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Dimethyl Sulfoxide Stabilize the Color of *Clitoria ternatea* Flower Extract at pH 6-8 by Preventing the Deacylation: A Spectrophotometric Evidence

Abdullah Muzi Marpaung*, Christine Suwandy

Department of Food Technology, Swiss German University, Indonesia

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Abstract The pursuit of stable natural food colorants arises from health concerns linked to artificial coloring agents. Polyacylated anthocyanins, known for their deep and stable color in various food pH conditions, have emerged as promising natural alternatives. Among these, the butterfly pea (*Clitoria ternatea*) flower contains polyacylated anthocyanins called ternatins. However, the color stability of butterfly pea flower extract diminishes at higher pH levels. Surprisingly, despite their conventional role in weakening intramolecular copigmentation, organic solvents have been found to enhance the color stability of butterfly pea flower extract at pH 7. This study focused on investigating the potential stabilizing effect of dimethyl sulfoxide (DMSO) on the color of butterfly pea flower extract at pH 6, 7, and 8 using spectrophotometric analysis and varying concentrations of DMSO. The results demonstrated that the addition of DMSO did not significantly alter the extract's spectrogram, indicating the absence of complex formation between DMSO and anthocyanins or flavonoids. Notably, the presence of DMSO remarkably improved color stability compared to DMSO-free extracts. The highest enhancement was achieved with 20% DMSO, extending the color retention period from 61.4 to 206.6 days, depending on the pH level. Analysis of spectrogram patterns over time revealed that color degradation in extracts with DMSO concentrations ≥ 20% was reversible, primarily attributed to the unfolding of hydrophobic interactions, while chemical degradation through deacylation of polyacylated anthocyanins had not yet occurred. Further analysis confirmed a significant decrease at anthocyanin levels in extracts without DMSO and those with 10% DMSO, while extracts with ≥ 20% DMSO maintained stable anthocyanin levels. This study enhanced our understanding of butterfly pea flower extract color stability and unveiled the unexpected stabilizing effect of DMSO. The findings provided valuable insights for the development of natural colorants, emphasizing the role of organic solvents in enhancing color stability. However, limitations included the sole focus on DMSO as the solvent and the need for further exploration of underlying mechanisms and practical implications. Nonetheless, this study represented a significant stride toward harnessing the potential of butterfly pea flower extract as a stable natural food colorant.

Keywords Butterfly Pea Flower Extract, Color Stability, DMSO, Polyacylated Anthocyanins, pH Levels

1. Introduction

The quest for stable natural food colorants continues due to the health risks posed by using artificial coloring for a long period. Among the most promising natural colorants are polyacylated anthocyanins, which possess a deep, stable color in food pH, unlike common anthocyanins that tend to fade in these conditions [1]. The butterfly pea

(*Clitoria ternatea*) flower, with its distinctive purple-blue color in low acidic solutions, is a particularly appealing source of polyacylated anthocyanins. There are 9 types of polyacylated anthocyanins called ternatins found in fully opened flowers [2], [3].

The stability of the color of butterfly pea flower extract has been studied for years. Generally, the butterfly pea flower extract is highly stable at pH ≤ 4, but its stability decreases dramatically at pH ≥ 6 [4], [5]. The color stability of the extract is also affected by temperature. At refrigerator temperatures, the color is very stable [6]. The addition of sugar has been reported to slightly increase the color stability of the flower extract at pH 5 and 6 [7]. An interesting discovery is that organic solvents can enhance the stability of butterfly pea flower extract at pH 7, with acetone showing better performance than ethanol and methanol [4]. This is unexpected as organic solvents are typically thought to weaken intramolecular copigmentation, resulting in color loss [8–10].

By analyzing spectrophotometric data, two mechanisms involved in the degradation of butterfly pea flower extract color were proposed: the unfolding of hydrophobic interaction and deacylation. The deacylation is then proven by the HPLC-DAD analysis [4], [5]. Organic solvents appear to cause the unfolding of hydrophobic interaction, but hinder deacylation. Degradation of color due to deacylation is more severe than hydrophobic interaction unfolding. As a result, organic solvents enhance the stability of butterfly pea flower extract color [4], [5].

