the best anthocyanins.

Figure 1A-1C is the three-dimensional plot of the three variables on the anthocyanin content of the extract (44.05 – 108.61 mg/100 g). Citric acid plays an important role in triggering the release of anthocyanins from the material, followed by the concentration of ethanol. Citric acid is a weak acid, the addition of an ethanol-water solvent generally results in a solution pH of about 2-3. It is not possible to adjust the pH of the extract to a lower level even if the concentration is increased. Under these conditions, anthocyanins will maintain the form of flavylium that has good stability (Yang *et al*., 2010; Le *et al*., 2019). Citric acid is also easier to diffuse into the plant matrix, which makes anthocyanin hydrolysis process easier (Kurtulbaş *et al*., 2020).

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The role of ethanol in increasing the anthocyanin extract was quite significant. However, when the ethanol concentration reached 57%, the anthocyanin extract decreased drastically. This is related to the higher solubility of anthocyanin molecules in a moderate ethanol medium (± 55%). In addition, the decrease in anthocyanin levels as the ethanol concentration increases is also caused by the presence of unwanted impurities being isolated from the material, which will affect the quality of anthocyanins (Jha *et al*., 2017; Le *et al*., 2019). However, the anthocyanin content at the highest ethanol concentration (60%), was still better than at the lowest ethanol concentration (50%). Based

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Please elaborate this statement according to the previous studies.

on these data, the prediction of the polynomial equation for the anthocyanin content of black rice extract is:

$$Y\ (anthocyanin) = 106.78 + 10.57X_1 + 16.52X_2 + 3.82X_3 - 5.13X_1X_2 + 5.51X_1X_3 - 2.38X_2X_3 - 18.10X_1^2 - 20.23X_2^2 - 14.35X_3^2$$

3.3 Effect of independent variables on flavonoids

Extraction temperature had the strongest positive linear effect among other variables which makes the flavonoid content of black rice extract increased significantly. Many researchers reported the effect of temperature on flavonoid extraction. Rajha *et al*. (2014) obtained the optimal temperature for solid-liquid extraction of flavonoids from grape by-products at 93°C. Meanwhile, the temperature of 94.66°C was the most optimal to obtain high flavonoid Flos populi extract (Sheng *et al*., 2013). Increasing the extraction temperature causes a decrease in solvent viscosity, which is followed by an increase in molecular movement that cause optimal release of bioactive compounds from black rice.

A high concentration of citric acid generally causes the pH of the solvent to decrease. Here, the concentration of citric acid is positively correlated with flavonoids, although the increase tends to be stable (Figures 3A and 3B). In previous studies, flavonoids are easier to extract at a high pH. The polarity of bioactive compounds at high pH will result in higher dissociation of -OH groups so that the solubility of bioactive compounds increases. Flavonoids will be optimal when the pH of the solvent is between 4.5 – 6 (Karvela *et al*., 2009). Recent reports support this study, flavonoids are more easily hydrolyzed at an acidic pH of 3.24 (Soquetta *et al*., 2019). Mai *et al*., (2020) also investigated the effect of solvent pH on the recovery of *Euonymus alatus* and suggested using a low pH (2.5 – 3.5). Each type of plant has a different amount and type of flavonoid. The position of the -OH group of each compound also affects the flavonoid content of the extract.

Different results were shown by the ethanol factor. Flavonoids increased until the ethanol concentration reached 55%, then began to slope and decrease rapidly when the ethanol concentration was increased further. Ethanol is known to be very efficient in the extraction of flavonoids and their glycosides (Sheng *et al*., 2013). The presence of water in the ethanol solvent has a positive impact on the extraction process, water will facilitate mass transfer between solids and liquids by increasing the permeability of the plant matrix so that the extraction efficiency is better. The type of flavonoid also affects the extraction process. Less polar flavonoids such as flavonols, aglycones, and flavanones generally will optimally decompose in chloroform, acetone, ethyl acetate, and hexane solvents (Chaves *et al*., 2020). After applying the response surface regression method to black rice extract flavonoids, the polynomial equations were as follows:

$$Y\ (flavonoids) = 24.23 + 0.28X_1 + 0.29X_2 + 2.23X_3 - 0.04X_1X_2 + 0.20X_1X_3 - 0.25X_2X_3 - 1.63X_1^2 - 0.26X_2^2 + 0.76X_3^2$$

3.4 Effect of independent variables on total phenolic compounds

The three independent variables had a very significant positive linear effect ($p < 0.01$) on the total phenol extract. Temperature is the main factor that affects the total phenol extract. Increasing the temperature could increase~~d~~ the total phenol extract by 37 mg GAE/ 100g (13.69%). Wang *et al*. (2016) also reported that increasing the extraction temperature from 50 – 70°C, succeeded in

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increasing the total phenolic extract of blueberries by 14.48% (43 mg GAE/ 100 g), and red pear peels by 23.33% (42 mg GAE/ 100 g). Higher temperature can separate the phenolic components into smaller structures which cause the viscosity and surface tension to decrease, thereby increasing the solubility in solvents. This condition does not apply when the extraction time is increased. Long extraction time in high temperatures cause degradation of phenolic compounds (Yilmaz and Toledo, 2006).

