The Analysis of Monomeric Anthocyanin by pH Differential Method Is Not Appropriate for Certain Anthocyanins

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Keywords: Anthocyanins, Color Intensity, Flavylium Cation, Hemiketal, pH Differential.

Abstract: The light absorbance at pH 1 and 4 of 22 anthocyanin-source plant extracts was studied. Each one gram of fresh sample macerated in 4 ml 0.1 N HCl-Ethanol 96% (1:9) for an hour, then diluted in buffer solution pH 1 and 4 with various dilution factor. The extract spectrophotometrically scanned at visible region (400 – 700 nm), then the λmax, color intensity, browning index (BI), and violet index (VI) determined. The λmax of extracts were widely vary from 508 nm to 548 nm. Based on the BI the relatively high color quality at pH 4 exhibited by *Clitoria ternatea* (CT), *Dendrobium sonia* (DS), *Ipomoea tricolor* (IT), *Dianella ensifolia* (DE) and *Melastoma malabathricum* (MM) extract. Based on the VI, CT and DE exhibited bluish-purple color at pH 4, while DS was redish-purple, IT was purplish-red, and MM was red. Based on the light absorbance, the extracts might be classified into three types. The A-type exhibited very low intensity of flavylium cation (AH^+) species at pH 4 because of the hydration to colorless hemiketal. The B-type had relatively high intensity of AH^+. The C-type showed the existence of purple and blue quinonoidal base species. The measurement of monomeric anthocyanin by pH differential method is based on assumption that the anthocyanin colorless at pH 4.5. Therefore, the method was not suitable for the B- and C-type anthocyanin-source plant extract.

1 INTRODUCTION

Anthocyanins are the largest water-soluble pigment that produce various color like red, purple, and blue. There are more than 900 types of anthocyanin found in plants (Yoshida, et al., 2009). They also provide beneficial health effects to the human body as an antioxidant (Gradinaru, et al., 2003; Patras, et al., 2010), antidiabetic (Belwal, et al., 2017), anticancer (Patras, et al., 2010) and anti-inflammatory (Lee, et al., 2017).

The anthocyanin content in a plant extract is commonly determined spectrophotometrically as monomeric anthocyanin by the pH-differential method (Lee, et al., 2005). The analysis based on the characteristics of monomeric anthocyanin that may appear as six different species depend on the pH. At pH 1, anthocyanin exists as the red flavylium cation species (**AH^+**). Meanwhile, at pH 4.5 the pigment exists as the colorless hemiketal (**B**). Hence, the difference in light absorbance represent the concentration of the pigment. The polymeric form – the product of anthocyanin degradation – is resistant to color change with change of pH and appear as red both in pH 1 and 4.5 and is not measured by the pH-differential method.

As the part of our research to find the potential source of anthocyanins for natural food colorant, we evaluated the light absorbance of various plant extract at pH 1 and 4. We found that several anthocyanins show relatively high color intensity that make them not suitable to be analyzed by the pH differential method.

2 MATERIALS AND METHODS

2.1 Materials

Twenty-two samples of anthocyanin-source flowers and fruits collected from various location in Indonesia. Seventeen flowers included in this research were *Agapanthes umbellatus*, *Antirrhinum majus*, *Bauhinia purpurea*, *Clitoria ternatea*, *Chrysanthemum x morifolium*, *Dendrobium sonia*, *Dianthus caryophyllus*, *Gladiolus hortunalus*, *Hydrangea macrophilla*, *Ipomoea tricolor*, *Kalanchoe blossfeldiana*, *Petunia integrifolia*,

Marpaung, A. and Tjahjadi, K.
The Analysis of Monomeric Anthocyanin by pH Differential Method is not Appropriate for Certain Anthocyanins.
DOI: 10.5220/0009985400002964
In *Proceedings of the 16th ASEAN Food Conference (16th AFC 2019) - Outlook and Opportunities of Food Technology and Culinary for Tourism Industry*, pages 26-30
ISBN: 978-989-758-467-1

Ruellia tuberosa, *Sinningia speciose*, *Stachytarpheta jamaicensis*, *Tibouchina semidecandra*, and *Torenia fournieri*. Five fruits included were *Clidemia hirta*, *Dianella ensifolia*, *Melastoma malabathricum*, *Rhodomyrtus tomentosa*, and *Vitex pinata*.

