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Unpredictable half-life of color in butterfly pea (*Clitoria ternatea* L.) flower extract

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Abstract

Butterfly pea (*Clitoria ternatea* L.) flower is a potential source of anthocyanin for food colorant. However, the stability of the extract in food pH range is still in question. A study was conducted to examine the color stability of butterfly pea flower extract (CTE) at pH 4 to 8. The study included numerous replicates to ensure the reliability of the results. The findings of the study revealed that the color of CTE degraded significantly during storage, and the degradation pattern involved three stages. The first two stages showed signs of intramolecular copigmentation unfolding, while the third stage involved deacylation of ternatins into unacylated anthocyanins. Interestingly, the degradation pattern varied between samples from the same extract. Although most of the degradation could be modeled using first-order degradation kinetics, the estimated half-life of the extract's color showed a wide range of values due to the large standard errors of the kinetic degradation rate. When 150 samples of pH 7 extract were stored for their average half-life of 9 days, their remaining color intensity varied greatly, with most falling outside the standard error limits. The study concluded that a better understanding of the factors contributing to CTE's color degradation is necessary to ensure its reliable use as a natural colorant in various food and beverage applications, given its uncertain stability.

1. Introduction

The butterfly pea (*Clitoria ternatea* L.) flower is an excellent source of polyacylated anthocyanins, known for its high stability and intense blue color in low acidic solutions, making it a valuable ingredient in various food and beverage applications (Marpaung *et al.,* 2019). In 2021, the FDA approved butterfly pea flower extract (CTE) as a food colorant. Studies have shown that the color of CTE may greatly vary after being stored at pH 6 to 8 (Marpaung *et al.,* 2018). One possible reason for this is that the butterfly pea flower extract contains not less than nine types of polyacylated anthocyanins, known as the ternatin series (Terahara *et al.,* 1998; Kazuma *et al.,* 2003), which may experience different degradation rates during storage. Marpaung *et al*. (2017a, 2019) proposed that the degradation of color in CTE occurs through two different pathways: the unfolding of intramolecular copigmentation and deacylation. These differences may contribute to the high variance in color stability of the flower extract during storage.

As CTE continues to gain popularity in the food and beverage industry, it is crucial to understand its stability during storage to ensure its quality and performance. A series of data on the color stability of CTE at pH 4 - 8 during various storage periods has been collected from 2017 to 2022 to (i) confirm the occurrence of high variation in its stability, (ii) assess degradation models to determine its half-life, and (iii) identify the causes of the high variation.

These data will provide valuable insights into the degradation mechanisms of butterfly pea flower extract and allow for the development of strategies to improve its stability during storage. Understanding the factors that contribute to the high variation in color stability will help ensure that CTE can be reliably used as a natural colorant in various food and beverage applications. By continually improving our knowledge of this valuable natural ingredient, we can harness its full potential and meet the growing demand for sustainable, healthy, and natural food ingredients. This study aims to assess the storage stability of butterfly pea flower extract at different pH levels and to clarify the factors and

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mechanisms underlying its high variation in color stability.

2. Materials and methods

The study was conducted over a period of five years from 2017 to 2022. The samples of fresh butterfly pea flowers were harvested from the same garden located in South Tangerang, Banten, Indonesia. The blue portion of the fully opened flower was separated from the white part, steam-blanched for 6 min (Marpaung *et al.,* 2017a), and then subjected to freeze-drying. The dried flowers were ground into a fine powder by a ball mill and sifted through a 250 µm screen. The powder was then stored in an airtight container and kept frozen until further use. Deionized water was procured from a local market called Amidis®, and buffer solutions with varying pH values were purchased from Merck®. The reagents used were all analytical grade and were used without any further purification. The pH 4 buffer solution contained citric acid, sodium hydroxide, and hydrogen chloride, while the pH 5 and 6 buffers contained citric acid and sodium hydroxide. The pH 7 and 8 buffer solutions consisted of disodium hydrogen phosphate and potassium dihydrogen phosphate.

2.1 Preparation of Clitoria ternatea extract

The extraction of butterfly pea petal powder was performed using a modified version of Marpaung *et al*. (2017a) and was consistent across all batches. One gram of powder was mixed with 40 mL of deionized water at a temperature of 60°C for 30 min while continuously shaking and protected from light exposure. The resulting suspension was filtered with a cloth, and the filtrate was collected and then centrifuged at 7000 rpm for 5 min.