The interaction of ethanol concentration with temperature gave the greatest contribution (F-value 2.24). However, the total phenol decreased when the ethanol concentration reached more than 55%. Ethanol concentration was reported to reduce the boiling point and polarity of the solution, resulting in a decrease in the isolation of phenolic compounds. This makes a balanced ethanol-water mixture to give a positive contribution to the total phenol extract. Ethanol plays a role in increasing the solubility of solutes, and the role of water is very important in the desorption process of the material matrix so that the extraction will be optimal (Mustafa and Turner, 2011).

Total phenol increased when the solvent medium became more acidic, this can be seen in Figures 3A and 3B. The addition of citric acid causes the pH of the solution to become acidic, this condition will support the separation of phenolic bound to protein and carbohydrate polymers. The solubility of hydrophobic phenolic compounds in micelles will increase, due to decreased proton activity in deprotonated phenols and their ionic characteristics. Thus, the amount of phenol extracted increased with the addition of citric acid (El-Abbassi *et al*., 2014; İlbay *et al*., 2014). The polynomial equation of the total phenol extract is predicted as follows:

$$Y\ (phenolics) = 287.81 + 1.87X_1 + 1.72X_2 + 12.85X_3 + 0.20X_1X_2 - 1.05X_1X_3 - 0.05X_2X_3 - 6.82X_1^2 + 1.78X_2^2 + 4.03X_3^2$$

3.5 Effect of independent variables on antioxidant activity

The concentration of ethanol and citric acid had a very significant ($p < 0.001$) positive linear effect on the antioxidant activity of the extract, while the extraction temperature factor had a positive linear effect with a weak significance ($p < 0.05$). The main factors affecting the antioxidant activity of the extract were the concentration of citric acid, then the concentration of ethanol, and finally the temperature. The interaction of ethanol with citric acid and citric acid with temperature is negatively correlated, while the interaction of ethanol - temperature produces a positive correlation. Based on a statistical analysis of the data obtained, the polynomial equation of antioxidant activity of the extract is predicted according to the following equation:

$$Y\ (antioxidant\ activity) = 61.90 + 1.19X_1 + 1.63X_2 + 0.41X_3 - 0.90X_1X_2 + 0.90X_1X_3 - 0.13X_2X_3 - 2.64X_1^2 - 2.56X_2^2 - 1.81X_3^2$$

The antioxidant activity of black rice extract ranged from 53.18 – 62.20% inhibition, higher than that reported by Pedro *et al*. (2016), namely 29.13 – 51.78% inhibition. The main antioxidant in black rice comes from anthocyanin compounds. Zhang *et al*. (2006) stated that the cyanidin-3-glucoside compound in black rice acts as an antioxidant hydrogen donor (AH_4). Its presence can disrupt the lipid autoxidation chain reaction so that further adipose oxidation can be stopped. The mechanism is by supplying hydrogen atoms four times (AH_4), offering H to (R-) and then returning R- to the original lipid (RH).

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Why the authors conducted the extraction process for 120 minutes?

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Why the antioxidant activity of black rice extract in this research showed higher than the result of Pedro et al (2016)?

3.6 Optimization of the parameters

Table 3 shows the optimum maceration conditions for the extraction process using RSM. Ethanol with a concentration of 56.11% as a solvent, added with 4.42% citric acid as a medium for reducing pH and the maceration process at 49.29°C can produce a functional extract of black rice which is rich in anthocyanins (109.64 mg/100 g), high in flavonoids (25.35 mg QE/100 g), total phenol (295.56 mg GAE/100 g), and antioxidant activity (61.96% of inhibition). The hypothesis fit the experimental results obtained based on the optimum conditions using RSM model.

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4. Conclusion

The extraction process of functional compounds of Indonesian varieties of black rice using the Box-Behnken design revealed a significant effect of ethanol concentration, citric acid concentration, and extraction temperature on the yield. Furthermore, the interaction between treatments showed good optimization results. The results support black rice as an important source of anthocyanin compounds, which can be extracted well using a simple method using a combination of ethanol-water solvents, citric acid with the help of controlled heat. This can provide new opportunities, especially for the beverage industry to develop functional drinks.