Dimethyl sulfoxide (DMSO) is a commonly used organic solvent to unfold the hydrophobic interaction of proteins. Its molecular structure is like acetone; however, it has a higher dipole moment at 3.96 D compared to acetone's 2.69 D. DMSO has been reported to increase the stability of anthocyanins [11], but it also has the potential to disrupt intramolecular copigmentation [8]–[10].

This study aimed to investigate the potential stabilizing effect of DMSO at different concentration levels on the color of butterfly pea flower extract at pH 6, 7, and 8. Spectrophotometric observations at wavelengths of 250 to 700 nm were applied as a basis for predicting how this stabilizing effect takes place.

2. Materials and Methods

2.1. Material

The main material was butterfly pea (*Clitoria ternatea*) flower (CT) that was obtained from a private garden in Tangerang, Banten, Indonesia. The fresh petal of CT separated from the sepal, steam blanched for 6 min [4], dried at 45 ℃ for 24 h (Oven Gravity WiseVen ® WON – 305 High Clean Air Oven), pulverized and sieved through a 250 μm screen. The dried powder was loaded in an airtight dark container and stored in a freezer (about -20 ℃) until used. Deionized water was Amidis® (local market, Tangerang, Banten, Indonesia). Buffer solutions of pH 6 (citric acid-sodium hydroxide), buffer solution pH 7 and 8 (disodium hydrogen phosphate-potassium dihydrogen phosphate), dimethyl sulfoxide (DMSO), and hydrochloric acid procured from Certipur® (Merck KGaA, Darmstadt, Germany).

2.2. Instrumentation

The fresh and stored extract of all samples was loaded into a quartz cuvette and scanned by a UV spectrophotometer (Genesys 10UV Thermo Electron Corporation, USA) at 250 to 700 nm. The spectrogram was analyzed to determine the appearance of absorption bands (absorption peaks and shoulders) and absorption shifts. The shifts were bathochromic (to a longer wavelength), hypsochromic (to a shorter wavelength), hyperchromic (to greater absorbance), and hypochromic shift (to lower absorbance).

Three absorption band near 265 to 275 indicates the presence of A ring of phenolic compound, including anthocyanin [12], [13]. The band near 310 denotes the hydroxycinnamic acid acylation [14], [15]. The band around 350-360 nm represents the non-anthocyanin flavonoids [16]. The band in the visible region shows the appearance of colored species that consist of flavylium cation (**AH**$^+$), quinonoidal base (**A**), and anionic quinonoidal base (**A**$^-$)

2.3. Extraction

One gram sample was added to 40 mL of deionized water (60 ℃) and placed in a water bath shaker (60 ℃) for 30 min with continuous shaking and without light exposure [4]. The extract was separated from the solid through Whatman #41 filter paper. The extract was diluted in pH buffer-DMSO solution with a dilution factor (DF) of 80. The pH of the buffer was 6, 7, and 8, and the fraction of DMSO was 0, 10, 20, 30, 40, and 50% (v/v).All 15 treatments each with two replicates were coded as T6-0, T6-10, T6-20, T6-30, T6-40, T6-50, T7-0, T7-10, T7-20, T7-30, T7-40, T7-50, T8-0, T8-10, T8-20, T8-30, T8-40, and T8-50.

The diluted extract was bottled in a dark glass container and stored in a dark cabinet at room temperature. Every three days, the light spectra and total anthocyanin of the samples were observed.

2.4. Analysis of Total Anthocyanin

Total anthocyanin (TA) content was determined as delphinidin-3-glucoside by measuring the light absorbance of the extract at pH 1, known as single pH method [17]. At the pH, anthocyanin only exists as flavylium cation (AH+) species.

$$A = (A_{\lambda max}) - (A_{700}) \times DF \quad (1)$$

$$TA\ (mg/L) = (A \times MW \times DF \times 1000)/(\varepsilon \times l) \quad (2)$$

MW is the molecular weight of delphinidin-3-glucoside (465.2 g/mol), DF is a dilution factor, ε is the molar absorptivity of delphinidin-3-glucoside (29000), and l is the cuvette width (1 cm).