2.2 Color stability test

There were three stages of testing for the color stability of CTE. The first stage involved testing the extract at different pH levels, ranging from 4 to 8, while the second and third stages only tested the extract at pH 7.

For the first stage, the extract was diluted in buffer solutions with a pH range of 4 to 8 and packaged in transparent glass bottles with a volume of 20 mL. Each pH extract in each batch consisted of three replicates. These samples were stored at room temperature with light, and their color intensity (CI) was periodically monitored using a UV-VIS Spectrophotometer. The pH 4 and 5 extracts were tested in 4 batches, while the pH 6 to 8 extracts were tested in 8 batches.

In the second stage, the CTE extract was diluted in buffer solution with a pH 7 and packaged in 150 glass bottles with a volume of 3 mL. The samples were then stored at room temperature with light for 9 days, and their CI was measured using a UV-Vis spectrophotometer.

In the third stage, the color stability of four replicate samples at pH 7 was evaluated by storing them at room temperature with light exposure for 9 days. The CI, violet index (VI), and hypsochromic shift of both fresh and stored extracts were measured by analyzing their spectrograms at wavelengths of 400-700 nm using a spectrophotometer. Additionally, any changes in the ternatin composition of both fresh and stored extracts were assessed by using HPLC-DAD.

The CI was determined by the modified formula of a previous study (Marpaung *et al.,* 2017b), while the VI was determined by the method of (Cisse *et al.,* 2012).

$$CI = (A_{550} - A_{700}) + (A_{580} - A_{700}) + (A_{620} - A_{700}) \quad (1)$$

$$VI = (A_{580} - A_{700}) / (A_{520} - A_{700}) \quad (2)$$

The absorbance readings at 550 nm, 580 nm, and 628 nm correspond to the red (AH^+), purple (A), and blue (A^-) species, respectively. To correct for haze, the absorbance readings were adjusted by subtracting the absorbance at 700 nm.

The extract's chromatogram was obtained using an HPLC system (Hitachi, ELITE LaChrom, Japan) with a Symmetry® C18 5.0 µm column (4.6 x 150 mm; Waters Corp., USA). The analytical HPLC was carried out with a flow rate of 1.0 mL/min and an injection volume of 10 µl. The Diode Array Detector L-2455 (Hitachi) was set at 530 nm. The solvent system consisted of a linear gradient elution for 30 min using 40% acetonitrile containing 0.05 M in solvent A (5% acetonitrile containing 0.05 M TFA) with a range of 14% to 86% solvent B.

2.3 Degradation kinetics

The degradation kinetics of CI were evaluated by applying a first-order reaction model and subsequently determining the half-life ($t_{1/2}$).

$$C = C_o.e^{-k.t} \quad (3)$$

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

C represents the percentage of remaining CI, Co represents the initial percentage of CI, which is 100%, and k represents the constant rate of the reaction (in units of day^{-1}).

2.4 Statistical analyses

The significance test of the degradation model was analyzed through regression analysis using Microsoft Excel® (Microsoft 365, USA). The significance level of the analysis was set at $\alpha = 0.05$.

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3. Results and discussion

3.1 Three stages of color degradation

Figure 1 represents the color degradation pattern of three batches of CTE during storage at pH 4-8. Each batch consisted of three replicates. The figure demonstrates a wide variation in the color intensity of CTE during storage, not only between batches but also within the same batch replicates, as reported in previous studies (Marpaung *et al.,* 2018). It is worth noting that the variation tended to increase as the pH increased.

Figure 1 also shows that the color degradation pattern of CTE was not simple. This indicates that the color had undergone a complex degradation mechanism. At least, there were three steps in the color degradation. First, the period of increasing color intensity (from 3 to 6 days), second, a period of slight degradation, and third, a period of sharp degradation. Each of these periods appeared to be related to a shift in the equilibrium between anthocyanin species, the unfolding of hydrophobic interactions, and deacylation, as reported in several previous studies (Marpaung *et al.,* 2017a; 2018; 2019).

