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Research Paper

Spectrophotometric Change of Butterfly Pea (*Clitoria ternatea L.*) Flower Extract in Various Metal Ion Solutions During Storage

Abdullah Muzi Marpaung[1*], Dania Pustikarini[1]

[1]*Food Technology Department, Swiss German University, The Prominence Tower Jl. Jalur Sutera Barat. No. 15, RT.003/RW.006, Panunggangan Tim., Kec. Pinang, Tangerang, 15143, Indonesia*

*__Corresponding author__: abdullah.muzi@sgu.ac.id

Abstract

This study aimed to investigate the effect of six chloride salts on butterfly pea flower extract's anthocyanins stability. The salts were NaCl, KCl, $CaCl_2$, $MgCl_2$, $FeCl_3$, and $AlCl_3$. The samples were analyzed using a UV-Vis spectrophotometer to observe color degradation and change in hue during storage. The extraction of anthocyanins was done using a modified method, and the solutions were stored in dark vials at room temperature. The degradation kinetics of benzene derivatives, acyl groups, non-anthocyanin flavonoid, flavylium cation, quinonoidal base and anionic quinonoidal base were evaluated using the first-order reaction, and the half-life was calculated. The effect of metal ions was studied by analyzing the change in absorbance of each band using regression analysis and a slope test. The results showed that monovalent (Na^+ and K^+) and divalent (Ca^{2+} and Mg^{2+}) ions did not result in a significant shift in the spectrogram. Trivalent metal ions (Al^{3+} and Fe^{3+}) had limited interaction with the anthocyanins, heightened the brown color, and decreased the overall color quality. K^+, Ca^{2+}, Mg^{2+}, Al^{3+}, and Fe^{3+} ions showed the ability to improve the stability of the extract's color, while Na^+ tended to accelerate color degradation. The pattern of changes in the spectrogram during storage suggests that color degradation occurs in two ways: the unfolding of hydrophobic interactions and the deacylation of anthocyanin. Trivalent metal ions showed the best stability performance, with Fe^{3+} preventing the unfolding of hydrophobic interactions and Al^{3+} hindering the deacylation. The combination of the two is highly likely to improve the color stability of the butterfly pea flower extract. However, both increase the browning index, thus decreasing color quality. This research highlights the potential of adding cations to improve the color stability of the butterfly pea flower extract, making it a more attractive food coloring agent.

Keywords

Anthocyanin, Butterfly Pea Flower, Color Degradation, Metal Ions, Stability, UV-Vis Spectrophotometer

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1. INTRODUCTION

The butterfly pea (*Clitoria ternatea L.*) flower extract is a rich source of anthocyanins that are well-known for their deep purple-blue color and their stability in low acidic solutions (Marpaung et al., 2017). This relatively high stability is due to the presence of polyacylated anthocyanins in the butterfly pea flower extract, which has intramolecular copigmentation that helps to prevent the loss of color.

The color of butterfly pea flowers can be used as food coloring, but further research is required to ensure the color remains stable. Factors such as pH, chemical structure, light, heat, and metal ions can impact the stability of the color in butterfly pea flowers. Previous studies have investigated the effect of metal ions on the stability of anthocyanins from different sources, with varying results. Some studies have found that cations can improve anthocyanin stability, while others have found that they can provoke instability. For example, Na^+ has been found to decrease (de Rosso and Mercadante, 2007; Hubbermann et al., 2006), improve (Dangles and Brouillard, 1992; Figueiredo and Pina, 1994; Peng et al., 2016), or have no effect on anthocyanin stability (Wang et al., 2010). K^+ increases the color intensity and improves stability (Czibulya et al., 2015), while others have found that Ca^{2+} enhances color Li (2014) without affecting stability (Ren et al., 2014), but in some cases, it decreases stability (Ratanapoompinyo et al., 2017). Studies have observed no impact on anthocyanin color from Mg^{2+} (Sigurdson et al., 2016). Fe^{2+} and Fe^{3+} enhance the color and stabilize the configuration of anthocyanins but also destroy anthocyanins (Li, 2014; Peng et al., 2016; Ratanapoompinyo et al., 2017). Al^{3+} has a positive effect on the stability of anthocyanins (Maylinda et al., 2019; Peng et al., 2016), while others have found no effect (Li, 2014).