2.5. Degradation Kinetics

The k-values and half-time ($t_{1/2}$) value for first-order reaction were calculated using the following equation.

$$ln[A]_t = -kt + ln[A]_0 \quad (3)$$

$$t_{(1/2)} = ln(2)/k \quad (4)$$

$[A]_t$ is the absorbance at a certain time, $[A]_0$ is the absorbance at the initial time, and k is the kinetic degradation constant (d^{-1}).

2.6. Statistical Analysis

The trend of the change in absorbance of each band was analyzed using regression analysis, and the difference in slopes between the two samples was determined using a slope test. Both statistical evaluations were conducted using Microsoft Excel® as part of Microsoft 365, Microsoft, Redmond, Washington, USA, with a significance level of $\alpha = 0.05$.

3. Results and Discussion

3.1. The Stabilizing Effect of DMSO

The distinctive features of 6 bands, 3 in the UV region and 3 in the Visible region, which is characteristic of CT extract at low acidic conditions [5] are prominent and easily noticeable at pH 6. Meanwhile, at extracts pH 7 and 8, only the band of $\mathbf{A^-}$ (peaking around 630 nm) is clearly visible as also reported by previous studies [4], [5].

There was no significant change in either vertical or horizontal shift due to the addition of DMSO in the CT extract at pH 6, 7, and 8. This indicated that DMSO does not form complexes with anthocyanins or other flavonoids in the flower.

The stabilization effect of DMSO on the color of the CT extract at pH levels of 6, 7, and 8 is evident in Figure 1. This contradicts previous findings that DMSO and organic solvents weaken the polyacylated intramolecular copigmentation of polyaclyated anthocyanin [9], [10], but aligns with [4] who found that organic solvents improve the color stability of CT extract. The effect is quantitatively presented as the degradation rate (k) and half-life ($t_{1/2}$) of the color of each extract in Table 1. Without DMSO, the CT extract at pH 6 to 8 was relatively easy to decolorize, as reported by previous studies [4], [5]. The color intensity decreased by half in just four days. The presence of DMSO noticeably prolonged the color stability. The addition of 20% DMSO showed the greatest improvement, with the color retaining 50% of its original intensity for periods ranging from 61.4 to 206.6 days, depending on the pH level. The stability of the color decreased as the pH increased.

A key element of the study was the observation of the pattern of changes in the spectrogram of the extract over time, to describe how DMSO stabilizes the color of the ternatins in CT extract. There are two possible pathways that might lead to color degradation: the unfolding of the hydrophobic interaction of intramolecular copigmentation and the deacylation of polyacylated anthocyanins [4], [5]. The occurrence of deacylation of polyacylated anthocyanins in the extract with 0% and 10% DMSO was indicated by two signs that can be observed through spectrophotometry. The first sign was the hypsochromic shift [4], [5]. As seen in Figure 1, the $\lambda_{max\text{-}vis}$ (wavelength with the highest absorbance at visible region) of the extract with 0% and 10% DMSO consistently shifted to a shorter wavelength. At pH 6, $\lambda_{max\text{-}vis}$ shifted by 10 nm in six days, then disappeared at day 9. At pH 7, $\lambda_{max\text{-}vis}$ shifted by 9 nm in three days, then disappeared at day 6. At pH 8, $\lambda_{max\text{-}vis}$ shifted by 27 nm in six days, then disappeared at day 9. The shorter hypsochromic shift was detected in the extract added with 10% DMSO. The second sign was the decrease in A_{310} absorbance, which is a band of hydroxycinnamic acid acylation [14], [15]. The time required for the A_{310} to reach $t_{1/2}$ was found to range from 3.3 days to 14.95 days, in extracts at all pH levels studied with 0% or 10% DMSO added. Additionally, significant degradation of the A ring of phenolic compounds [12], [13] was also visible at 265 nm in all extracts with 0% or 10% DMSO added. Conversely, in extracts with DMSO $\geq$ 20%, no significant trends in degradation of A_{310} and A_{265} were observed.

