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Thermal Degradation of Anthocyanins in Butterfly Pea (*Clitoria ternatea* L.) Flower Extract at pH 7

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Abstract The degradation of anthocyanins from *Clitoria ternatea* L. flower (CT) extract at pH 7 bottled with 0% and 50% volume of headspace (HS0 and HS50, respectively) were studied at various temperatures (7, 30, 45, 60, 75, 90°C). The extract was stable at 7°C up to 56 days. The effect of the presence of headspace to accelerate the degradation was significance at ⩾30°C. The color and chemical degradation were adequately be described by the first order reaction kinetics. However, the degradation at 30°C was faster than at 45°C. The activation energy for the chemical degradation of HS0 and HS50 extracts at 45-90°C were 83.21 and 101.15 kJ/mol. The decrease of A_{628} was the fastest, followed by A_{580} and A_{550}, respectively. By the evidence collected, it was proposed that the degradation of anthocyanins in CT extract was initiated by the unfolding and the deacylation of anionic quinonoidal base species.

Keywords: *anthocyanins, butterfly pea, degradation, heat, headspace*

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1. Introduction

Polyacylated anthocyanins are anthocyanins containing two or more aromatic acyl groups [1,2]. The aromatic acyl groups configure an intramolecular stacking with the anthocyanidin chromophore that protects the dehydration of flavylium cation to colorless hemiketal [1]. Therefore, the stability of polyacylated anthocyanins at low acidic and neutral conditions is much higher than monoacylated or unacylated anthocyanins, made them as a potential source of natural food colorant [3].

Butterfly pea or *Clitoria ternatea* L. (CT) is one of the most interesting sources of polyacylated anthocyanins because it provides blue color at a low acidic or neutral solution [4,5,6]. Its stability was reported better than the heavenly blue anthocyanin from morning glory (*Ipomoea tricolor*) flower [7]. Like other anthocyanins, the stability of polyacylated anthocyanins from CT extract was affected by many factors, especially pH and temperature.

Reference [4] reported that the stability of CT extract at pH 4 or below was much higher than at pH 5 or above. The comparable results were also reported by the other researchers [6,8]. At low temperature, the CT extract exhibited a very high stability. The extract at pH ≤ 6.0 remained about 80-90% of its color when kept in the dark at 7°C for 60 days [8]. At temperature 25-90°C, the color stability the extract at pH 3 decreased as temperature increased and fit to the first order kinetic degradation with an activation energy (E_a) 28.66 kJ/mol [4]. The E_a value was relatively low, indicating that the degradation rate of anthocyanin of CT extract was less susceptible to temperature increase. Other than pH and temperature, our previous study has shown that the volume of container's headspace also gave a significant effect to accelerate the color degradation of CT extract at pH 7 [9].

The anthocyanin degradation consists of two different terms, which are color and chemical degradation. The color degradation related to reversible equilibrium change between colored and colorless forms of anthocyanin [10]. The colored species was consisted of the red flavylium cation (AH^+), purple quinonoidal base (A) and blue anionic quinonoidal base (A^-), while the colorless species consisted of hemiketal (B), cis-chalcone (Cc) and trans-chalcone (Ct) [10]. In consequence, the color degradation is preferably studied by determining the decrease of [AH^+] + [A] + [A^-], even though it might be depicted by the decrease of color intensity. Reference [11] determined the color intensity as the absorbance at λ_{max} within the visible region, but for this research, we proposed to determine the color intensity by the total absorbance of the wavelength that represents red, violet, and blue color.

The chemical degradation related to the decrease of total anthocyanin due to the irreversible degradation of pigments, mainly arising from cleavage of the chromophore to form benzoic acid and an aldehyde derivative [10]. In general, at below pH 2, the only anthocyanin species exist is the red flavylium cation. Therefore, the total anthocyanin can be represented by the absorbance at λ_{max} of anthocyanin-source extract at pH 1 [12].

The aim of this research was to study the thermal degradation rate of both color and anthocyanin from

butterfly pea flower extracts at pH 7 that were stored in the glass bottle with 0% and 50% headspace, respectively.

