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Submission date: 21-May-2023 07:38PM (UTC+0700)
Submission ID: 2098284529
File name: Ultrasonication_Treatment_Ijaseit,_Q2_anggota.pdf (1.31M)
Word count: 6183
Character count: 32085

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Article *in* International Journal on Advanced Science Engineering and Information Technology · October 2021

DOI: 10.18517/ijaseit.11.5.11010

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International Journal on Advanced Science Engineering Information Technology

Vol.11 (2021) No. 5
ISSN: 2088-5334

Ultrasonication Treatment Improves Butterfly Pea Flower's (*Clitoria ternatea* L.) Anthocyanin Extraction as a Natural pH Indicator

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***Abstract*— Butterfly pea flower (*Clitoria ternatea* L.) is rich in anthocyanin, which can be utilized as a natural colorant. The color of anthocyanin changes based on pH values. Separating anthocyanin can be done by ultrasonication with ethanol solvents. This study aims to determine the effect of ultrasonication in butterfly pea flower extraction towards its yield, the value of pH extract, total anthocyanin, and color change sensitivity at different pH values. Butterfly pea extraction was performed with four different ultrasonication durations, i.e., 0 (without ultrasonication), 5, 10, and 15 minutes with five replications. The yield was evaluated using the gravimetry method. The value of pH extract was acquired using a pH meter. The total anthocyanin was determined by a differential pH method. Color change sensitivity was evaluated based on its red, green, and blue value (RGB). The study was conducted over 2 months. Analysis of variance (ANOVA) with a significance level of 5% and Duncan post-hoc analysis was used to evaluate the data. The results showed that the yield and total anthocyanin significantly increased as the ultrasonication duration increased and the color intensity became stronger. Whereas the value of pH extract slightly decreased with a longer ultrasonication duration. The best result was observed in 15 minutes of ultrasonication treatment with a yield of 71.03%, pH extract value of 6.69, and total anthocyanin of 16.99 mg/L. The extract also showed high color change sensitivity in different pH values, indicating its potential as a good pH indicator.**

***Keywords*— Anthocyanin; butterfly pea; *Clitoria ternatea* L.; pH indicator; ultrasonication.**

Manuscript received 03 Feb. 2020; revised 18 Jul. 2020; accepted 25 Feb. 2021. Date of publication 31 Oct. 2021.

I. INTRODUCTION

The butterfly pea flower (*Clitoria ternatea* L.) is a decorative plant belongs to the *Fabaceae* or *Leguminosae* family [1]. Butterfly pea flower (BPF) has been utilized in many food applications. For example, it has been used as blue natural colorant in sticky rice in Malaysia and consumed as a vegetable in India. Those facts prove that butterfly pea is safe to consume and can be utilized as natural colorants, due to its blue color pigments in BPF that come from anthocyanin [2].

Anthocyanin is a natural dye that acts as an antioxidant found in plants. Anthocyanin is stable at pH 3.5 and temperature 50°C, susceptible to light and degraded at temperatures above 70°C [3]. Anthocyanin has a base structure 2-phenyl-benzophyrylium from flavylium salts [4]. The color of anthocyanin changes based on different pH values. It can be red in an acidic solution and blue in an alkaline solution. Thus, anthocyanin can be utilized as a natural pH indicator. However, anthocyanin degradation could occur during extracting, processing, and storaging. Some factors that affect anthocyanin stability is structure change, pH, metal ions, oxygen, sugar, enzymes, and sulfur dioxide [5]. Volden *et al.* [6] revealed that high-temperature processing could decrease anthocyanin content in red cabbage to 59%, 41%, and 29%, respectively. While Brownmiller *et al.* [7] found that 6 months storaging could decrease total monomeric anthocyanin in blueberry purees to >50%. If the temperature rises too high, the glycosidic bond in anthocyanin will hydrolyze [8].