To date, there has been no study on the effect of metal ions

on the stability of butterfly pea anthocyanins. Therefore, the aim of this study is to investigate the impact of six different chloride salts (NaCl, KCl, $CaCl_2$, $MgCl_2$, $FeCl_3$, and $AlCl_3$) on the stability of butterfly pea flower extract anthocyanins. The UV-Vis spectrophotometer would be employed to monitor the color degradation and change in hue of the extract during storage over a wavelength range of 250-700 nm.

2. EXPERIMENTAL SECTION

2.1 Materials

The study utilized fully opened flowers of butterfly pea (*Clitoria ternatea L.*) sourced from a private plantation in Tangerang, Banten, Indonesia. The petals were carefully separated from the sepals and steam-blanched for 6 minutes (Marpaung et al., 2012). Various chemical reagents were utilized in the research, including Aluminum Chloride (AnalaR BDH, England), Calcium Chloride (Brataco, Indonesia), (Brataco, Indonesia), Iron (III) Chloride (Eterna, Indonesia), Magnesium Chloride (Brataco, Indonesia), Potassium Chloride (Brataco, Indonesia), and Sodium Chloride (AnalaR BDH, England).

2.2 Extraction and Addition of Metal Ion

The extraction of anthocyanins from butterfly pea (*Clitoria ternatea L.*) flowers was conducted using a modified version of the previous research (Marpaung et al., 2017). The extraction process involved mixing 20 grams of the fresh petals with 80 milliliters of deionized water at 60°C for 30 minutes in the absence of light and under constant agitation. The resulting suspension was then filtered (Filter paper (Whatman 41)), with the filtrate collected and centrifuged at 7,000 revolutions per minute for 5 minutes to separate the pigments.

The chloride salts used in this study were NaCl, KCl, $CaCl_2$, $MgCl_2$, $FeCl_3$, and $AlCl_3$, and each had a concentration of 0.025 M. 0.6 mL of the salt solution was added to 1 mL of butterfly pea flower extract, while 0.6 mL of deionized water was added to the control solution. The sample of each extract (2 mL) was placed in a quartz cuvette and then scanned using a UV-Vis spectrophotometer (Genesys 10uv Thermo Electron Corporation, U.S.A.) at a wavelength range of 250 - 700 nm with a 1 nm interval.

2.3 Stability Test

Each of the 20 mL sample was repeated twice and placed in a dark vial, then stored in a room that was kept away from light at room temperature. The spectrogram of each sample was scanned with the spectrophotometer at an interval of every two days. The changes in each band in the spectrogram were then analyzed.

2.4 Kinetic Formulation and Statistical Analysis

The degradation kinetics of all the bands were evaluated by the first-order reaction.

$$A = A_o.e^{-kt} \quad (1)$$

$$t_{0.5} = ln(2)/k \quad (2)$$

A is the final absorbance, A_o is the initial absorbance, k is the constant of degradation rate (per day), t is the storage time (in days), and $t_{0.5}$ is the half-life (in days).The trend of the change in absorbance of each band was analyzed using regression analysis, with a significance level of $\alpha = 0.05$. The slope difference between two samples was assessed using a slope test. Both statistical evaluations were conducted using Microsoft Excel® as part of Microsoft 365, which was developed by Microsoft in Redmond, Washington, USA, with a significance level of $\alpha = 0.05$.

3. RESULTS AND DISCUSSION

3.1 The Effect of the Metal Ions on the Spectrogram

Six bands appeared in the spectrogram of the aqueous extract of the butterfly pea flower (Figure 1a). The UV region featured three bands, representing benzene derivatives (at ± 265 nm), acyl groups (at ± 310 nm), and non-anthocyanin flavonoids (at ± 350 nm). The visible light region featured three bands of the colored species of anthocyanins, at ± 550 nm representing the red flavylium cation AH^+, at ± 574 nm, representing the purple quinonoidal base A, and at ± 617 nm representing the blue anionic quinonoidal base A^- (Marpaung et al., 2019).