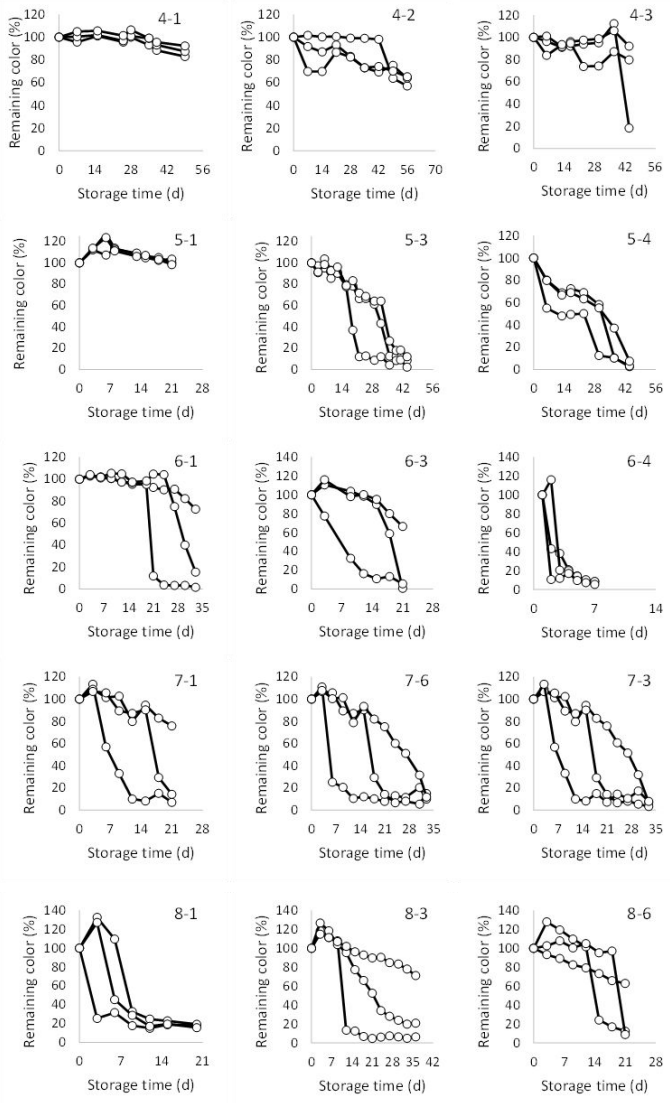

Figure 1. Remaining color (%) of three batches of butterfly pea flower extract pH 4-8, during storage at room temperature. The y-axis is the remaining color, the x-axis is the storage time (d). Each batch consists of three replications.

At pH > 2, anthocyanin exists in an equilibrium state between six species: $C_t \leftrightharpoons C_c \leftrightharpoons B \leftrightharpoons AH^+ \leftrightharpoons A \leftrightharpoons A^-$, with different configurations depending on the pH (Trouillas *et al.,* 2016; Marpaung *et al.,* 2017a). C_t and C_c are trans- and cis-chalcone, respectively, which are yellow in color, B is a colorless hemiketal, AH^+ is a red-colored flavylium cation, A is a purple-colored quinonoidal base, and A^- is a blue-colored anionic quinonoidal base.

At pH 4 to 6, B is the predominant species, so anthocyanin is generally colorless or loses most of its color at that respective pH. In polyacylated anthocyanins, such as the ternatin series in CTE, intramolecular copigmentation blocks the $AH^+ \rightarrow B$ reaction, so it does not experience severe color loss. However, Figure 1 clearly shows that the blocking is not perfect, as some AH^+ is still hydrated into B. This was evident from the increase in color intensity of the extract at all studied pH levels during the early storage period. Even at pH 4 and 5, the color intensity fluctuated repeatedly until the next period, indicating that the $AH^+ \leftrightharpoons B$ reaction continued to occur in the extract during storage. In other words, the folding-unfolding of intramolecular copigmentation occurred repeatedly during storage. $AH^+ \leftrightharpoons B$ is a thermodynamic event, and one of the factors that affect its equilibrium is temperature. The extract was stored at room temperature, and the possibility of inconsistent temperature is very high, thus promoting this folding-unfolding.

During the second stage, a gradual trend of color degradation was observed in all extracts at pH 6-8, and some extracts at pH 4 and 5. During this period of gradual color degradation at pH 4 and 5, the $AH^+ \leftrightharpoons B$ reversible reaction continued to occur. At this stage, it appears that some B did not convert back to AH^+ but underwent tautomerization to become C_c and C_t. Subsequently, C_t underwent deglycosylation to produce anthocyanidin, which then further degraded into simpler compounds, and the color permanently disappeared (Sun *et al.,* 2011; Trouillas *et al.,* 2016). The duration of the second stage varied greatly, but generally lasted longer in extracts with lower pH.