Due to its unstable nature, an effective extraction process is imperative to preserve anthocyanin content and quality. Common extraction techniques include maceration, percolation, and Soxhlet require a lot of time and a lot of solvents. Anthocyanin, which is sensitive towards high temperature, oxygen, and light, should ideally be extracted through a shorter and more efficient process. Ultrasonication is a method that can improve extraction efficiency. It utilizes ultrasonic waves with a frequency of >20 kHz that can propagate in a solid, liquid, and gas medium [9]. Ultrasonication does not affect the primary component from its sample; even ultrasonication can extract more components compared to the Soxhlet method [10].

Therefore, ultrasonication is a promising method to increase the anthocyanin extraction efficiency. Kusrini *et al.* [11] researched the activity tests of BPF extract as an anti-cataract agent, which was sonicated with one duration treatment, but no one has examined the improvement of BPF extract based on different ultrasonication durations. The current study employed ultrasonication treatment in BPF extraction and aimed to determine the effect of ultrasonication duration on its yield, pH extract value, total anthocyanin, and color change sensitivity.

II. Materials and Method

A. Materials

The fresh BPF (one-week-old) was obtained from Crispy Farm, Semarang, Central Java, which was harvested at 08.00–10.00 am, with ethanol 96% as the solvent. Other supporting materials were potassium chloride, natrium acetate, acetic acid, hydrogen chloride, natrium hydroxide, natrium dihydrogen phosphate for the making of the pH buffer, filter paper, and aluminum foil.

B. Equipment

The equipment used in this study were bath ultrasonicator (Branson, USA), vacuum rotary evaporator (Biobase, China), analytic scale (Shimadzu, Japan), pH meter (Ohaus, USA), and spectrophotometer UV-vis (Shimadzu, Japan).

C. Experimental Design

The study followed a completely randomized design (CRD) with four factors and five replications. Four different treatments were extraction with 0, 5, 10, and 15 minutes of ultrasonication with flow chart shown in Fig. 1. The obtained data were statistically analyzed using one-way ANOVA with a significance level of 5% and continued with Duncan post-hoc whenever it was significant. The data were expressed as mean.

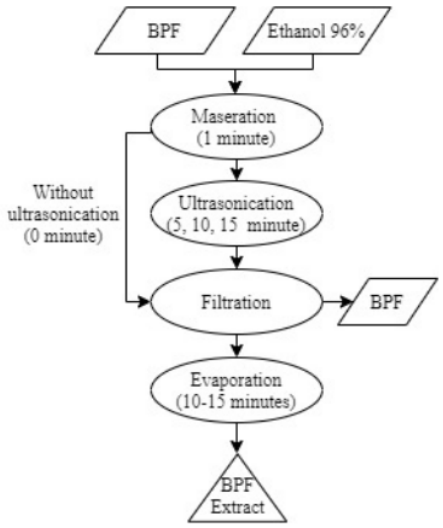

Fig. 1 Flow chart of BPF extract

D. Extraction of BPF

The fresh BPF was cut into small pieces and soaked in ethanol 96% in a ratio of 1:10 for 1 minute, according to Marpaung [12] and followed ultrasonication treatment at 40 kHz. The 0-minute treatment was carried out by macerating the BPF in ethanol for 1 minute. The extract was filtered using filter paper and evaporated at 40°C using a vacuum rotary evaporator until the volume was constant [13]. The concentrated extract was stored in a refrigerator at 10°C for further evaluation [14].

E. Yield Analysis

Yield is a ratio between the product weight to raw material weight. If the yield increases, the compound will be obtained [15]. Yield can be calculated with this formula:

$$Yield\ (\%) \frac{BPF\ extract\ weight\ (gr)}{BPF\ weight\ (gr)} \quad (1)$$

F. Value of pH Extract Analysis

The value of pH extract analysis was measured with a pH meter. The equipment was calibrated with pH 4.0, 7.0, and 10.0 buffer solutions before measurement [16].