This observation suggested that color degradation in the extract with DMSO $\geq$ 20% was merely a reversible reaction between the colored and colorless species of anthocyanins, which can only occur if the hydrophobic interaction of intramolecular copigmentation unfolds. Meanwhile, chemical degradation in the form of deacylation of ternatins into unacylated anthocyanins, which is then followed by degradation into anthocyanidins and finally ends with the formation of simple phenolic compounds as described by [18], had not yet occurred. The decrease in color intensity during storage with the addition of up to 50% DMSO indicates that DMSO tended to disrupt the intramolecular copigmentation of polyacylated anthocyanins in CT extract, but the chemical structure remains stable without undergoing deacylation.

The phenomenon of color fading in CT extract while A_{310} remains stable is also observed when the extract is added with Al^{3+} ions (personal data). The decolorization of CT extract's anthocyanin due to the unfolding of hydrophobic interaction was also proposed for CT extract at pH 7 added with acetone [4] and for CT extract at pH 4 [5] due to the absence of a hypsochromic shift. However, in both studies, observations at 265 and 310 nm were not conducted.

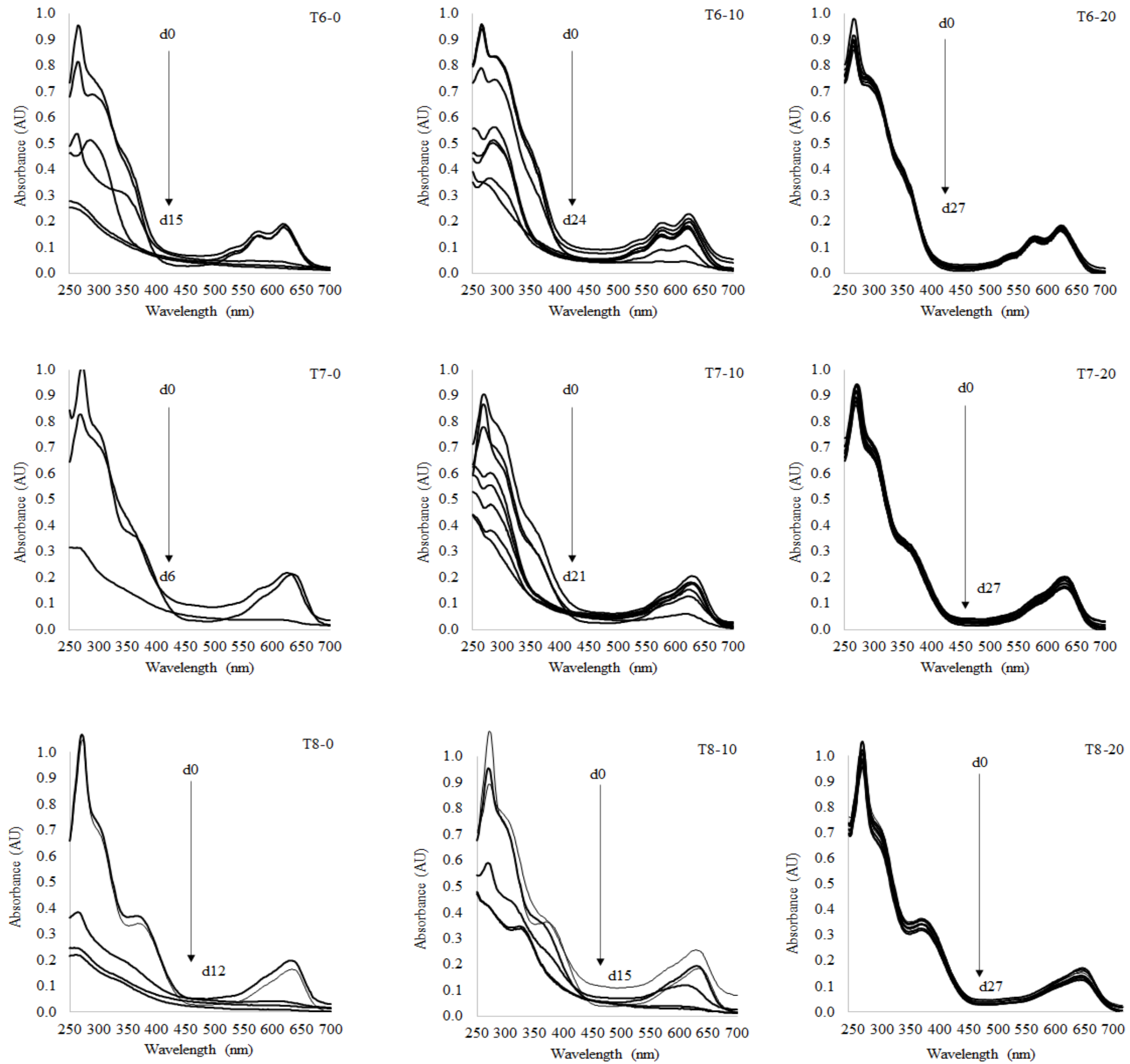

Figure 1. The spectrogram of CT extract at pH 6 added with 0, 10, 20% DMSO (T6-0, T6-10, T6-20, respectively), at pH 7 added with 0, 10, 20% DMSO (T7-0, T7-10, T7-20, respectively), and at pH 8 added with 0, 10, 20% DMSO (T8-0, T8-10, T8-20, respectively) during storage at room temperature with no light. The extract added with 30, 40, and 50% were included in the research, but excluded from this figure.

Table 1. Degradation rate (k) and half-life of color of butterfly pea flower extract at pH 6 – 8 with various concentration of DMSO

DMSO	Degradation Rate, k (d^{-1})*		
	pH 6	**pH 7**	**pH 8**
0%	0.197 ±0.050^{d}	0.212 ±0.053^{e}	0.203 ±0.048^{d}
10%	0.069 ±0.039^{c}	0.063 ±0.020^{d}	0.135 ±0.043^{c}
20%	0.003 ±0.001^{a}	0.004 ±0.001^{a}	0.011 ±0.003^{a}
30%	0.004 ±0.002^{ab}	0.007 ±0.003^{b}	0.013 ±0.003^{ab}
40%	0.006 ±0.002^{b}	0.008 ±0.002^{bc}	0.014 ±0.005^{ab}
50%	0.007 ±0.003^{b}	0.012 ±0.003^{c}	0.017 ±0.004^{b}