The hydrochloric acid, ethanol 96%, potassium chloride, buffer solution pH 4 (citric acid-sodium hydroxide-hydrogen chloride) were obtained from Merck®. All reagents were analytical grade and used without further purification. The buffer pH 1 made from hydrochloric acid and potassium chloride.

2.2 Maceration and Extract Preparation

One gram of fresh sample macerated in 4 ml 0.1 N HCl-Ethanol 96% (1:9) for an hour. The suspension was filtered through filter paper Whatman 40 (8 μM). The extract was diluted with buffer solution pH 1 and 4 with various dilution factor (DF) (two to twenty) depend on the initial intensity of the extract.

2.3 Light Absorbance

The light absorbance at visible region (400 nm - 700 nm) of all extracts was scanned by UV-Vis spectrophotometer (T60 visible PG Instrument) to determine the wavelength with maximum absorbance (λ_{max}), relative color intensity (RCI), browning index (BI) and violet index (VI).

The CI was ($A_{\lambda max} - A_{700}$) x DF (Cisse, et al., 2012). The RCI was the CI at pH 4 divided by the CI at pH 1. The BI determined by (A_{420}-A_{700})/ ($A_{\lambda max}$ - A_{700}) (Cisse, et al., 2012). The VI determined by (A_{580}-A_{700})/(A_{520}-A_{700}) (Cisse, et al., 2012). $A_{\lambda max}$ was the absorbance at wavelength with maximum absorbance, A_{420} was absorbance at wavelength 420 nm, A_{580} was the absorbance at 580 nm, A_{520} was the absorbance at 520 nm, A_{700} was the absorbance at 700 nm for haze correction.

3 RESULTS AND DISCUSSION

3.1 λ_{max}, Browning Index, and Violet Index

All anthocyanin-source extracts showed red color at pH 1 that represent the presence of flavylium cation ($\mathbf{AH}^+$) species. The λ_{max} varied from 508 nm to 548 nm. The shortest λ_{max} exhibited by the *Chrysanthemum x morifolium* (CM) extract, while the longest λ_{max} belonged to the *Clitoria ternatea* (CT) extract (Table 1). The variation was affected by the type of anthocyanin aglycon (Bueno, et al., 2012), the number of glycosyl group, acylation and copigmentation (Gauche, et al., 2010) and the presence of metal complexation (Yoshida, et al., 2009). The presence of acyl group tends to increase the λ_{max}. The CT extract, for instance, contains 9 types of anthocyanin that have two to four acyl groups (Kazuma, et al., 2003). The TS extract contains anthocyanin with one acyl group (Lowry, 1976). Meanwhile, the main anthocyanins in MM extract have no acyl group (Aishah, et al., 2013). The λ_{max} of the extracts were 548, 534 and 514 nm, respectively.

Figure 1 depicted the chemical structure change of simple anthocyanin as the change of pH (Trouillas, et al., 2016). The red $\mathbf{AH}^+$ in most anthocyanin-source extract thermodynamically hydrated to colorless B as the pH of solution increase to 4 to 5. As the result, the color intensity of the extract is dramatically decrease and the λ_{max} disappear. Eight of twenty-two extracts studied exhibited no λmax at pH 4 (Table 1). Six extracts had λ_{max} that similar with the λ_{max} at pH 1. The hypsochromic shift (the shift of λ_{max} toward a shorter wavelength) occurred in two extracts. Meanwhile, the bathochromic shift (the shift of λ_{max} toward a longer wavelength) appeared in six extracts. The wide bathochromic shift occurred in CT, DS, and DE extracts: 24, 17, and 36 nm, respectively. The wide shift indicated the kinetic deprotonation of $\mathbf{AH}^+$ to form purple quinonidal base **A** (Trouillas, et al., 2016).