In the third stage, a rapid degradation of color occurs in CTE, indicating the involvement of another color degradation mechanism. This massive color degradation seems to occur through deacylation of ternatins into unacylated anthocyanins, as previously proposed by several studies (Marpaung *et al.,* 2017a, 2018, 2019).

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This stage of color degradation has a variable duration among batches with the same pH, and even within the same batch.

3.2 Degradation kinetics

CTE is a complex material. Various types of ternatins contribute to the color (Terahara *et al.,* 1998; Kazuma *et al.,* 2003). In addition, color degradation can be reversible, caused by the unfolding of intramolecular copigmentation, or irreversible, caused by deacylation and deglycosylation (Yoshida *et al.,* 2009; Sun *et al.,* 2011; Trouillas *et al.,* 2016; Marpaung *et al.,* 2017a, 2019). Therefore, it is reasonable that the degradation pattern of the CTE is complex and should be described through more than one degradation kinetics model. The challenge is whether it is possible, for quality control and determination of half-life or shelf-life, to represent this complexity with only one degradation kinetics model.

Twelve distinct degradation patterns were observed across four batches of color stability experiments conducted at pH 4 and 5. Of these, eight and nine patterns at pH 4 and 5, respectively, were successfully modeled using first-order degradation kinetics, as indicated by a p-value of regression ≤ 0.05. For the extract at pH 6 to 8, 24 degradation patterns were observed, and almost all of them could be modeled using first-order degradation kinetics. However, despite being statistically significant, most of the k values (kinetic degradation rate) had relatively large standard errors, leading to a wide range of values for the 95% confidence interval of half-life. For instance, at pH 4, the estimated half-life of the extract ranged from 11 to 1945 days, at pH 5, it ranged from 6 to 4332 days, and at pH 6, it ranged from 2 to 169 days.

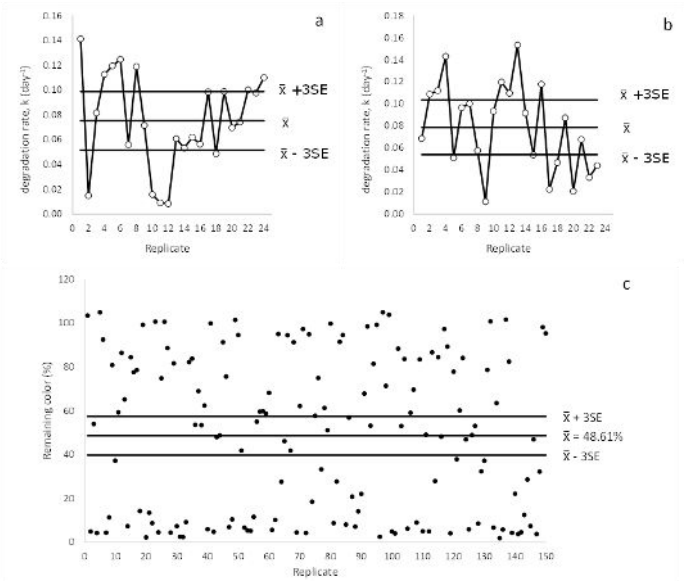

Figure 2. The degradation rate (k) of the color of the butterfly pea flower extract at pH 7 (a) and 8 (b) and the remaining color of the extract at pH 7 after being stored for 9 days at room temperature (c) exhibited a wide range of values, indicating that determining the half-life of the butterfly pea flower extract with high precision is challenging.

Figure 2 illustrates the variation in the rate (k) of color degradation of CTE at pH 7 and 8. As seen in the figure, most of the k values are outside the mean k ± 3 SE (standard error). This large variation emphasizes the difficulty in accurately estimating the half-life of the color of butterfly pea flower extract with high precision.

3.3 Observing remaining color close to the half-life point

The average degradation rate (k) of the color of CTE at pH 7 was 0.0752/day, which means that its half-life on average is 9.22 days. Therefore, this study proceeded to observe the remaining color of 150 samples of CTE at pH 7 after being stored for 9 days at room temperature with light exposure.