	half-life, $t_{1/2}$ (d)**		
	pH 6	**pH 7**	**pH 8**
0%	3.53	3.27	3.42
10%	10.01	11.06	5.15
20%	206.64	194.53	61.38
30%	173.42	92.64	52.73
40%	111.81	82.39	50.07
50%	92.97	59.33	41.84

*) average ±95% confidence interval, ** average

*siginificantly different, α = 0.05

3.2. Total Anthocyanin: Additional Evidence?

Analysis of total anthocyanins can also be used to reinforce suspicions about whether deacylation occurs in the extract during storage. Modeling with first-order degradation kinetics showed a trend of anthocyanin degradation in all extracts without DMSO and those added with 10% DMSO (p-value of regression <0.05). The t1/2 of anthocyanins in the extract without added DMSO at pH 6, 7, and 8 was 3.53, 2.93, and 2.28 days, respectively. Meanwhile, the t1/2 of anthocyanins in the extract with 10% DMSO added at pH 6, 7, and 8 was 32.80, 49.05, and 25.13 days, respectively. In all extracts with ≥ 20% DMSO added, there was no significant decrease in the total anthocyanin, with modeling of 0th order, 1st order, or quadratic (R2 <0.5 and the p-value of regression >0.05).

The fact that there was a slight but statistically significant color degradation in the extract with ≥ 20% DMSO while the anthocyanin remains stable, suggested that there had not been any chemical degradation in the extract. The plausible explanation for the color degradation is through hydrophobic interaction unfolding that causes the **AH^{+}** to be hydrated to the colorless **B** [5].

3.3. Absorbance at 550, 580, and 620 nm

Additional understanding may be extracted from observing the changes in light absorption at 550, 580, and 620 nm, each of which represents AH+, A, and A-, respectively (Figure 2). Firstly, despite a slight decrease in A550, A580, and A620 in the extract with 20% DMSO, there was also a pattern of periodic ups and downs in their absorbance. This phenomenon indicated that during storage, reversible reactions occur among anthocyanin species: B ⇌ AH+ ⇌ A ⇌ A-. In other words, the folding-unfolding of hydrophobic interaction occurred regularly in the CT extract during storage. Secondly, in the extract with 10% DMSO, where evidence of deacylation occurs, a more complex pattern was observed.

