FOODSCITECH
Food Science and Technology Journal

Optimization of Time, Temperature, and pH for the Extraction of Anthocyanin from *Buni* (*Antidesma bunius*) Fruit

William Juhadi[1], Abdullah Muzi Marpaung[1]*

[1]Food Technology Department, Swiss German University, The Prominence Tower Alam Sutera, Jl. Jalur Sutera Barat Kav 15, Kota Tangerang, Banten 15143; Telp: +6221 2977 9596
Email: abdullah.muzi@sgu.ac.id

ABSTRACT

The Box-Behnken design to find the optimum condition for anthocyanin extraction of Buni (Antidesma bunius) fruit powder was applied. Color intensity (CI), total monomeric anthocyanin (TMA), total phenolic content (PC), and antioxidant activity (AA) were the parameters selected to express optimality resulted from the extraction process. The three studied factors were temperature (30-90^{o}C), time (30-90 minutes), and pH of solvent (1-3). The extraction was conducted in a water bath shaker under no light by HCl-acidified water as a solvent with a 1:25 (w/v) powder to solvent ratio. The quadratic model described the effect of factors on CI, TMA, and AA of buni fruit extract, while the linear model suitable for the total phenolic content. All factors influenced the CI and TMA, but time was insignificant for PC and antioxidant activity ($p < 0.05$). The optimum parameter for extraction was at 68^{o}C for 30 minutes with pH 1 solvent. The 95% confidence interval of CI, TMA, PC, and AA were 40.29 ± 0.91, 555.17 ± 13.34 mg/l, 1084.4 66.845 ± 66.85 mg GAE/l, and 45.00 ± 2.56 % inhibition, respectively. The color and anthocyanin stability test showed that the extract was almost colorless at pH 5-6, but the anthocyanin was relatively stable with half-life 28 and 17.6 days, respectively. At pH 7-8 the stability of purple-blue color and anthocyanin of the extract was extremely low. The half-life of the color and anthocyanin was less than 2 and 10 hours, respectively.

Keywords: *Anthocyanin; Antidesma bunius; extraction; Response Surface Methodology; stability*

INTRODUCTION

There is an increasing demand in the development of food colorants from natural sources to replace synthetic food colorants due to concerns on the impact to health (Bakowska-Barczak, 2005). One of the most potential natural pigment are anthocyanins that are widely distributed in plant and yield a variety of colors. In addition, several functional benefits have been reported in anthocyanin such as antioxidant, anti-carcinogenic property, hepato-protection capacity, and ability to enhance memory (Turturica, Oancea, Bahrim, & Rapaenu, 2015).

Antidesma bunius fruit, known as *buni* in Indonesia, could be considered as the potential source of anthocyanin. Traditionally, the ripe buni fruit has been commonly used as Indonesian salad called rujak buni or as an herbal medicine (Amalia, et al., 2013). *Buni* reported to contain high amount of anthocyanin with 141.94 mg/100 g that was higher than those reported in red grape cultivar Cabernet Sauvignon with 99.08 mg/100 g (Butkhup & Samappito, 2011). However, this potential has not been fully explored even in the extraction process.

Previous studies on *buni* extraction had been done in long extraction period. The extraction arranged at room temperature for 72 hours (Amalia, et al., 2013) and 24 hours (Barcelo, Nullar, Caranto, Gatchallan, & Aquino, 2016), respectively, with the latter reported the extract with the highest color intensity and total anthocyanin used acidified ethanol 70% as the solvent. Previous studies have found that anthocyanin is better extracted at low pH environment. The

long extraction hours may be reduced by increasing the extraction temperature, with a note that excessive heat exposure may degrade the anthocyanin. Combination of temperature and time determines how much heat is exposed to the extraction material. Therefore, extraction temperature, extraction time, and pH of solvent are the influential factors in the extraction of anthocyanin. In the case of *buni*, the effect of these factors to the resulting extract was yet to be studied.

One of the methods to optimize process parameters like extraction is Response surface methodology (RSM), especially when many factors and many interactions influence the response. The main advantage of RSM is the reduced number of experimental runs to produce acceptable result. Consequently, RSM is a faster and less expensive than the classical method. RSM generates a mathematical model which can be graphically interpreted to define the effect of factors, either alone or in combination, on the process then allow it to be optimized. The graphical representation of the model is visualized in the form of surface which follows the nature of the model's function. Therefore, the objective of this study is to determine the optimum condition for anthocyanin extraction from *buni* based on the desired parameters and evaluate the stability of the best extract.