The average remaining color is 48.61%, which is close to 50%. However, as predicted, the percentage of remaining color varied greatly, as shown in Figure 2c. Like the distribution of k values previously presented, most of the percentage of remaining color fell outside the range of the average remaining color ± 3 standard errors (SE).

In addition to the high variability in the remaining color, the differences in color were also visually noticeable. This issue highlighted a serious concern regarding the use of butterfly pea flower extract as a food colorant for products that are intended to be displayed for extended periods of time.

3.4 The cause of the uncertain stability

The initial study by Marpaung *et al.* (2018) reported a high variability of remaining colors in CTE at pH 6 to 8 after storage, which they attributed to random deacylation. Another study by Marpaung *et al.* (2019) found evidence of deacylation in CTE at pH 7 after storage. However, there have been no reports on random deacylation.

Various ternatins in CTE are theoretically susceptible to random deacylation. The three most abundant ternatins found in butterfly pea flower extract are ternatin B2 (17.2%), B1 (14.2%), and D1 (13.9%), each of which contains 3, 4, and 4 acyl groups, respectively, as shown in Figure 3 (Kazuma *et al.,* 2003). When water attacks acyl groups at positions 1 and 2, the intramolecular copigmentation of ternatin is lost, resulting in rapid color fading. However, if acyl groups at positions 3 and 4 are attacked, the intramolecular copigmentation configuration remains, which may lead to greater color stability. Consequently, different CTE samples may exhibit varying levels of color stability.

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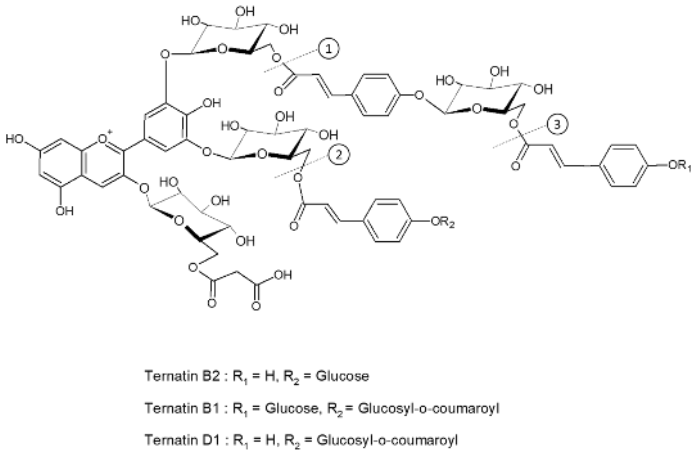
Figure 4 shows the results of analyzing the fresh extract at pH 7 (F) and 4 samples of extract at pH 7 (R1 to R4) that were stored for 9 days at room temperature using spectrophotometric and HPLC DAD techniques. The spectrogram revealed a wide range of the remaining color intensity (hypochromic shift) among the 4 replicates, ranging from 40.42% to 89.28%. Furthermore, there was a shift in absorbance towards shorter wavelengths (hypsochromic shift) with a λmax shift ranging from 4 to 12 nm. The violet index (A_{580}/ A_{520}) also exhibited a varied decrease, indicating a tendency for the extract to become redder in color after storage.



Ternatin B2 : R_1 = H, R_2 = Glucose

Ternatin B1 : R_1 = Glucose, R_2 = Glucosyl-o-coumaroyl

Ternatin D1 : R_1 = H, R_2 = Glucosyl-o-coumaroyl

Figure 3. Chemical structure of Ternatin B2, B1, and D1, showing the three positions of acyl groups that can be attacked by water, resulting in deacylated or unacylated ternatins.