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Good conclusion consists of main conclusion, key findings, implication, and future directions.

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How about the halal issues in relation to use of ethanol for extraction of anthocyanin?

Conflict of interest - Disclose any potential conflict of interest appropriately.

The authors declare no conflict of interest.

Acknowledgments

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406 Table 1. The Box–Behnken design and experiment data

No	Independent variables and coded			Response variables			
	Ethanol (%)	CA* (%)	T** (°C)	Anthocyanin [(a)]	Flavonoids [(b)]	Phenolics [(c)]	AA*** [(d)]
1	50 (-1)	3 (-1)	50 (0)	44.05 ± 1.42	21.60 ± 0.14	277.48 ± 0.85	53.48 ± 0.41
2	50 (-1)	4 (0)	55 (1)	65.57 ± 2.12	25.43 ± 0.19	270.28 ± 0.85	56.16 ± 0.25
3	50 (-1)	4 (0)	45 (-1)	59.06 ± 0.00	21.13 ± 0.24	299.08 ± 0.85	56.10 ± 0.16
4	50 (-1)	5 (1)	50 (0)	74.58 ± 0.71	22.13 ± 0.14	281.28 ± 1.13	57.84 ± 0.16
5	55 (0)	3 (-1)	45 (-1)	48.05 ± 1.42	22.03 ± 0.28	279.88 ± 0.85	55.52 ± 0.16
6	55 (0)	3 (-1)	55 (1)	50.55 ± 0.71	26.73 ± 0.05	304.68 ± 1.41	55.57 ± 0.25
7	55 (0)	5 (1)	45 (-1)	98.60 ± 0.71	23.23 ± 0.00	282.68 ± 1.41	59.76 ± 0.08
8	55 (0)	5 (1)	55 (1)	91.60 ± 0.71	26.93 ± 0.14	307.28 ± 1.13	59.29 ± 0.08
9	60 (1)	3 (-1)	50 (0)	72.58 ± 0.71	22.63 ± 0.09	283.88 ± 0.28	57.38 ± 0.33
10	60 (1)	4 (0)	45 (-1)	72.07 ± 1.42	20.90 ± 0.38	273.08 ± 1.41	56.97 ± 0.08
11	60 (1)	4 (0)	55 (1)	100.60 ± 0.71	26.00 ± 0.05	297.68 ± 1.13	60.63 ± 0.00
12	60 (1)	5 (1)	50 (0)	82.59 ± 0.71	23.00 ± 0.14	288.48 ± 0.57	58.13 ± 0.08
13	55 (0)	4 (0)	50 (0)	105.61 ± 0.71	24.27 ± 0.24	288.08 ± 1.70	61.79 ± 0.33
14	55 (0)	4 (0)	50 (0)	108.61 ± 0.71	24.37 ± 0.09	287.68 ± 3.96	62.20 ± 0.08
15	55 (0)	4 (0)	50 (0)	106.11 ± 1.42	24.07 ± 0.14	287.68 ± 0.00	61.73 ± 0.08

407 (*) Citric Acid; (**) Temperature; (***) Antioxidant Activities; [(a)] mg/100 g; [(b)] mg QE/100 g; [(c)] mg GAE/100 g; [(d)] % of Inhibition
408

409

Table 2. Significance level of ANOVA and regression coefficient value of quadratic model

Coefficients		Estimated coefficients			
		Anthocyanins	Flavonoids	Phenolics	AA
Intercept		106.78	24.23	287.81	61.90
Linear	X_1 ethanol (%)	10.57**	0.28**	1.87**	1.19**
	X_2 Citric acid (%)	16.52**	0.29**	1.72**	1.63**
	X_3 Temperature (°C)	3.82	2.23**	12.85**	0.41*
Interactions	$X_1 X_2$	-5.13	-0.04	0.20	-0.90**
	X_1X_3	5.51	0.20*	-1.05	0.90**
	X_2X_3	-2.38	-0.25*	-0.05	-0.13
Quadratic	X_1^2	-18.10**	-1.63**	-6.82**	-2.64**
	X_2^2	-20.23**	-0.26*	1.78*	-2.56**
	X_3^2	-14.35**	0.76**	4.03**	-1.81**
R^2		0.921	0.987	0.978	0.962
F-value (model)		24.78**	165.07**	92.60**	53.33**

*significant at 0.05 level; **significant at 0.01 level.