METHODS

Materials

The fully riped *buni* (*Antidesma bunius*) fruits were collected from Bekasi, West Java, Indonesia. The fruits were washed, steam-blanched for six minutes, freeze-dried for 96 hours, pulverized and sieved through 250 µm screen. The moisture content of the powder (gravimetry) was less than five percent. The powder packed in a dark and tight container and kept in a freezer (-20^{o}C) until used. The chemicals used were 2,2-diphenyl-2-picrylhydrazyl (DPPH), buffer solution pH 5 and 6 (citric acid-sodium hydroxide, pH 7 and 8 (disodium hydrogen phosphate-potassium dihydrogen phosphate), concentrated HCl, ethanol, Folin-ciocalteu reagent, gallic acid (Merck®, Germany), citric acid, potassium chloride, sodium citrate (Brataco, Indonesia), distilled water (Amidis®, Indonesia), and sodium carbonate (AnalaR BDH, England).

Anthocyanin Extraction and Stability Test

The anthocyanin extraction followed the method of Marpaung et al. (2013) with modification. One gram of *buni* powder extracted in 25 ml of solvent under no light with continuous shaking. The extract immediately cooled down in a cold water-bath, then centrifuged at 8,000 rpm at 4^{o}C for 10 minutes. The liquid separated using filter paper and stored in dark bottles prior to analysis. The analyses were color intensity (CI), total monomeric anthocyanin (TMA), total phenolic content (TP) and antioxidant activity (AA). The extract from optimum extraction method was diluted in buffer solution, then stored in dark bottles at room temperature. The CI and TMA were determined per day for extract pH 5 and 6, and per hour for extract pH 7 and 8.

Chemical Analysis

The CI determined by a UV-Vis spectrophotometer (Genesys 10uv Thermo Electron Corporation, USA) as ($A_{\lambda max}$ - A_{700}) ×DF (Cisse, Vaillant, Kane, Ndiayea, & Dornier, 2012). $A_{\lambda max}$ was an absorbance at a wavelength with maximum absorbance, A_{700} was an absorbance

at 700, and DF was a dilution factor.

The TMA determined as cyanidin 3-glucoside by pH-differential method (Wrolstad & Giusti, 2001). Sample diluted in buffer solution pH 1 (potassium chloride and hydrochloric acid) and 4.5 (sodium acetate and hydrochloric acid). The DF was 10. The absorbance of both samples measured at λ_{max} and 700 nm. The difference between absorbancies found as $A = (A_{\lambda max} - A_{700})_{pH1.0} - (A_{\lambda max} - A_{700})_{pH4.5}$. The TMA was $(A \times MW \times DF \times 1000)/(\varepsilon \times l)$. MW was molecular weight (449.2 g/mol), ε was molar absorptivity (26,900), and l was the cuvette width.

The TP assessed by the Folin–Ciocalteu method (Marpaung, Andarwulan, & Prangdimurti, 2013). A 0.1 ml sample reacted with 0.5 ml Folin-Ciocalteau reagent and 0.4 ml sodium carbonate (7.5% w/v). After one hour, the absorbance (A) measured at 765 nm. A gallic acid standard curve was constructed beforehand with various concentration of gallic acid to find the slope (m). The results were calculated as gallic acid equivalent (GAE): TP (mg/L GAE) = A/m.

The AA determined by DPPH (2,2-diphenyl-1-pycrilhydracyl) radical assay (Prior, Wu, & Schaih, 2005) with a little modification. A 0.05 ml of extract with the respective DF was added with 0.85 ml ethanol 96%, and 0.10 ml DPPH solution, then incubated for 30 minutes in dark condition before measured for its absorbance. Blank sample was prepared without extract and DPPH solution addition while control sample was prepared by replacing extract with deionized water in the same amount. The DPPH solution was prepared by diluting 11 mg DPPH in 20 ml ethanol 96%. The AA (%) was $(A_{control}-A_{sample})/A_{control} \times 100\%$.

Experimental Design and Statistical Analysis

The Box-Behnken design was applied to study the effect of temperature (30-90^{o}C), time (30-90 minutes) and solvent (water acidified with HCl solution) pH (1-3) to the CI, TMA, TP, and AA of *buni* extract. There were 13 combinations and five replications in center point to build 17 treatments. The data were processed by Design-Expert® 7.0.0 (Stat-Eease Inc.) software. The data were fitted to the first or second order equation as a function of the factors at 5% confidence level ($\alpha = 0.05$). Verification procedure conducted by repeating the extraction at optimum condition five times.