Browning index (BI) is a common parameter to measure the color quality of an anthocyanin source extract (Cisse, et al., 2012). The increase of browning index indicates the decrease of desirable color (red, purple or blue) and or the increase of undesirable pale yellow color (A420) that contributed by the chalcone species (Reyes & Cisneros-Zevallos, 2007). The relatively small BI (< 0.5) exhibited by CT, DS, IT, DE, and MM extracts. The smallest BI belonged to CT extract.

The other common parameter to determine the color quality of anthocyanin is violet index (VI) that measure the ratio of intensity of purple color (represented by the absorbance at 580 nm) to the intensity of red color (represented by the absorbance at 520 nm). Twenty extracts exhibited red color at pH 4, that represented by the relatively low VI (< 1). The CT, DS, and DE had VI >1 and exhibited purple to purple blue color.

Table 1: The λ_{max} of all anthocyanin-source extract studied at pH 1 and 4 and their color quality at pH 4.

Plant	Code	λ_{max} (nm)		Color quality at pH 4		
		pH 1	pH 4	RCI	BI	VI
Flower						
Agapanthes umbellatus	*AU*	536	536	**0.96**	0.91	0.56
Antirrhinum majus	*AM*	530	537	**0.85**	0.63	0.51
Bauhinia purpurea	*BP*	522	522	0.22	1.19	0.59
Clitoria ternatea	*CT*	548	572	**1.28**	0.16	1.94
Chrysanthemum x morifolium	*CM*	508	-	0.41	1.76	0.48
Dendrobium sonia	*DS*	526	543	**0.89**	0.42	1.12
Dianthus caryophyllus	*DC*	525	-	0.18	1.67	0.38
Gladiolus hortunalus	*GH*	520	-	0.07	2.79	0.48
Hydrangea macrophilla	*HM*	525	525	0.15	1.55	0.47
Ipomoea tricolor	*IT*	536	539	**0.71**	0.28	0.49
Kalanchoe blossfeldiana	*KB*	520	-	0.50	2.36	0.42
Petunia integrifolia	*PI*	531	-	0.43	1.77	0.58
Ruellia tuberosa	*RT1*	526	-	0.11	2.03	0.60
Sinningia speciose	*SS*	520	-	0.07	2.54	0.42
Stachytarpheta jamaicensis	*SJ*	527	521	0.15	1.62	0.60
Tibouchina semidecandra	*TS*	534	-	0.15	1.94	0.65
Torenia fournieri	*TF*	532	525	0.30	1.24	0.50
Fruit						
Clidemia hirta	*CH*	521	526	0.14	0.67	0.35
Dianella ensifolia	*DE*	532	568	**1.17**	0.29	1.59
Melastoma malabathricum	*MM*	514	513	0.29	0.48	0.19
Rhodomyrtus tomentosa	*RT2*	511	511	0.27	0.61	0.16
Vitex pinata	*VP*	516	517	0.35	0.79	0.41

3.2 Classification of Anthocyanin-source Extract based on the Light Absorption

The light absorption of the anthocyanin-source extracts studied might classified into three types as shown in Figure 2. The A-type represented the most common anthocyanin that show very low color intensity at pH 4 as the result of the conversion of red $\mathbf{AH}^+$ to **B** (Lee, et al., 2005). The pale red color in the extract at pH 4 was the polymeric form of anthocyanin that is resistant to color change because of the pH change (Lee, et al., 2005).

At the B-type, the CI of the extracts at pH 4 was slightly lower than the CI at pH 1. There were three extracts include in this group: AU, AM, IT. The retaining color of the three extracts at pH 4 were 96%, 85%, and 71%, respectively. Probably, the relatively high intensity indicated that the hydration of $\mathbf{AH}^+$ to **B** was blocked because of the presence of intramolecular copigmentation. The occurrence of intramolecular copigmentation involving three acyl groups and the anthocyanin chromophore in heavenly blue anthocyanin of IT extract was already determined (Yoshida, et al., 2009).