2. Materials and Methods

2.1. Materials

The butterfly pea flower (CT) was harvested from a garden at South Tangerang, Banten, Indonesia. The blue part of the fully opened flower was separated from the white part, steam-blanched for 6 minutes [6] then freeze-dried. The dried flower was pulverized by a ball mill and sieved through 250 μm screen. The powder was packed in a tight container and kept in a freezer until used. The deionized water was obtained from a local market (Amidis®). The buffer solution pH 7 (disodium hydrogen phosphate-potassium dihydrogen phosphate), hydrochloric acid (HCl) 1 M, and trifluoroacetic acid (TFA) were obtained from Merck®. All reagents used were analytical grade without further purification.

2.2. Preparation of CT Extract

The CT was extracted by the method of reference [6] with modification. One gram powder was extracted by 40 ml of deionized water at 60°C for 30 minutes with continuous shaking and without light exposure. The suspension was filtered and the filtrate was collected and centrifuged at 7000 rpm for 5 minutes. Finally, the extract was diluted with buffer solution pH 7 with dilution factor equal to 20.

2.3. Stability Test

The fresh extract, with three replications, was scanned in a quartz cuvette with 1 cm path length using UV-VIS Spectrophotometer (Genesys 10uv Thermo Electron Corporation, USA). The λ_{max}, λ_{peak} and $\lambda_{shoulder}$ of the extract were determined and used to calculate the color intensity.

The fresh extract was divided into 2 groups. The first group was bottled without headspace (HS0) and the second group with 50% headspace (HS50). All samples were stored in the dark at 6 temperature levels: 7 (refrigerator), 30, 45, 60, 75, and 90°C. Each sample consisted of three replications.

The color intensity (CI) was $(A_{550} - A_{700}) + (A_{580} - A_{700}) + (A_{628} - A_{700})$. The A_{550}, A_{580}, and A_{628} were the absorbance at 550, 580, and 628 nm that represent the red (AH^+), purple (A), and blue (A^-) species, respectively. The absorbance was subtracted by the absorbance at 700 nm for haze correction.

The total anthocyanin was calculated based on single pH method [12]. The sample of extract (1.5 ml) was adjusted with HCl 1 M to reach pH 1. The absorbance at λ_{max} was determined and subtracted by the absorbance at 700 nm for haze correction.

The samples stored at 7°C were monitored for every 7 days. The samples stored at 30, 45, and 60°C were monitored for every 3, 2, and 1 days, respectively. The samples stored at 75 and 90°C were monitored for every 6 and 1 hrs, respectively.

2.4. Degradation Kinetics

The degradation kinetics of color and anthocyanin were evaluated using zero and first order reaction (Eq. 1 and Eq. 3), then the $t_{1/2}$ was determined.

$$C = Co - k.t\,(\text{zero order}) \quad (1)$$

$$t_{1/2} = Co / 2.k \quad (2)$$

$$C = Co.e - k.t\,(\text{first order}) \quad (3)$$

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

C was the final concentration, Co was the initial concentration, k was constant of reaction rate (h^{-1} or day^{-1}). The activation energy was determined using Eq. 5.

$$k = koe - Ea / RT \quad (5)$$

ko was a pre-exponential factor, E_a was activation energy ($j.mol^{-1}$), R was the ideal gas constant (8.314462175 $j.mol^{-1}.K^{-1}$), T was the absolute temperature (K).

2.5. Statistical Analyses

The statistical analysis used to see the significant effect of headspace to the color intensity and anthocyanin intensity decrease during storage was Wilcoxon signed-rank test with the help of Openstat® software. The regression analyses were examined by using Microsoft Excel software.

3. Results and Discussion

There are three species in equilibrium that contribute to the color of anthocyanin at pH > 2, which are the red flavylium cation (AH^+), purple quinonoidal base (A), and blue anionic quinonoidal base (A^-). However, most anthocyanins only exhibit one band at a visible region that represents a predominant colored species. Thus, the color intensity of an anthocyanin source extract is measured as the absorbance at the peak of the band. The CT extract in the buffer solution pH 7 exhibited one band (λ_{max}) and two shoulder band ($\lambda_{shoulder}$) at the visible region. The λ_{max} appeared at 628 ± 1 nm, while the $\lambda_{shoulder}$ appeared at around 550 and 580 nm, respectively. Therefore, the color intensity was calculated as the sum of the absorbance of λ_{550} (red), λ_{580} (purple), and λ_{628} (blue).