G. Total Anthocyanin Determination

Total anthocyanin was determined with a pH differential method using pH 1.0 buffer and pH 4.5 buffer solutions [17]. Buffer solutions were prepared as follows:

- pH 1 (25 mL KCl 0,2 M and 67 mL HCl 0,2 M)
- pH 4.5 (3 gr $C_2H_3NaO_2$ diluted in aquadest until 100 mL, then its added CH_3COOH 0,1 M until value of pH reaches 4.5)

Each sample of 0.2 ml BPF extract was diluted in 1.3 ml buffer solutions and its absorbance was measured at wavelengths (λ) of 520 dan 700 nm. The difference in absorbance was calculated with this formula:

$$A = (A_{520} - A_{700})_{pH\ 1.0} - (A_{520} - A_{700})_{pH\ 4.5} \quad (2)$$

Total anthocyanin is defined as total monomeric anthocyanin, which is calculated with the below formula:

$$Total\ Anthocianin\ (mg/L) = \frac{AxMWxDFx1000}{\varepsilon x1} \quad (3)$$

MW= cyanidin-3-glucoside molecular weight (449.2 g/mol)
DF = dilution factor (7.5)
ε = molar absorptivity (26900)

H. Color Change Sensitivity

Color change in different pH values was observed by dropping BPF extract in pH 1.0, 3.0, 7.0, 9.0, 11.0, and 13.0 buffer solutions for further color analysis. Red, green, and blue values (RGB) were obtained using "*Color Grab*" application (ver. 3.6.1). It was determined by the descriptive method to get a black and white index (I_{bw}), which was calculated with this formula [18]:

$$I_{bw} = \frac{Red+Green+Blue}{3} \quad (4)$$

Each pH buffer was made with this method:

- pH 1.0 (25 mL KCl 0.2 M and 67 mL HCl 0.2 M)
- pH 3.0 (0.27 gr $C_2H_3NaO_2$ diluted in aquadest until 100 mL, then added with 15 mL CH_3COOH 0.1 M)

- pH 5.0 (3 gr C2H3NaO2 diluted in aquadest until 100 mL, then its added with 10 mL CH3COOH 0.1 M)
- pH 7.0 (49 mL NaH2PO4 0.1 M, then added with 40 mL HCl 0.1 M)
- pH 9.0 (84 mL NaH2PO4 0.1 M, then added with 0.5 mL HCl 0.1 M)
- pH 11.0 (84 mL NaH2PO4 0.1 M, then added with 7.2 mL NaOH 0.1 M)
- pH 13.0 (84 mL NaH2PO4 0.1 M, then added with 10 mL NaOH 0.1 M)

III. Results and Discussion

The description of yield, the value of pH extract, and total anthocyanin are displayed in Fig. 2, 3, and 4. Color change sensitivity is presented in Table 1. The table presents the data, such as captured images that represent color changes as they respond from BPF extract in different pH buffers, and present I_{bw} values for each treatment.