RESULTS AND DISCUSSION

By employing RSM with four responses (CI, TMA, TP, and AA), the optimum parameters of the extraction were determined. The effect of three parameters (time and temperature of extraction and pH of solvent) on each response studied based on the suggested models provided by Design-Expert software (Table 1). The models were selected at a significant level of 5% ($\alpha = 0.05$). Lack-of-fit must also be considered. A significant lack-of-fit means the models are not ideal to describe the actual data. As seen in Table 1, all models suggested were significant with insignificant lack of fit. However, several sources of variance were insignificant and could be removed from the equation to obtain a better model. The equation generated for each model by excluding the insignificant factor terms is shown in Table 2.

FOODSCITECH, Vol. 4 No. 1 July 2021
ISSN 2622-1985 (print) | ISSN 2622-4127 (online)
DOI: http://dx.doi.org/10.25139/fst.v4i1.3319

Table 1. List of p-value of ANOVA for response (CI, TMA, PC, and AA) surface model suggested by the Design Expert Software

Source	p-Value			
	CI (Quadratic)	TMA (Quadratic)	PC (Linear)	AA (Quadratic)
Model	<0.0001*	<0.0001*	0.0003*	0.0021*
A	<0.0001*	<0.0001*	0.0001*	0.0080*
B	0.0364*	0.0020*	0.4789	0.9928
C	<0.0001*	<0.0001*	0.0047	0.0001*
AB	0.0023*	0.0121*	-	0.5601
AC	0.0051*	0.1467	-	
BC	0.4096	0.0008	-	0.9547
A^2	0.0002*	<0.0001*	-	0.0214*
B^2	0.3436	0.2506	-	0.158
C^2	0.0001*	0.2394	-	0.0080*
Lack of Fit	0.0861	0.3569	0.1515	0.0877

A: Temperature (30 - 90ºC), B: Time (30 - 90 minutes), C: pH (1 - 3); *) Significant at α = 0.05

Table 2. Selected models and generated equations to describe the effect of extraction factors on each response

Response	Model	Equation in coded factors (-1, +1)*
CI	Quadratic	$Y_1 = 31.70 - 2.41A - 0.64B - 11.50C - 1.64AB + 1.42AC - 2.47A^2 - 2.65C^2$
TMA	Quadratic	$Y_2 = 536.17 - 48.30A - 25.05B - 40.00C - 16.12AB + 26.77BC - 58.45A^2$
TP	Linear	$Y_3 = 995.07 + 138.91A - 89.33C$
AA	Quadratic	$Y_4 = 34.63 + 2.95A - 6.22C + 3.37A^2 - 4.16C^2$

*) A: Temperature (30 - 90ºC), B: Time (30 - 90 minutes), C: pH (1 - 3)

Effect on Color and Total Monomeric Anthocyanin

Anthocyanin can exist as six different species depend on the pH: red flavylium cation (**AH^+**), purple quinonoidal base (**A**), blue anionic quinonoidal base (**A^-**), colorless hemiketal (**B**), cis-chalcone (**C_c**) and trans-chalcone (**C_t**). The color intensity (CI) represents the light absorbance of **AH^+**, **A**, and **A^-**, while total monomeric anthocyanin (TMA) counts all six species. In general, an anthocyanin-source extract exhibits the lowest CI at pH around 4-5 as the colorless **B** starts to predominate at pH > 2 (Malien-Aubert, Dangles, & Amiot, 2001). The equation in Table 2 showed that pH performed as the most affecting factor to CI. The higher the pH, the lower the CI (Figure 1a).

HCl reported to result in less anthocyanin content than other solvent systems (Amalia, et al., 2013). However, the higher TMA in *buni* extract obtained when it extracted at the lower pH (Figure 1b). The effectivity of the lower pH was probably due to the role of acid in assisting the hydrolysis of the cell wall matrix. Hence, the release of the phytochemicals was more effective (Amalia, et al., 2013).