3.1. The Effect of Headspace

The degradation of anthocyanin is comprised of color and chemical degradation that represented by the color intensity (CI) and total anthocyanin (TA) decrease, respectively. The CT extract exhibited very high color and chemical stability in the refrigerator by remaining more than 97% of its color after 56 days (Figure 1). There was no significant difference between the extract bottled without headspace (HS0) and with 50% headspace (HS50). Our work was in accordance with reference [8] that CT extract at pH ≤ 6.0 remained about 80-90% of its color on day 60 when stored at 7°C. Therefore, it is a potential colorant for cold food systems.

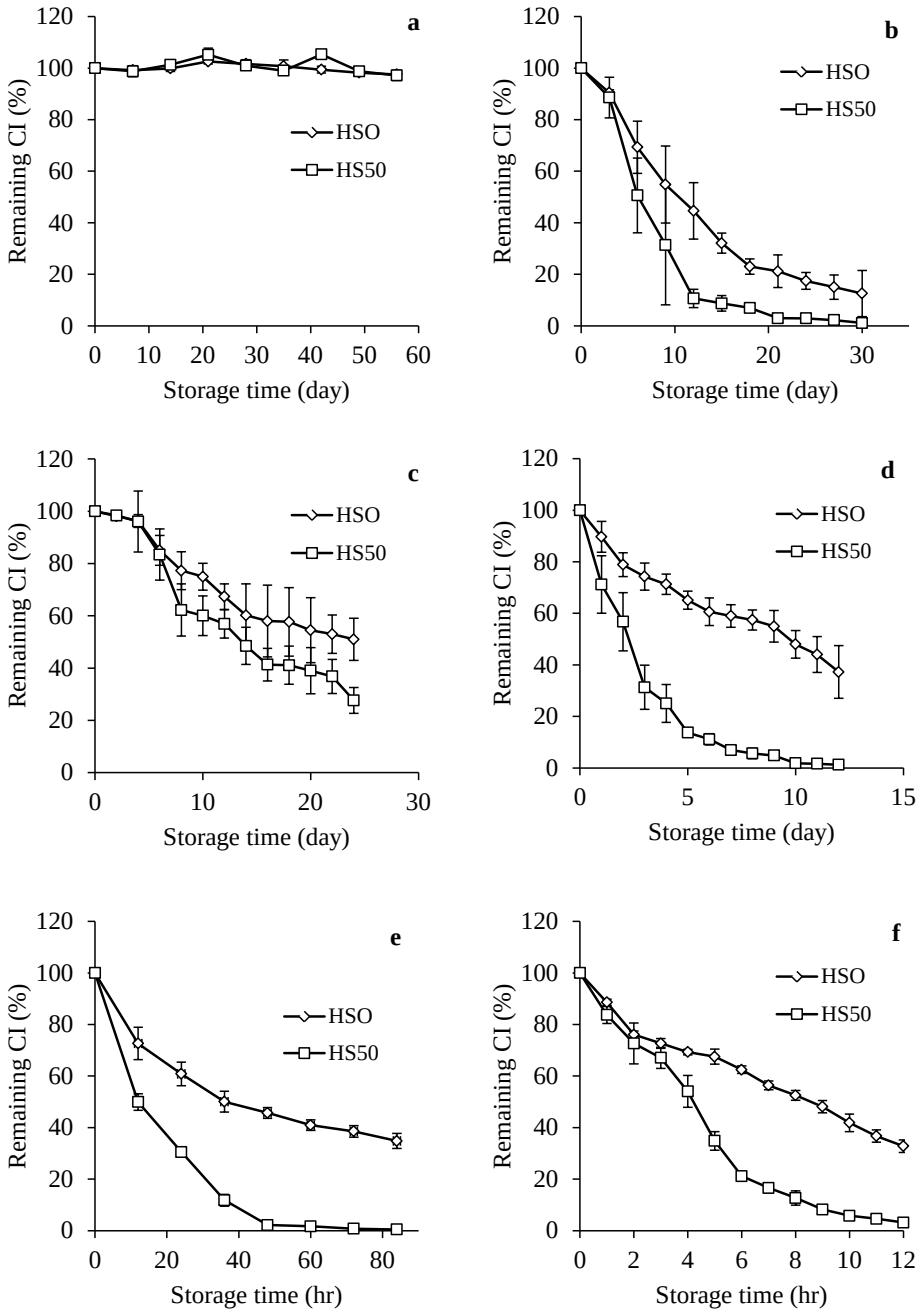

Figure 1. The remaining color intensity (CI) of CT extract at pH 7 during storage in the dark at various temperature (a = 7°C, b = 30°C, c = 45°C, d = 60°C, e = 75°C, and f = 90°C)

At ≥ 30°C, both color and chemical stability of the extracts bottled with 50% headspace (HS50) were significantly lower than those without headspace (HS0) (p-value < 0.01). This was in accordance with our other work that the headspace between the extract's surface and container's cap provoked the color stability of CT extract at various solvent systems at pH 7 [9]. Our other work demonstrated that the effect of headspace was not in association with the gas type in the headspace (unpublished). Hence, we propose that the presence of headspace caused the formation of a hydrophobic surface that led to the unfolding of hydrophobic interactions of the polyacylated anthocyanins.

As already well-known, water tends to minimise its contact with the hydrophobic surface to maintain its hydrogen bond as much as possible (increase the entropy). In this case, water force the hydrophobic group of polyacylated anthocyanins to interact with the hydrophobic surface and the intramolecular copigmentation disrupted. The occurrence of hydrophobic interactions unfolding caused by the hydrophobic surface had been reported in protein [13,14,15,16]. In addition, the presence of headspace in phials or container was reported to accelerating the recombinant fusion protein [17].