The addition of monovalent (Na^+ and K^+) and divalent (Ca^{2+} and Mg^{2+}) did not give a significant shift in the spectrogram of the extract (Figure 2b). The absence of the shift caused by the addition of monovalent and divalent metal ions was also observed in various anthocyanin source extracts, such as blueberries (Wang et al., 2010), red cabbage (Guo et al., 2017), black peanuts (Shao et al., 2015), *Bunius antidesma* (Mustika and Marpaung, 2020), cyanidin-derived anthocyanin (Sigurdson et al., 2017; Sigurdson et al., 2016), *Ribes nigrum* (Buchweitz et al., 2013), dark maize (Mei et al., 2014), and fungus *Pycnoporus sanguineus* (Zhang et al., 2019). In contrast, sodium (Na^+) enhances the color of malvidin-derived anthocyanin (Dangles and Brouillard, 1992; Figueiredo and Pina, 1994), potassium (K^+) increases the intensity of anthocyanin in red wine (Czibulya et al., 2015), and calcium (Ca^{2+}) has a color-enhancing effect on blueberry anthocyanin (Li, 2014) and a slight impact on the color of red cabbage anthocyanin (Ratanapoompinyo et al., 2017).

There was also limited evidence of interaction between trivalent metal ions (Al^{3+} and Fe^{3+}) and the anthocyanins in the butterfly pea flower extract (Figure 1c). The spectrogram of the butterfly pea flower extract added with Al^{3+} showed only a minor increase in absorbance at ± 550 nm and a slight decrease at ± 617 nm, indicating a possible contribution of Al^{3+} to the protonation reaction $A^- \rightleftharpoons A \rightleftharpoons AH^+$. A similar outcome was observed in extracts containing Fe^{3+} ions. Although there was a noticeable shift towards red in hue, it appears to be the same effect exhibited by Al^{3+}, where Fe^{3+} drove the protonation. Fe^{3+} had a more intense protonation effect than Al^{3+}.

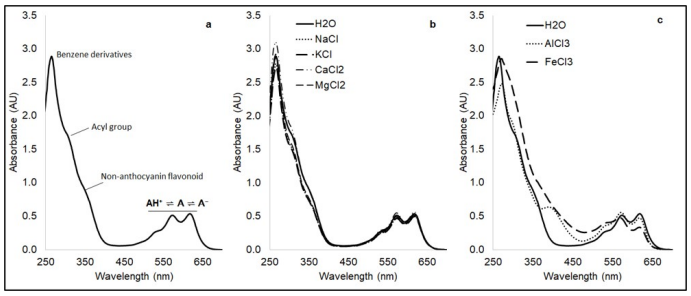

Figure 1. UV-Vis Light Spectra of Butterfly Pea Flower Aqueous Extract (a), In Divalent Metal Ion (b), and Trivalent Metal Ion Solution (c)

Aside from intensifying the red color, Al^{3+} and Fe^{3+} also heightened absorption at 420 nm, which reflects yellow-brown color. The average absorption at 420 nm (A_{420}) in the extracts added with trivalent metal ions was ± 0.5, while in other extracts it was ± 0.06. The high A_{420} significantly increased the browning and decreased the overall color quality of the extract (Cisse et al., 2012).

The reason why metal ions interact with anthocyanins is not known, but the case of butterfly pea flower extract provides a clear understanding. Metal ions typically interact with anthocyanins via hydroxyl groups on the B-ring. However, the anthocyanins in butterfly pea flowers, the polyacylated anthocyanins known as ternatins, lack free hydroxyl groups on the B-ring (Kazuma et al., 2003).

Significant changes occurred in the UV region in the extract added with Al^{3+}, including a new band around 380 nm, which appears to be a shift to a longer wavelength from the 350 nm absorption, which is the absorbance of non-anthocyanin flavonoids. This new band indicates the formation of a complex between the non-anthocyanin flavonoid and Al^{3+}. Flavonol glycosides are the most found flavonoids in butterfly pea flowers, with kaempferol 3-glycoside being the main compound (Kazuma et al., 2003). The interaction between kaempferol and Al^{3+} has been reported recently (Sun et al., 2021).