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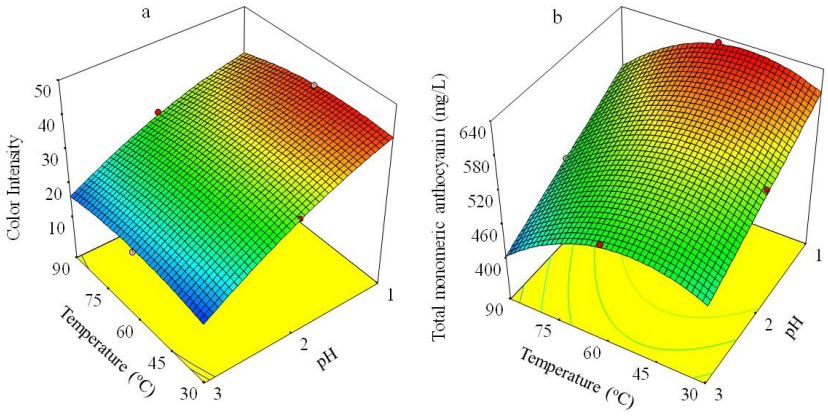

Figure 1. 3D surface plot describing the effect of temperature and pH on color intensity (a) and total monomeric anthocyanin (b) at 30 minutes of extraction time

Total heat exposed to the *buni* fruit depended on the temperature and the duration of the extraction. The equation showed that heat exposure tended to decrease the CI and TMA. This result followed the common knowledge that anthocyanins are a heat-sensitive substance. Figure 1 showed that in the range of 30^{o}C and 90^{o}C, the CI and TMA increased to the highest around the starting range and declined towards the end of the range. The significant effect of temperature and pH confirmed the previous research on anthocyanins and phenolics from litchi fruit pericarp tissue (Uma, Ho, & Wan Aida, 2010).

Effect on total phenolic content and antioxidant activity

The extraction time was not significant to the TP and AA (Table 1). Therefore, it excluded from the model (Table 2). A similar result also occurs in henna (*Lawsonia inermis*) leaves extracted at 90-450 minutes (Uma, Ho, & Wan Aida, 2010). The higher extraction time might need to be applied to see the effect of heat exposure on the polyphenols, both for the recovery and degradation. The role of heat, in this case, was only limited to the cell wall matrix destruction to allow the phenolic compounds to extracted by the solvent. In other words, the cell wall matrix will only release the contained phenolic compounds when it can no longer withstand the temperature regardless of how long the heat exposure is applied. The *buni* extract tended to have higher TP and AA at lower pH and higher temperature (Figure 2). This behavior followed the previous work that the decline in phenolic content extracted using water-HCl solvent occurred as the pH of solvent increase (Meireles, 2009). The level of acidity provided by HCl contributed to the hydrolysis of the cell wall and allowed more polyphenols to extracted.

As temperature increased, the TP and AA increased as well. This finding was not in agreement with the TMA. As the temperature increased, the TP and AA increased as well. This finding was not in agreement with the TMA. It could be deducted that phenolic compounds were more resistant to heat, and the antioxidant activity mainly contributed by the phenolic compounds. Although the anthocyanin content decreased towards the high level of extraction temperature, the phenolic experienced a steady increase. The possible reason was that the anthocyanins, despite degraded at high temperature, still recognized as phenolic compounds. Anthocyanin degraded into benzaldehyde and benzoic acid derivative (Marpaung, Andarwulan, Hariyadi, & Faridah, 2017).

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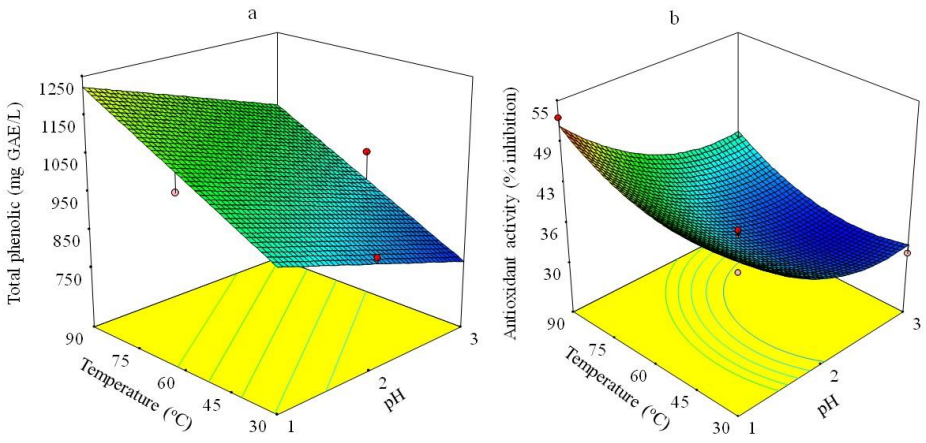

Figure 2. 3D surface plot describing the effect of extraction temperature and pH on total phenolic content (a) and antioxidant activity (b) at 30 minutes of extraction time