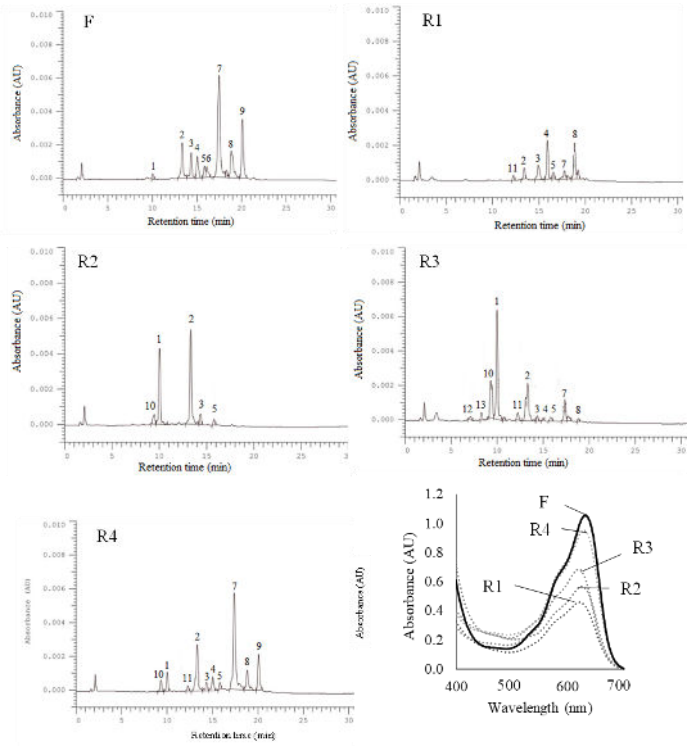


Figure 4. Comparison of chromatogram and spectrogram of fresh butterfly pea flower extract at pH 7 (F) and after 9 days of storage (R1 to R4). The chromatogram demonstrates a significant variance in the ternatins profile, while the spectrogram indicates various hypochromic and hypsochromic shifts.

The chromatogram of the fresh CTE displayed 9 peaks, which represent the 9 common ternatins found in fully bloomed butterfly pea flowers (Terahara *et al.,* 1998). By comparing these peaks to the chromatogram reported by Terahara *et al*. (1998), it can be estimated that peaks 1-9 are ternatin A3, B4, A2, B3, A1, B2, B1, D2, and D1, respectively. Unlike the CTE from other studies (Terahara *et al.,* 1998; Kazuma *et al.,* 2003), which reported ternatin B2 as the most dominant anthocyanin, the CTE used in this study contained ternatin B1 as the most dominant (43%). Ternatins D1 and D2 followed, each comprising 16.11% and 12.96%, respectively.

After 9 days of storage, the total anthocyanin content decreased to around 28.5% to 80.5% of its initial level. The slightly lower color intensity compared to the remaining anthocyanin indicates that color degradation of the butterfly pea extract has reached the stage of chemical degradation (Sun *et al.,* 2011; Trouillas *et al.,* 2016). The higher remaining color compared to remaining anthocyanin indicates the formation of colored species (AH^+, A, and A^-) from colorless species (B).

Valuable information can be obtained from the variation in the chromatogram of the 4 replicate samples after storage. Each sample has a very different chromatogram from the others. For example, peak 7 (Ternatin B1) is still quite high in R4, remaining slightly in R1 and R3, and disappearing in R2. Meanwhile, peak 1 (Ternatin A3) was not found in R1 and instead increased more than 16 times in R2 and R3. Overall, ternatins with more acyl groups (A1, B1, B3, D1, D2) tended to decrease in content. In other words, more complex ternatins were deacylated into simpler ternatins. Based on their chemical structures, the deacylation of ternatin can occur as follows:

A1 → A2 → A3 → C4 → C5

B1 → A2 → A3 → C4 → C5

B3 → B4 → C4 → C5

B3 → C2 → C4 → C5

D1 → B2 → A3 → C4 → C5

D2 → C1 → C4 → C5

D2 → B4 → C4 → C5

The chromatogram shows an increase in the content of ternatin A2, A3, B2, and B4. In addition, new peaks appear at shorter retention times (peaks 10-13), which

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represent simpler ternatins. Referring to Terahara (1998), peak 10 is likely to be ternatin C4. Meanwhile, ternatin C5, which is the end point of all deacylation scenarios, is an unacylated ternatin that is very unstable and quickly degrades into non-anthocyanin compounds, so it is not visible on the chromatogram.

4. Conclusion

In conclusion, this study investigated the color stability of CTE by analyzing a large number of replicates. The degradation of color extract was found to exhibit a complex pattern, consisting of three stages that cannot be accurately represented by a single degradation kinetics model. Furthermore, a wide variation in remaining color was observed in CTE with pH values ranging from 4 to 8, making the determination of the half -life highly imprecise. The uncertainty in the color stability of CTE was shown to be caused by random deacylation, which was proven through chromatographic analysis.

Conflict of interest

The authors declare no conflict of interest.

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