3.2. Degradation Kinetics

The degradation rates of CI and TA of CT at ≥ 30°C were can adequately be described by the zero order (data is not showed) and first-order degradation kinetics. However, the better fit was provided by the first order with coefficients of determination (R^2) ≥ 0.90. This was in agreement with other works that anthocyanins degradation can be modelled with the zero or first order kinetics [18,19,20].

Table 1. Kinetic parameters for thermal degradation of CT extract at pH 7

Samples	Temp (°C)	CI	TA	A_{550}	A_{580}	A_{628}
		k, day-1				
HS0	30	0.061 ± 0.0060	0.060 ± 0.0060	0.055 ± 0.0062	0.063 ± 0.0064	0.073 ± 0.0065
	45	0.023 ± 0.0015	0.023 ± 0.0016	0.013 ± 0.0017	0.021 ± 0.0010	0.028 ± 0.0025
	60	0.052 ± 0.0051	0.068 ± 0.0059	0.031 ± 0.0038	0.046 ± 0.0046	0.064 ± 0.0058
	75	0.211 ± 0.0216	0.226 ± 0.0192	0.120 ± 0.0096	0.190 ± 0.0168	0.269 ± 0.0288
	90	1.219 ± 0.1848	1.183 ± 0.1608	0.300 ± 0.1128	0.948 ± 0.1656	1.754 ± 0.2208
HS50	30	0.104 ± 0.0061	0.098 ± 0.0057	0.090 ± 0.0077	0.119 ± 0.0081	0.146 ± 0.0100
	45	0.042 ± 0.0021	0.040 ± 0.0028	0.026 ± 0.0032	0.041 ± 0.0020	0.051 ± 0.0027
	60	0.312 ± 0.0137	0.323 ± 0.0134	0.227 ± 0.0132	0.311 ± 0.0135	0.375 ± 0.0149
	75	1.109 ± 0.1176	1.008 ± 0.1368	0.710 ± 0.0936	1.111 ± 0.1128	1.603 ± 0.0912
	90	4.879 ± 0.8952	5.2820 ± 0.8112	3.346 ± 0.8832	4.596 ± 0.9312	5.748 ± 0.9096
		$t_{1/2, day}$				
HS0	30	11.40	11.55	12.60	11.00	9.47
	45	30.27	30.54	51.73	32.85	24.76
	60	13.33	10.16	22.80	15.07	10.86
	75	3.28	3.07	5.78	3.66	2.58
	90	0.57	0.59	2.31	0.73	0.40
HS50	30	6.67	7.05	7.73	5.84	4.74
	45	16.39	17.24	26.56	17.07	13.56
	60	2.22	2.15	3.05	2.23	1.85
	75	0.63	0.69	0.98	0.62	0.43
	90	0.14	0.13	0.21	0.15	0.12
		E_a, kJ.mol-1				
HS0		84.71	83.21	68.37	81.61	88.01
HS50		99.52	101.15	100.79	99.26	100.33