A. Yield

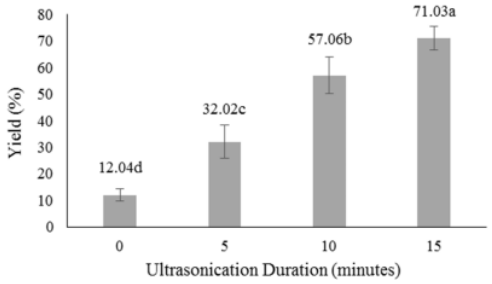

Fig. 2 The yield of BPF extract in various ultrasonication duration

Fig. 2 shows the ultrasonication duration increase caused higher extract yield. Based on the ANOVA one-way test, the significance value was 0.000, which indicates that the treatment has a significant effect on BPF yield. There was a big difference in the 4 treatments because we used maceration in 0-min treatment to compare with ultrasonication. Therefore, material in 0-min treatment was immersed in a solvent without homogenizing and heating [19]. The solvent possibly forms a layer around the cell that could inhibit the extraction. Ultrasonic waves could break this layer due to cavitation bubbles formed during extraction [20]. Besides the duration, the yield is affected by other factors such as material and solvent ratio, solvent types, and temperature. Mehmood *et al.* [20] performed their ultrasonication using dried BPF and aquabidest with a ratio of 1:15; the obtained yield is 29%. Therefore, fresh BPF and ethanol 96% with a ratio of 1:10 was proved effective; with 15 minutes, the treatment's yield reached 71.03%.

Based on Fig. 2, the yield continued to increase until the 15 min treatment, indicating the treatment still optimal for anthocyanin extraction and the solvent had not reached its saturation point. However, the yield increase is less as long as ultrasonication duration increases (5 min–0 min = 20; 10 min–5 min = 25; 15 min–10 min = 14). Thus, there is a possibility that if the ultrasonication duration increased to 20 min, it will be less effective because there will not be a further significant increase. Cheok *et al.* [24] proved that ultrasonication duration 20 min of anthocyanin extraction from mangosteen hull was not resulting in significant yield. Therefore, the ultrasonication of 15 min is the optimum duration.

Yield is the percentage of raw material used and represents the process efficiency [21]. Yield is calculated based on gravimetry, which isolates and weighs a certain compound [22]. The weighed extract was in a concentrated form. Thus, it was assumed as an isolated compound because the solvent had been evaporated. The evaporation process of BPF extract occurred in a vacuum to evaporate ethanol below its boiling point, so the evaporation process can run quickly with the lower temperature (40°C) and result in a higher yield. There are two main processes in extraction, namely: washing out and diffusion [23]. While cutting the BPF into small size, the cells will be broken, and the solvent will dissolve the compounds that come out during the process called washing out. After that, the extraction will enter the diffusion stage. The gradient concentration causes compounds in cells that have the same solubility (in this case, anthocyanins are polar, same as ethanol) to be dissolved by the solvent. The solvent will bring the compound out of the cell. The solvent will stop attracting the compound when it reaches its saturation point, and the gradient concentration will not occur again.

B. Value of pH Extract

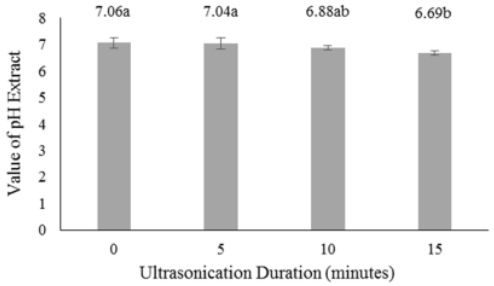

Fig. 3 The value of pH BPF extract in various ultrasonication duration

Value of pH or acidity degree is used to state the level of acidity or alkalinity of a substance. Normal value of pH is 7.0, value of pH > 7.0 is alkaline, whereas value of pH < 7.0 is acid [25]. The value of pH 0 indicates the highest acidity degree, while the value of pH 14 indicates the highest alkaline degree. The value of pH can be measured using a pH meter that works based on electrolyte or the conductivity principle of a solution. The graph in Fig. 3 shows the ultrasonication duration increase caused by the value of pH extract and decrease due to more anthocyanin extracted. This was caused by the lysis of the vacuole cell wall that releases the anthocyanin and decreases the value of pH [26]. Some types of anthocyanin are carried acid groups, such as ternatin, which could affect the value of pH extract. Rodrigues *et al.* [27] stated that some water-soluble phenolic compounds in plant cells, which are also soluble in ethanol, could decrease the value of pH because they are weak acids. One of the phenol compounds present in BPF is ellagic acid. Based on the ANOVA one-way test, the significance value is 0.006. Thus, the treatment has a significant effect on the value of pH BPF extract.