At the early stage of degradation, there was a rise in intensity of the three colored species, showing a consecutive reaction of B → AH+ → A → A-. This requires two things: the intramolecular copigmentation must unfold and there must be a disturbance to the equilibrium among the anthocyanin species. The direction of the reaction showed that the disturbance began with a decrease of A-. It is crucial to keep in mind that this A- refers to polyacylated A-. The decrease in polyacylated A- would only occur if there was deacylation of polyacylated A- to be deacylated or unacylated A-. Hence, it can be suggested that the degradation of ternatins began with the deacylation of unfolded A-.

In the next stage of degradation, there was a drastic decrease in A550, A580, and A620, with A620 experiencing the worst decline. The explanation is as follows: the deacylation of A- eventually resulted in unstabilized unacylated A- which immediately deprotonated to A, then to AH+. The unacylated AH+ then was hydrated to unacylated B, causing decolorization. This cycle continued until the extract loses most of its color.

All the descriptions above can be summarized into a scheme that illustrates the suggested mechanism of the color degradation of ternatin and the role of DMSO in inhibiting the color degradation (Figure 3).

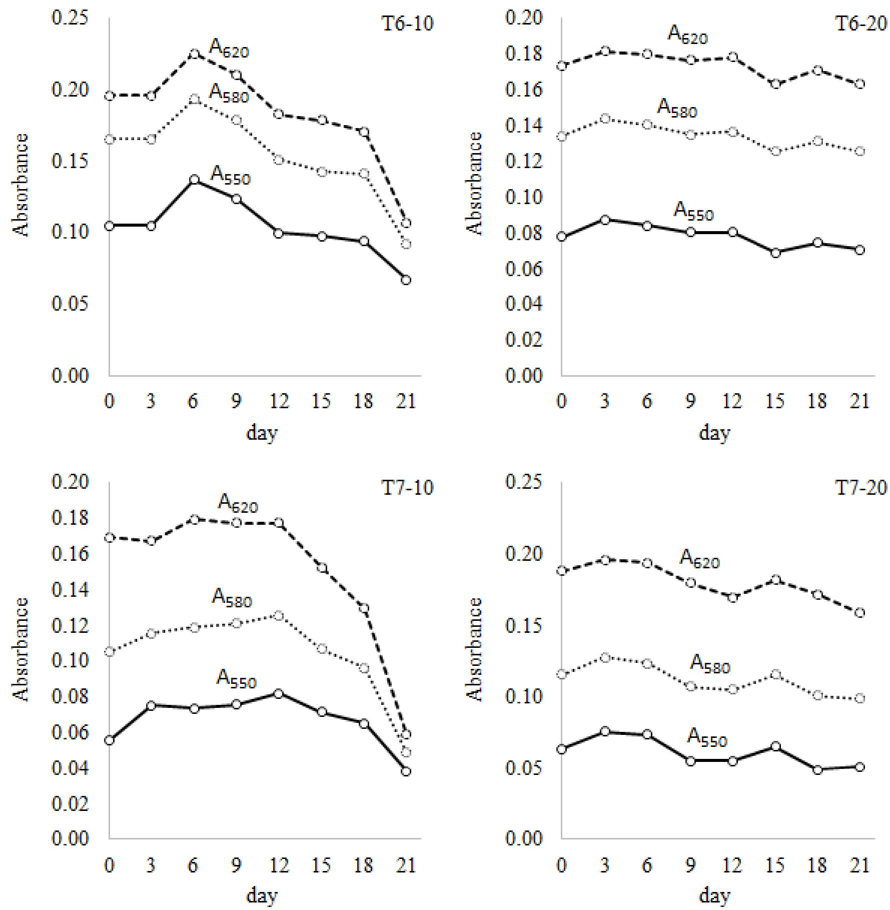

Figure 2. The evolution of the absorbance at 550, 580, and 620 nm of CT extract at pH 6 added with 10% and 20% DMSO (T6-10 and T6-20, respectively) and at pH 7 added with 10% and 20% DMSO (T7-10 and T7-20, respectively)