A significant shift to longer wavelengths was also observed in the UV region of the extract with Fe^{3+}, indicating the potential interaction between Fe^{3+} and non-anthocyanin phenolic compounds.

3.2 The Effect of the Metal Ions on the Color Stability During Storage

Scanning of butterfly pea flower extract's light absorption (250-700 nm) during storage revealed changes in benzene derivatives, acyl groups, non-anthocyanin flavonoids, and anthocyanins over time (Figure 2). Overall, a degradation of color ($A_{550} + A_{574} + A_{617}$) occurred in all extracts during the 12 days of storage. The rate of color degradation can be modeled by first-order degradation kinetics satisfactorily (p-value of the regression < 0.05). A slope test was carried out with an alpha level of 0.05 to assess the differences in degradation rate between the control extract and extracts containing various metal

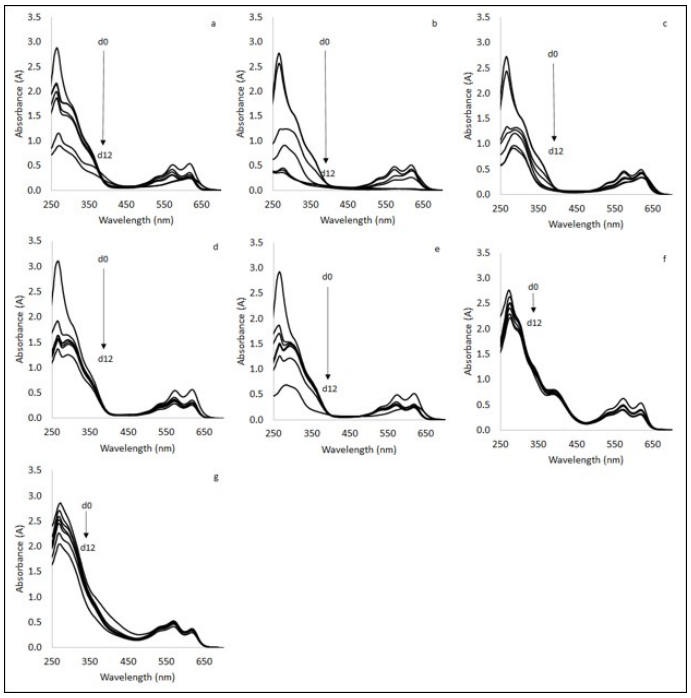

Figure 2. UV-Vis Spectrogram of Butterfly Pea Flower Aqueous Extract in Various Metal Ion Solutions During 12 Days Storage (a), Control (b), Na^{+} (c); K^{+}, (d) Ca^{2+}, (e) Mg^{2+}, (f) Al^{3+}, and (g) Fe^{3+}

ions, as seen in Table 1. The test indicated that K^{+}, Mg^{2+}, Ca^{2+}, Al^{3+} and Fe^{3+} significantly prolonged the half-life of the flower extract's color. Meanwhile, Na+ tended to shorten the half-life.

Table 1. The Degradation Kinetics (k) and Half-life ($t_{0.5}$) of the Color of Butterfly Pea Flower Extract in Various Metal Ion Solution

Extract	R^2	p-value	k (day-1)*	$t_{0.5}$ (days)*
H_2O	0.96	<0.01	0.068	10.23^{b}
NaCl	0.88	<0.01	0.305	2.27^{a}
KCl	0.90	<0.01	0.039	17.72^{c}
$CaCl_2$	0.93	<0.01	0.030	23.20^{d}
$MgCl_2$	0.79	<0.05	0.026	26.48^{d}
$AlCl_3$	0.80	<0.05	0.024	28.91^{d}
$FeCl_3$	0.80	<0.05	0.017	41.17^{e}

*stated as mean of two replications, different letters above data points indicate significant differences

The effect of metal ions on anthocyanin stability is inconsistent, with some studies showing a negative effect of Na^{+} (Chen et al., 2019; de Rosso and Mercadante, 2007; Hubbermann et al., 2006), while others show no effect (Dangles and Brouillard, 1992; Figueiredo and Pina, 1994; Peng et al., 2016). The destabilization by Na^{+} is thought to result from increased solvation of the flavylium cation, leading to dissociation and