The decrease of pH extract was not affected by the solvent because ethanol is alkaline and contains hydroxide ions [28].

Ethanol plays a role as a quality determinant of the extract and has great power to dissolve compounds. Anthocyanin can be isolated by the extraction process, with polar solvents such as aquadest, methanol, and ethanol. However, Ramdan *et al.* [29] revealed that ethanol is better than aquadest. The solvent is selected by some factors, such as selectivity, solubility, density, and reactivity. The solvent should dissolve only targeted compounds. It has large power to dissolve compounds and has a large difference in density to ease the isolation of compounds. Resistance or barrier that inhibits dissolving compounds should be small, since it is affected by the amount and fineness of substance capillaries. Resistance can be done with size reduction to make the capillaries' path shorter [30]. The extracted compounds, including anthocyanin, caused a decrease in pH extract. The low pH extract is an advantage because it will stabilize the anthocyanin [8]. Li *et al.* [31] proved that the added acid compound to the solvent will result in lower pH value, such as tartaric acid and methanol. The presence of tartaric acid will stabilize the anthocyanin.

Moreover, the presence of acid compounds will extract more anthocyanin because of its ability to lysis more cell wall. For example, tartaric acid has a double role as an anthocyanin chelate agent and cell wall destroyer. However, chloride acid is only able to destroy the cell wall. Demirdoven *et al.* [32] performed their research about anthocyanin ultrasonication from red cabbage with ethanol and 1% formic acid. The pH extract that was obtained is 3.53 because they added acid to increase antimicrobe potential from anthocyanin. In this research, BPF extract will be used as a pH indicator. The value of the pH extract is expected to be low but tends to be neutral to keep the blue color and will not affect the color changes as can be seen in Table 1, hence this research did not add acid in ethanol.

TABLE I
COLOR CHANGE SENSITIVITY FROM BPF EXTRACT IN VARIOUS ULTRASONICATION DURATION

Ultrasonication Duration	pH Buffer							
	pH 1	pH 3	pH 5	pH 7	pH 9	pH 11	pH 13	
0 min								I_{bw} means
I_{bw}	199	203	180	193.67	203.33	210.33	222.67	201.71
5 min								I_{bw} means
I_{bw}	207.33	208.33	197.67	198	195	204.67	227	205.42
10 min								I_{bw} means
I_{bw}	211.33	213	190.33	187.33	198.67	205.67	220	203.76
15 min								I_{bw} means
I_{bw}	188.67	210	195.33	166.33	179.67	163	192.33	185.04

Note: I_{bw} = black and white index

C. Total Anthocyanin

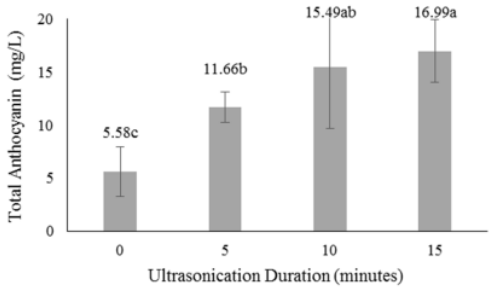

Fig. 4 Total anthocyanin of pH BPF extract in various ultrasonication duration

The graph in Fig. 4 shows that total anthocyanin will increase as the ultrasonication duration increases. The increase in total anthocyanin from 0 min to 5 min treatment was quite drastic because there was a difference in extraction methods. However, the increase in total anthocyanin from 10 min to 15 min treatment is not as high as the increase from 0 min to 10 min. It is indicated that the increase in ultrasonication duration will make the solvent more saturated, therefore the extraction ability will decrease [33]. Kusrini *et al.* [11] did their ultrasonication from dried BPF and aquadest with a ratio of 1:62 for 15 min, which resulted in a yield of 10.42 mg/L. Therefore, fresh BPF and ethanol 96% with ratio 1:10 is more effective, proved by the anthocyanin yield of T3 which reached 16.59 mg/L. An increase in total anthocyanin is linear with an increasing yield which can be seen in Fig. 2. Based on the ANOVA one-way test, the significance value is 0.001, thus the different ultrasonication duration has a significant effect on the total anthocyanin of BPF extract.