Table 3. Optimum conditions of anthocyanins, flavonoids, and phenolics extraction and antioxidant activity ~~for~~ of Indonesian black rice

Factor		**Optimal Condition**
Ethanol (%)		56.11 (%)
Citric Acid (%)		4.42 (%)
Temperature (°C)		49.29 (°C)
Respons	**Prediction value**	**Experimental value**
Anthocyanins (mg/100 g)	109.64	112.10
Flavonoids (mgQE/100 g)	25.35	25.43
Phenolics (mgGAE/100 g)	295.56	296.95
Antioxidant Activity (% of Inhibition)	61.96	61.24

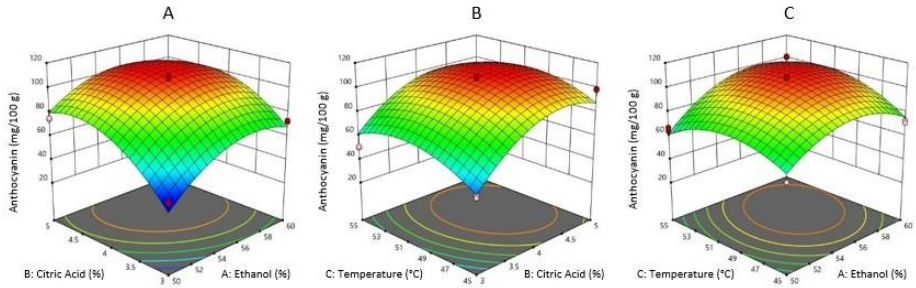

Figure 1. Response surface graphs and contour plots for the effects of ethanol concentration, citric acid concentration, and temperature on anthocyanin content of black rice extract

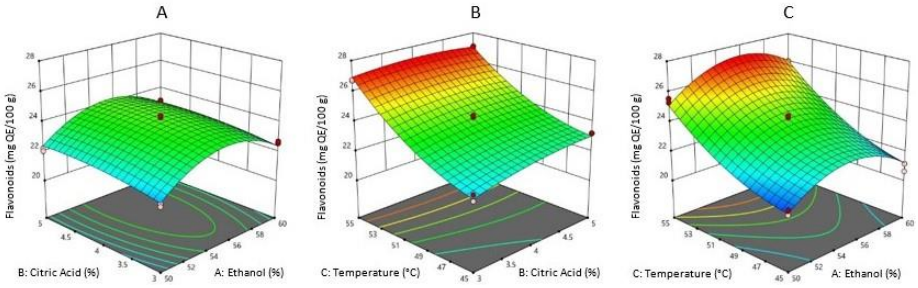

Figure 2. Response surface graphs and contour plots for the effects of ethanol concentration, citric acid concentration, and temperature on flavonoid content of black rice extract

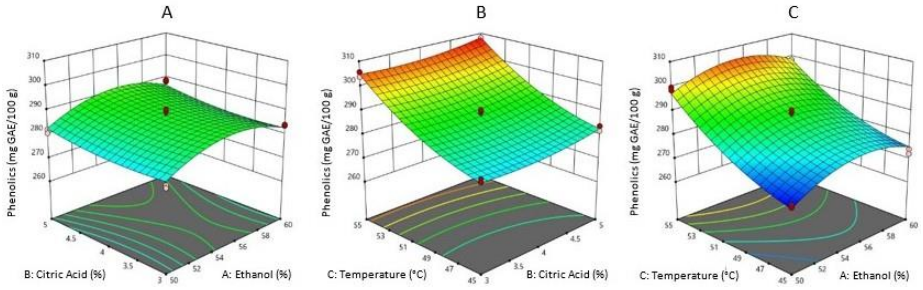

Figure 3. Response surface graphs and contour plots for the effects of ethanol concentration, citric acid concentration, and temperature on phenolics content of black rice

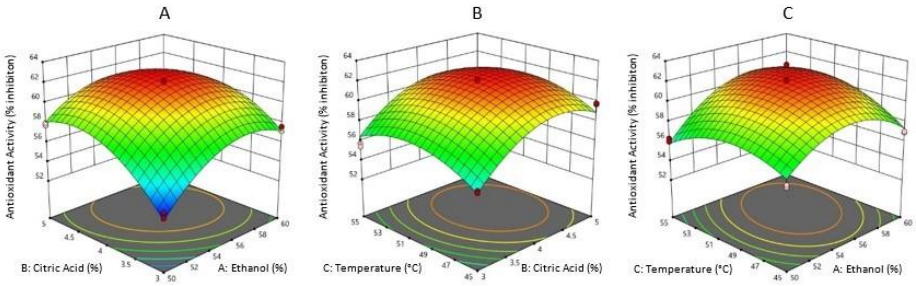

Figure 4. Response surface graphs and contour plots for the effects of ethanol concentration, citric acid concentration, and temperature on antioxidant activity of black rice extract