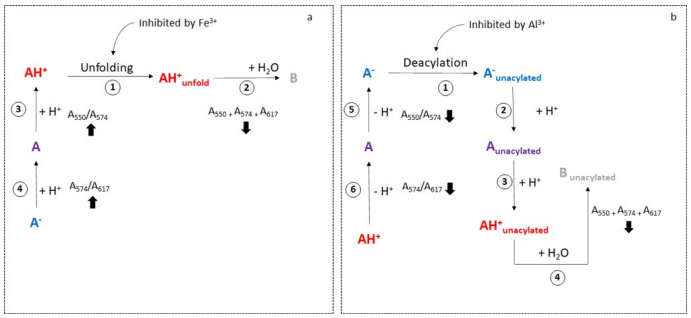

Figure 3. Two Possible Pathway of Color Degradation of a Polyacylated Anthocyanin Like Ternatin in Butterfly Pea Flower Extract, (a) the First Way Initiated by the Unfolding of Flavylium Cation (AH^+), (b) the Second Way Initiated by the Diacylation of Anionic Quinonoidal base (A^-)

color loss (Hubbermann et al., 2006). The positive effect of K^+ has been reported (Czibulya et al., 2015), but differs from other findings (Guo et al., 2017). The positive effect of Ca^{2+} observed in this study is consistent with Czibulya et al. (2015), but differs from other studies that saw no significant impact (Buchweitz et al., 2013; Guo et al., 2017). This study showed a significant positive impact from Mg^{2+} on anthocyanin stability. Meanwhile, other studies show that Mg^{2+} give no effect (Peng et al., 2016; Sigurdson et al., 2016) or even decreased stability (Mei et al., 2014). Peng et al. (2016) found a negative impact of Fe^{3+} and a positive impact of Al^{3+} on anthocyanin stability. The negative impact of Fe^{3+} was also reported by Guo et al. (2017) and Ratanapoompinyo et al. (2017). This study supports the improvement of stability by both trivalent metals.

In the aqueous system, polyacylated anthocyanins such as ternatins exist in a state of equilibrium between red, purple, and blue species: $AH^+ \rightleftharpoons A \rightleftharpoons A^-$. The hydration of AH^+ into the colorless hemiketal B does not occur because it is prevented by the intramolecular copigmentation that forms through the hydrophobic interaction between p-coumaric acid and the anthocyanin molecule (Terahara et al., 1998).

Theoretically, two pathways can cause a polyacylated anthocyanin to lose its color, as depicted in the scheme in Figure 3 (Marpaung et al., 2017; Marpaung et al., 2019). In the first pathway, the hydrophobic interaction in AH^+ undergoes unfolding, causing AH^+ to hydrate into B. This reaction disrupts the equilibrium of $AH^+ \rightleftharpoons A \rightleftharpoons A^-$ and drives protonation of $A \rightarrow AH^+$ and $A^- \rightarrow A$ to reach a new equilibrium (Figure 3a). In the second pathway, A^- as the least stable species Terahara et al. (1998) is deacylated to $A^-_{unacylated}$, then deprotonated to Aunacylated, and then $AH^+_{unacylated}$, which immediately hydrated to Bunacylated. This series of reactions disrupt the equilibrium of $AH^+ \rightleftharpoons A \rightleftharpoons A^-$ (polyacylated) leading to deprotonation $A \rightarrow A^-$ and $AH^+ \rightarrow A$ (Figure 3b). In the next stage, after the loss of color in anthocyanin, degradation continues to de-glycosylation to anthocyanidin and further degradation to benzaldehyde derivatives and 4-hydroxybenzoic acid (Sun et al., 2011).

The first event of the color loss can be observed in the spectrogram as a hypochromic shift and the constant or increasing concentration of AH^+ relative to A (A_{550}/A_{574}) and the ratio of A^- to A (A_{574}/A_{620}). Meanwhile, the second event can be observed as a hypochromic and bathochromic shift, as well as a decrease in A_{574}/A_{620} and A_{550}/A_{574}.