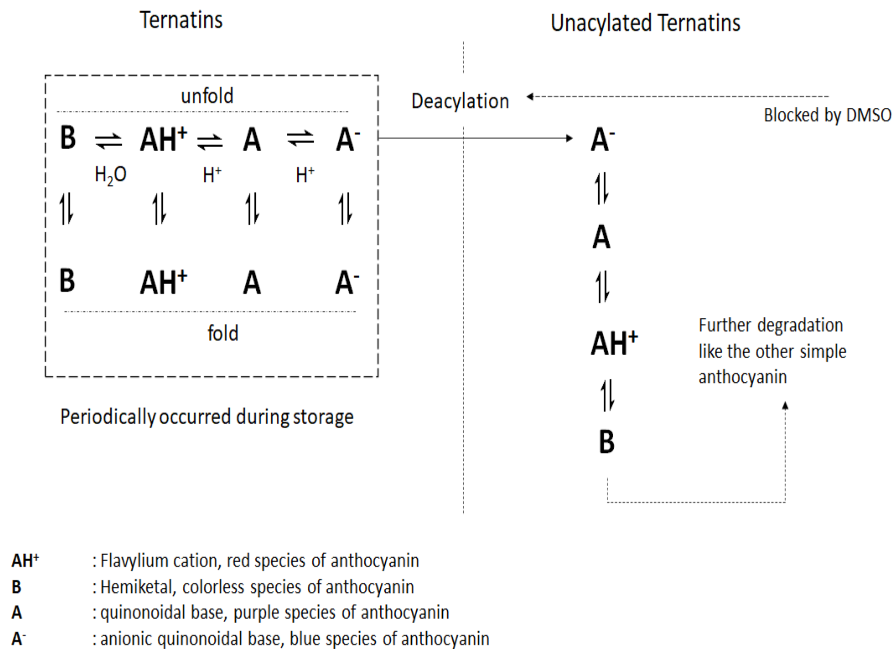

Figure 3. Proposed degradation mechanism for the color of ternatins in CT extract at pH 6 to 8 based on spectrophotometric data

3.4. How DMSO Prevented Diacylation?

Deacylation of polyacylated anthocyanins, including ternatins, has been extensively reported [5], [12], [13]. The hydroxide ion of water acts as a nucleophile and attacks the carbonyl group of the acyl group to remove it from the ternatins. The reactivity of OH- increases with increasing pH [14].

Spectrophotometric evidence in the study suggests the potential of DMSO to inhibit deacylation in ternatins. Two possible mechanisms are proposed: 1) DMSO interacts with the carbonyl group of the acylated anthocyanin, forming a hydrogen bond that hinders its interaction with the hydroxide ion. The protective effect is expected to be more pronounced at higher concentrations of DMSO. 2) DMSO may form an ion pair with the hydroxide ion, reducing the reactivity of OH- towards the carbonyl group of the acylated anthocyanin. However, further experimental and computational studies are needed to confirm the exact mechanism by which DMSO protects anthocyanin and to optimize the conditions for its use.

4. Conclusions

Two types of color degradation were observed in butterfly pea flower extract at pH 6 to 8: the unfolding of hydrophobic interaction and deacylation. Color degradation caused by deacylation was more severe than that caused by the unfolding of hydrophobic interaction. Although DMSO tends to unfold hydrophobic interaction, it prevents more severe color degradation by blocking deacylation. This is evidenced by the absence of chemical degradation in ternatins in the form of a decrease in anthocyanin content and absorbance at 310 nm in extract samples with the addition of DMSO $\geqslant$ 20%. As a result, the addition of DMSO is an effective way to improve the color stability of butterfly pea flower extract at this pH range. The addition of 20% DMSO showed the greatest improvement, with the color retaining 50% of its original intensity for periods ranging from 61.4 to 206.6 days, depending on the pH level. The stability of the color decreased as the pH increased.

The ability of DMSO to inhibit deacylation appears to be related to its property as a polar solvent that can provide cations to interact with the carbonyl group on anthocyanins or inhibit the aggressiveness of OH- in water. Therefore, it is recommended to conduct research on the stabilizing effect of various polar organic chemicals on polyacylated anthocyanins, such as various types of amino acids.

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