An interesting characteristic shown by the C-type extracts (CT, DS, and DE). At pH 4, all extracts exhibit two λ_{peak} and one $\lambda_{shoulder}$ that represent all the colored species of anthocyanin: red $\mathbf{AH}^+$, purple **A**, and blue $\mathbf{A}^-$. This unique light absorption profile was reported as the unique characteristic of anthocyanin that has acyl group located at the ring B (Baublis, et al., 1994). The presence of acyl group at the ring B of anthocyanin was identified in CT and DE extract (Yoshida, et al., 2009; Kazuma, et al., 2003).

The CI of CT and DE extract at pH 4 was higher than at pH 1, while the relative CI of DS extract was 0.89. The higher intensity was possible because the purple and blue species absorb light more intense than

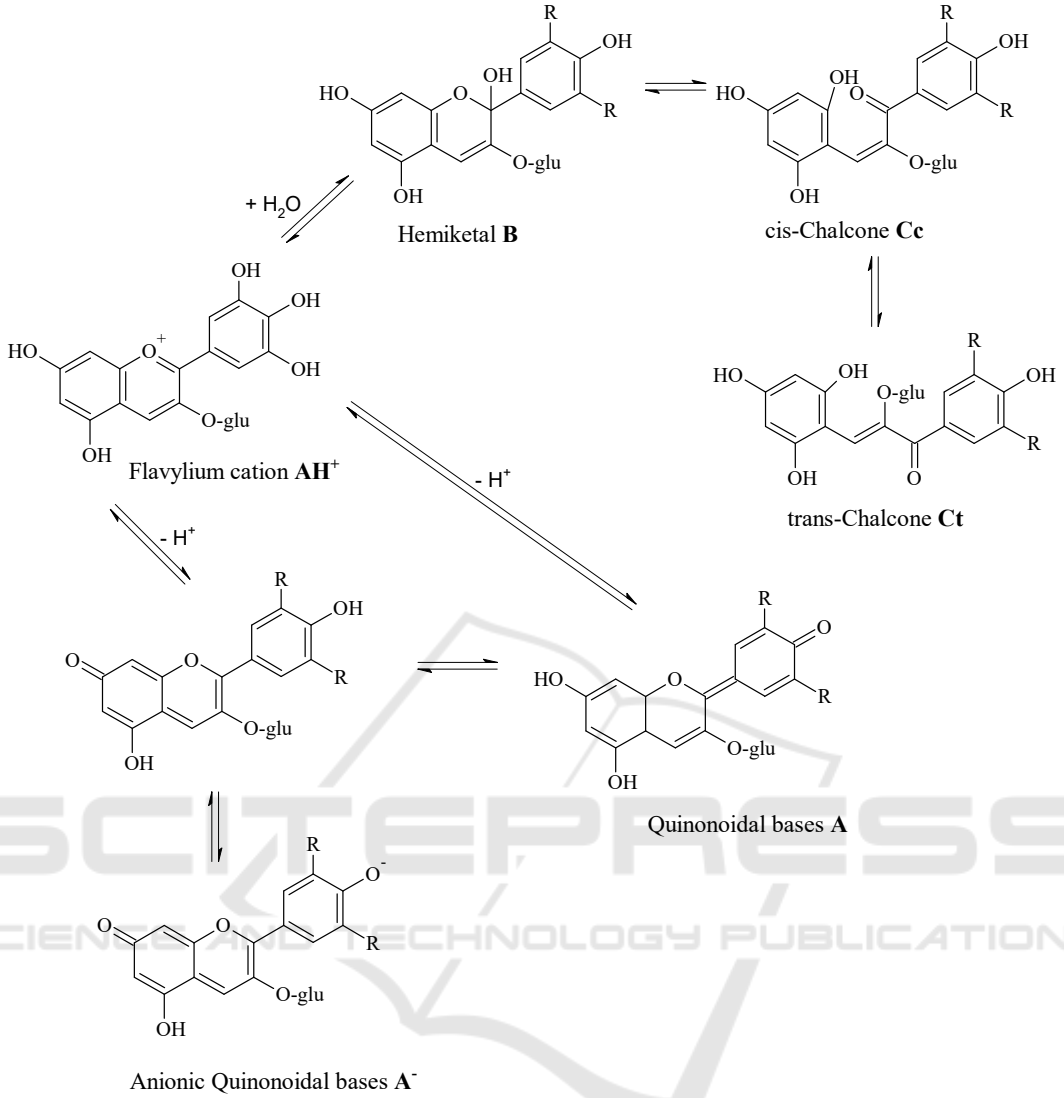

Figure 1: Chemical structures of six simple anthocyanins in aqueous solution (Trouillas, et al., 2016).