The result of the hydrolysis process in the esterification reaction of one anthocyanidin (aglycone) with one or more sugar groups (glycone) [34] is anthocyanin. Some types of anthocyanin contained in BPF are ternatin, delphinidin and cyanidin-3-sophorosida. Ternatin is a polyacilation group of delphinidin derivatives that consist of delphinidin 3, 3', 5'-triglucosides, bound to malonic acid, glucose, and p-coumaric acid [35]. Santoso and Estiasih [36] stated that types of anthocyanin could change if there is an addition of hydroxyl groups, formation of glycoside, or formation of methylation. The addition of hydroxyl groups will shift blue color (cyanidin to delphinidin), whereas glycoside formation and

methylation formation will shift red color (cyanidin to peonidin). Anthocyanin consists of 3 carbon atoms that bond with 1 oxygen atom to connect 2 benzene aromatic rings. All anthocyanins have differences based on R3' and R5' bonds with aromatic rings. The anthocyanin structure base can be seen in Fig. 5.

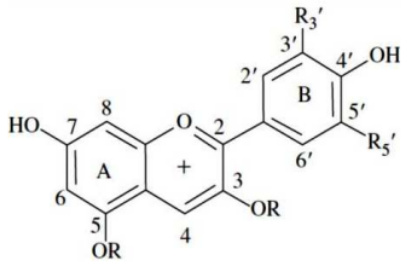

Fig. 5 R3' and R5' groups as determinants of anthocyanin types [37]

From 20 types of anthocyanin in total, each has 15 carbon atoms (C15) outside of the substitution group. R3' and R5' are substitution groups that were formed from cyanidin pigments. Those groups created by addition or reduction of hydroxyl groups, hydroxyl group position; methylation of hydroxyl groups, the number and location of sugars which are attached to the molecule; and the presence of aliphatic acids (malonic acid, acetic, malic, succinic and oxalic), or aromatic acids (p-kumaric, caffeic, ferulic, sinapic and gallic acid) in the sugar. This affects the color that will be expressed by anthocyanin and also affects its stability [38]. Anthocyanin can specifically absorb light in the absorption area of ultraviolet to violet and is stronger in the visible area of the spectrum. Anthocyanin is absorbed in a wavelength of 250–700 nm, with 2 peaks as the sugar group at a wavelength of around 278 nm, and the main peak as anthocyanin around a wavelength of 500 nm [39].

Total anthocyanin in BPF extract was considered total cyanidin, since cyanidin is the dominant anthocyanin type in plants and is usually used as the reference in total anthocyanin calculation [40]. Different types of anthocyanin will result in different color spectrums. For example, pelargonidin has reddish-orange color, while cyanidin has a red color and delphinidin has blue color [41]. The total anthocyanin calculation is based on the absorbance of the anthocyanin in the visible area. According to Tiwari *et al.* [42], conjugated double bonds in the anthocyanin groups allow absorbing light in the visible light region, thus enabling the analysis of these pigments spectroscopically. The longer the conjugated double bonds in the anthocyanin structure, the stronger the colors produced in plants. If this double bond is degraded, the color intensity will decrease. The principle of total anthocyanin determination with pH differential is dissolving the material in a buffer of pH 1 and pH 4.5 to form oxonium (orange to purple) at pH 1 and form hemiketal (colorless) at pH 4.5. Before starting to dilute, the appropriate dilution factor for the sample is determined by ensuring the absorbance value is less than 0.8 at a wavelength of 520 nm. A wavelength of 520 nm is the maximum wavelength for cyanidin-3-glucoside while a wavelength of 700 nm is used to correct deposits that still present in the sample.