Selection and Verification of Optimum Extraction Condition

The maximum value of all responses was the desired criteria for the optimization, with the order of priority was CI, TMA, AA, and PP. Based on the criteria, extraction with a pH 1 solvent at 68^{o}C for 30 minutes chosen. The 95% confidence interval of CI, TMA, PC, and AA at the optimum parameters were 40.29 ± 0.91, 555.17 ± 13.34 mg/l, 1084.4 66.845 ± 66.85 mg GAE/l, and 45.00 ± 2.56 % inhibition, respectively. Meanwhile, the 95% predicted intervals of the responses were 38.47 to 42.11, 527.33 to 582.99 mg/l, 914.31 to 1254.49 mg GAE/l, and 39.73 to 50.27 % inhibition, respectively. The extraction repeated five times as a verification procedure. The average result of CI, TMA, TP, and AA were 39.96, 622.11, 1060.53 mg GAE/l, and 48.43 % inhibition, respectively. These results were within the range of predicted values. Therefore, the constructed models and equations were all valid.

After conversion from wet basis to dry basis (db), the phenolic content of the optimum extract was 2651.32 mg/100 g. This amount is higher than *buni* fruit from Bagulin, Philippines, with 1978.38 mg/100g of total phenolic extracted at room temperature for 72 hours (Barcelo, Nullar, Caranto, Gatchallan, & Aquino, 2016).

Stability of the Buni extract

The *buni* extract showed a low CI at pH 5 and 6, because of the presence of colorless **B** as the predominant species of anthocyanin. The species is known as relatively stable at a low acidic condition. As a result, there was no significant decrease in CI and a slight decrease of TMA of the extract at pH 5 and 6. The $t_{0.5}$ (time needed to reach 50% of the initial content) of the TMA at pH 5 and 6 were 28 and 17.6 days, respectively. The decrease indicated that the part of **B** chemically degraded to colorless degradation products. It is known that the end products of anthocyanin degradation are benzaldehyde and benzoic acid derivative (Marpaung, Andarwulan, Hariyadi, & Faridah, 2017). This result showed that despite not potential as a coloring agent, the *buni* extract could be a potential source of anthocyanin in a functional drink.

The presence of **A** species provided a deep purple-blue color to the extract at pH 7 and 8. However, the species was unstable and converted **B** within hours. The $t_{0.5}$ of the color at pH

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7 and 8 were 1.2 and 1.6 hours, respectively. The stability of anthocyanin at the respective pH was also considerably low. The $t_{0.5}$ of the TMA at pH 7 and 8 were 9.4 and 2.4 hours, respectively. The short $t_{0.5}$ showed that **B** was unstable at pH 7-8 and underwent further degradation to form non-anthocyanin substances. Consequently, the *buni* extract was not a potential source of both color and anthocyanin at a neutral and basic condition.

CONCLUSIONS

The color intensity (CI) and total monomeric anthocyanin (TMA) extracted from *buni* fruit powder could be determined by a quadratic model or equation involving three factors (time, temperature, and pH of solvent) at a significant level of 0.05 (α = 0.05). Meanwhile, the total phenolic content (TP) and antioxidant activity were suited to be modelled by a linear equation.The anthocyanin of *buni* fruit was relatively sensitive to heat, while the phenolic compound exhibited relatively high resistance to heat. The optimum condition to extract the anthocyanin to yield the maximum CI, TMA, PC and AA from *buni* fruit was at 30 minutes extraction at 68^{o}C using a pH-1 solvent. The 95% confidence interval of CI, TMA, PC, and AA were 40.29 ± 0.91, 555.17 ± 13.34 mg/l, 1084.4 66.845 ± 66.85 mg GAE/l, and 45.00 ± 2.56 % inhibition, respectively.

The stability test showed that *buni* extract was not potent as a food colorant at pH 5 to 8. At pH 5 and 6, the *buni* extract was almost colorless, while 50% of the deep purple-blue color of the extract at pH 7 and 8 disappeared in 1.2 and 1.6 hours, respectively. The buni extract was also not potent as a source of anthocyanin at pH 7 to 8, due to the low stability. The half-life of the anthocyanin at respective pH was 9.4 and 2.4 hours. In contrast, the anthocyanin in *buni* extract was relatively stable at pH 5 and 6, with the half-life 28 and 17.6 days, respectively. Therefore, the *buni* extract might be considered as the source of anthocyanin for a functional beverage.

Investigation on stabilization technique to improve the color stability of *buni* fruit extract at food pH range advised for further study.

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