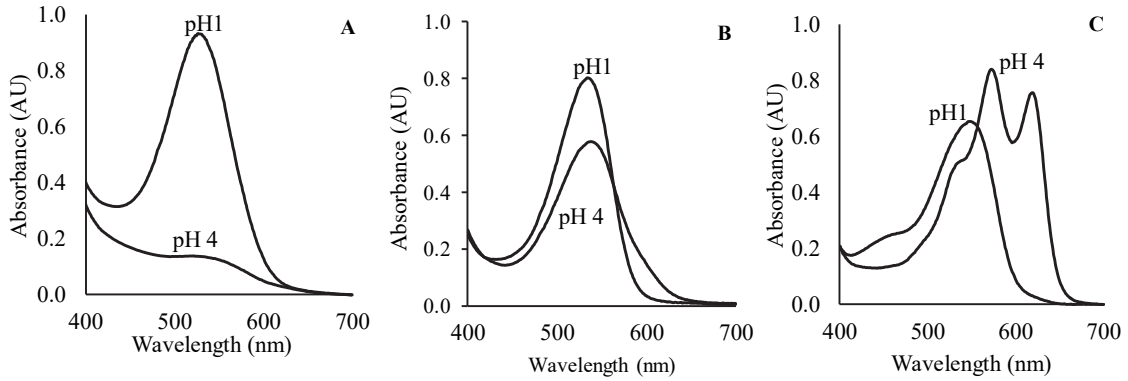

Figure. 2: Three different light absorption profile of 22 anthocyanin-source extract studied at pH 1 and 4. The A-type, consisted of 16 sources, exhibit very low intensity at pH 4. The B-type, consisted of three sources, exhibit a slight lower intensity at pH 4. The C-type, consisted of three sources, showed two λ_{peak} and one $\lambda_{shoulder}$. The color intensity at pH 4 was higher than at pH 1.

the red species (Yoshida, et al., 2009). 4, that represented by the relatively low VI (< 1). The CT, DS, and DE had VI >1 and exhibited purple to purple blue color

3.3 Determination of Monomeric Anthocyanin

The determination of monomeric anthocyanin by pH differential method is a rapid method that widely accepted to determine the anthocyanin content in a plant extract or juice (Lee, et al., 2005). In the method, an assumption made that the monomeric anthocyanins exhibit little or no light absorbance at pH 4.5. Meanwhile, the polymeric anthocyanins will absorb at the pH.

As demonstrated in Figure 2, the A-type of anthocyanin-source extracts we studied fit the assumption. Hence, the monomeric anthocyanin might appropriately be determined. However, the B-type and C-type exhibit relatively high light absorption at pH 4 that probably because they contain polyacylated anthocyanins. Consequently, the use of pH differential method to determine the monomeric anthocyanin content in B-type and C-type anthocyanin-source extract was not suitable.

4 CONCLUSIONS

The twenty-two anthocyanin-source plant extract exhibit different light absorption at pH 4 that can be classified into three types. The A-type exhibited very low light absorption of flavylium cation ($\mathbf{AH}^+$) species, the B-type showed relatively high intensity of $\mathbf{AH}^+$, while in the C-type the significant amount of purple quinonoidal base (**A**) and blue anionic quinonoidal base ($\mathbf{A}^-$) observed. Therefore, the use of pH differential method to determine the monomeric anthocyanin content was not appropriate to be applied to B- and C-type of anthocyanin source extract.

The spectrophotometric scan at visible light region, both at pH 1 and 4, of an unidentified anthocyanin-source plant extract is suggested before the examination of pH differential method.

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