The heat exposure, both during process and storage, was reported as one of the most influencing factors for accelerating the anthocyanin degradation [11,19,21]. Consequently, the anthocyanin stability decreased as temperature increased. However, an unusual characteristic was demonstrated by the CT extract at pH 7 by exhibiting a lower color and anthocyanin stability (higher k value) at 30°C than at pH 45°C, as seen in Table 1. This unusual characteristic was not appeared in CT extract at pH 3 [4] but occurred at pH 5.7 as reported by [5]. They suspected that the higher degradation rate of CT extract at 27°C and 37°C than at 45°C was probably related to the work of glycosidase enzyme that more active at that temperature range than at the higher temperature. However, our research did not support their claim, as the CT used in this research was steam-blanched for 6 minutes that enough to inactivate the glycoside enzyme [22,23].

We propose that the higher stability at 45°C than at 30°C was in association with the unfolding of hydrophobic interaction. Probably, the temperature increase caused the disruption of hydrophobic surface. Hence, it was not necessary to water to force the polyacylated anthocyanin to interact with the surface. This proposal was supported by the evidence that the smallest effect of headspace occurred at 45°C. The occurrence of the protein denaturation at lower temperature was reported by several studies [24,25].

The dependence of the anthocyanins degradation on temperature was represented by the activation energy (E_a). The high activation energy value indicates a higher sensitivity of the reaction rate to temperature. The E_a of color degradation at 45 to 90°C of the HS0 and HS50 samples were 84.71 and 99.52 kJ/mol, respectively, while the E_a of anthocyanin degradation were 85.02 and 97.51 kJ/mol, respectively. It was clear that the headspace provoked the anthocyanin of CT extract to be more susceptible to temperature elevation. It was also interesting that the E_a of anthocyanin degradation of CT extract at pH 7 was much higher than at pH 3 that only 28.66 kJ/mol [4]. Hence, the CT extract was more sensitive to heat in neutral solution than in acidic solutions.

3.3. The Decrease of A_{550}, A_{580}, and A_{628}

In the most anthocyanins, at a low acidic or neutral condition, the formation of colorless hemiketal is thermodynamically favourable. However, the anthocyanins themselves are fairly stable [10,26]. Therefore, an anthocyanin source extract may lose the color, but the anthocyanin content is retained. In other words, the degradation rate of CI is bigger than the degradation rate of TA. Interesting that the CT extracts performed differently. The degradation rate of CI and TA of the extracts at 30 to 90°C were similar, as represented by the close degradation rate (k) as shown in Table 1. The close degradation rate indicated that the color fading was irreversible, so the colorless substances was not anthocyanins or anthocyanidins but probably had already degraded to benzaldehyde and hydroxybenzoic acid. By this result, it is proposed that the intramolecular copigmentation disruption was not only due to the hydrophobic interaction unfolding, but also caused by the chemical degradation. This was in alignment with our prior work that the degradation of CT extract at pH 7 occurred with the presence of hypsochromic shift (the shift of λ_{max} towards lower wavelength). The hypsochromic shift indicated the occurrence of deacylation [9].

The observation on the degradation rates of A_{550}, A_{580} and A_{628} exhibited an interesting phenomenon. The degradation rate of the absorbance at 628 nm was higher

than the rate of th absorbance at 580 and 550 nm. This result brought to a proposal that the degradation of a polyacylated anthocyanin in CT extract at pH 7 is initiated by the unfolding and the deacylation of the blue anionic quinonoidal base (A^-).

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