D. Color Change Sensitivity

Anthocyanin is a pigment that is able to change its color in different values of pH. This ability will represent anthocyanin sensitivity to different values of pH [43]. The color changing occurs because anthocyanin is present in four forms of equilibrium depending on the pH conditions. The four forms are cavity flavylium, carbinol pseudo-base, quinoidal base, and chalcone. Abdullah et al. [8] proved that the color of BPF extract changed to reddish-orange, red, purple, blue, green, and brownish yellow at the pH range of 0.04–12.00. Those colors can be measured with various methods, one of them is RGB, which based on the calculation includes three primary colors, such as red, green, and blue. Those colors can mix to form other colors. If the highest intensity of each color is mixed, white color will be obtained. Meanwhile, the lower intensity of each color mixed will produce a black color. Measurement of the color can be affected by four factors, (1) light as a source of lighting; (2) the absorption and reflection spectrum of the illuminated product; (3) environmental conditions; and (4) the condition of the subject who sees the object [44]. Color has wavelength and light intensity parameters. If an object is illuminated, the incident ray will be absorbed (absorption), passed on (transmission), reflected (reflection), or emitted (emission).

Based on the data in Table 1, various pH buffers will result in the same color in each duration. The pH 1 buffer gave a reddish color. This happened since anthocyanin formed the flavilium cation, which is the most stable form [45]. The pH 3 buffer gave a purple color, then turned bluish-purple at pH 5 buffer due to the OH^- substitution, which changes the anthocyanin structure to carbinol pseudo base. More substitution of OH^- will cause the color to become blue, while methylation causes the color to become red. The purple color appeared as an intermediate color from red to blue. The pH 7 buffer did not change the color of the extract. The dominant type of anthocyanin caused the blue color of the extract in the BPF, delphinidin [35]. The pH 9 buffer changed the color of the extract to a greenish-blue because anthocyanin formed the quinoidal base. The extract is greener, and the blue color begins to disappear as its buffer pH value increases to pH 11 because the quinoidal base degraded to a colorless chalcone. The pH 13 buffer changes the color of the extract to yellow because all of the anthocyanin is not stable at high alkaline; therefore, it has been degraded and the color disappears [46]. The green color that appeared in pH 9 until pH 11 buffer is chlorophyll pigment. Rudra *et al.* [47] stated that chlorophyll is stable in an alkaline solution and easily degradable in an acidic solution. Otherwise, anthocyanin is stable in acid. Chlorophyll will produce chlorophyllide that bonds with magnesium ions in alkaline solution, but it will change to pheophorbide in acid solution, causes degradation in chlorophyll structure. Fasakin *et al.* [48] also stated that chlorophyll is easily dissolvable both in polar solvents like ethanol, methanol, acetone and nonpolar solvents like ether and chloroform.

Table 1 also shows that the color intensity is different even though the color of each treatment is the same. The average of I_{bw} value was decreased as ultrasonication duration increased. It proved that the longer the ultrasonication duration will result in a more concentrated extract and higher anthocyanin yield. Red value, green value, blue value (RGB)

can be averaged to determine I_{bw} value, which states black and white intensity, a decrease in I_{bw} value indicates that the object is getting darker and getting concentrated [49]. Meanwhile, an increase in I_{bw} value shows the opposite because the greater the I_{bw} value leads to the white color spectrum.

IV. Conclusion

The different ultrasonication duration in the extraction of BPF significantly affects the yield, value of pH extract, and total anthocyanin. The color becomes more concentrated as the ultrasonication duration increases. Thus, the color changes at various pH values are more pronounced. The best result was obtained in 15 minutes of ultrasonication with a yield of 71.03%, pH extract value of 6.69, and total anthocyanin of 16.99 mg/L. It also has high color change sensitivity in different pH values, hence indicating its potential to be used as a natural pH indicator.

Acknowledgment

The authors are grateful to the Diponegoro University for funding this research.

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