The extract added with Al^{3+} was the only sample that consistently shows an increase in A_{550}/A_{574} and A_{574}/A_{620}. Conversely, the extract added with Fe^{3+} consistently showed a decrease in A_{550}/A_{574} and A_{574}/A_{620}, although the bathochromic shift was not clearly visible. Meanwhile, in the control sample and those added with monovalent or divalent ions, there was an increase in A_{550}/A_{574} and A_{574}/A_{620} at the beginning of degradation, followed by a decrease in both ratios in the subsequent degradation stage. As an addition, a clear bathochromic shift is also seen in the control extract and those added with Na^+, K^+, and Mg^{2+} (Figure 2a, 2b, 2c, and 2e).The trend of changes in both ratios suggested that color degradation in the extract added with Al^{3+} occurred due to the unfolding of hydrophobic interaction, while in the extract added with Fe^{3+} the color degradation occurred due to deacylation. Meanwhile, color degradation in the control sample and those added with monovalent and divalent metal ions occurred due to the unfolding of hydrophobic interaction and followed by deacylation.

3.3 The Effect of the Metal Ions on Benzene Derivatives, Acyl Groups, Non-anthocyanin Flavonoids

The light absorption at 265 nm (A_{265}) represents benzene derivatives, including anthocyanins and other flavonoids. Figures 2 clearly shows that the addition of trivalent metal ions inhibited the degradation of benzene derivatives. With a first-order degradation kinetics model (p-value), the degradation rate (k) of A_{265} in the extract added with Al^{3+} or Fe^{3+} was significantly lower compared to the k in other extracts. In line with the degradation of colored anthocyanins, the stability of benzene derivatives in the extract added with Na^+ was lower compared to the control extract. However, there was no significant difference in the stability of benzene derivatives between the control extract and those added with K^+, Ca^{2+}, and Mg^{2+}.

The evolution of light absorption at 310 nm (A_{310}), which represents the acyl group, offered compelling evidence in support of the hypothesis regarding the mechanisms of anthocyanin degradation. The exponential regression analysis (1^{st} order degradation kinetics) revealed that degradation of A_{310} in the extract added with Al^{3+} was the only one that was not statistically significant ($p > 0.05$). This supported the notion that deacylation did not occur in the extract containing Al^{3+}, thereby reinforcing the idea that the degradation of colored anthocyanins in this sample was a result of the unfolding of hydrophobic interaction.

The fact that degradation of A_{310} was not significant and the degradation rate of A_{265} in the extract containing Al^{3+} was low suggests that the color degradation in this extract has only

reached the stage of transforming colored ternatin species into a reversible colorless one. There had not been any chemical degradation of the ternatins to unacylated anthocyanin and further to anthocyanidin, etc.

The extract added with Al^{3+} is also the only sample that did not experience significant degradation of non-anthocyanin flavonoids (A_{350} and A_{380}). It seemed that Al^{3+} successfully formed a stable complex with the non-anthocyanin flavonoids in the butterfly pea extract. Whether there is a connection between the stability of this complex and the inhibition of deacylation in butterfly pea flower anthocyanin, further study is needed.

4. CONCLUSION

Scanning the UV-Vis spectrophotometer at the wavelength range of 250-700 nm on the butterfly pea flower extract in various metal ion solutions during storage has provided a broader perspective on how color degradation occurs. K^+, Ca^{2+}, Mg^{2+}, Al^{3+}, and Fe^{3+} ions showed the ability to improve the stability of the butterfly pea flower extract color, while Na^+ tended to accelerate color degradation.

The pattern of changes in the extract spectrogram during storage suggests that the color degradation of the extract proceeds through two mechanisms: the unfolding of hydrophobic interactions that establish the intramolecular copigmentation between ternatin and p-coumaric acid, and the deacylation of ternatin. Trivalent metal ions showed the best stability performance. Fe^{3+} prevented the unfolding of the hydrophobic interaction, while Al^{3+} hindered the occurrence of deacylation. The combination of the two is highly likely to improve the color stability of the butterfly pea flower extract. However, both increase the browning index, thus decreasing color quality. This research highlights that the addition of cations is a potential method for improving the color stability of the butterfly pea flower extract, making it a more attractive food coloring agent.

5. ACKNOWLEDGMENT

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