EFFECT OF MICELLES AND pH ON STABILITY OF *Clitoria ternatea* COLOR EXTRACT

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ABSTRACT: Petal of *Clitoria ternatea* L. is an interesting source of natural colorants for pharmaceutical use. This work was to study the influences of pH and micelles on the color stability of *Clitoria ternatea* extract. The color changed from red to blue over pH 4 to 10. Degradation rate at various pH was in the order: pH 10 > 7 > 4 (% color remaining of <30, 50 and 80 at day 60, respectively). At pH 10 and 7 in the presence of micelles, color instability was in the order: cationic micelles > anionic micelles > nonionic micelles > buffer. At pH 4 in the presence of micelles, color instability followed the order: cationic micelles > nonionic micelles ~ buffer > anionic micelles (% color remaining of 47, 80, 80 and 85 at day 60, respectively). The color extract is stable at pH 4 but unstable in the presence of cationic micells at all pH.

Keywords: *Clitoria ternatea* color extract, Natural colorant, Micelles, pH, Stability

INTRODUCTION

Natural colorants are also good candidates in pharmaceutical use. Anthocyanins are a very popular group of natural colorants which are used in food and cosmetic products. They do not only provide beautiful red to blue color but also provide antioxidant ability [1, 2]. Naturally, different forms of anthocyanins coexist in equilibrium depending on temperature, pH and solvent of the system and consequently provide different shade and intensity. The concurrently existing forms are the quinonoidal base (A) providing blue color, the flavylium cation (AH^+) giving red color, the coloress carbinol pseudobase (B) and the off-yellow chalcone (C) as shown in schemes 1-3 [3, 4]:

$AH^+ \rightleftharpoons B + H^+$ hydration equilibrium scheme 1

$B \rightleftharpoons C$ tautomeric equilibrium scheme 2

$AH^+ \rightleftharpoons A + H^+$ acid-base equilibrium scheme 3

Each source of anthocyanins provides anthocyanins containing different aglycone structures and conjugated sugars. According to the molecular structure, some anthocyanins are more or less stable than others [5, 6]. Butterfly pea *(Clitoria ternatea* L.) is widely grown in Thailand. Butterfly pea petal contains ternatins, (poly) acylated anthocyanins with delphinidin skeleton (Figure 1). Colorant from the petal has been used as a colorant in food and cosmetic products which contained surfactants. Chemical stabilities are altered in surfactant solutions where micelles are formed due to complexity of the systems [7-12]. However, stability of this colorant in the presence of surfactants has never been reported.

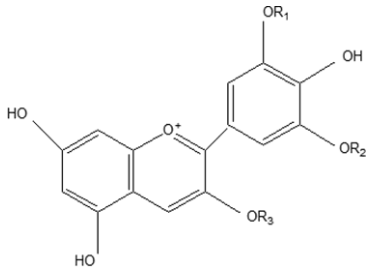

Figure 1 Anthocyanins found in butterfly pea petal; R1 and R2 are glucose with combinations of CGCG-CGCG, CGCG-CG, CGCG-CGC, CGC-CG, CGC-CGC, CGC-C; C= p-coumaric and residue; G=D-glucose residue; R3= glucose-CO-C-CO-OH

The purpose of this work was to study the stability of color from the aqueous extract of *Clitoria ternatea* petal. Factors under investigation included (1) pH (under acidic, neutral and alkaline conditions) and (2) the effect of cationic, anionic and nonionic micellar systems on color stability under various pH values. This information will be employed in order to design appropriate condition for applying this colorant in food, pharmaceutical and cosmetic products.

MATERIALS AND METHODS

Chemicals

Cetyltrimethylammonium bromide (CTAB) and polyoxyethylene 20 sorbitan monooleate (Tween 80) were purchased from Sigma Chemical Co., MO, USA. Monobasic sodium phosphate monohydrate, dibasic sodium phosphate and sodium bicarbonate were purchased from Merck KGaA, Darmstadt, Germany. Sodium lauryl sulfate (SLS) was purchased from Fluka, Japan. Succinic acid was purchased from Carlo Erba, Rodano, MI, Italy. Sodium hydroxide was received from Mallinckrodt, Mexico. The chemicals were analytical grade and used as received.

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Preparation of color extract

Clitoria ternatea flower was collected from the botanical garden at the Faculty of Pharmaceutical Sciences, Chulalongkorn University, Bangkok, Thailand. Freeze dried colorant powder was prepared from an aqueous extract of the petal. Thirty grams of fresh *Clitoria ternatea* petal were ground and 50 mL ultra purified water was added. The ground petal was soaked for 1 hr and filtrated through a filter paper No. 1 (Whatman International Ltd., Maidstone, England). The residue was soaked for 1 hr using another 50 mL ultra purified water and filtrated through the filter paper. Both filtrated extracts were collected and freeze dried (Dura-dry FTS Systems™, Cambridge, USA).

UV characteristics of *Clitoria ternatea* color extract at various pH

The freeze dried colorant was dissolved in and diluted to a 2.5 mg/mL concentration using 0.1 M succinic acid. Fifty milliliters of the solution was placed in a double jacketed vessel in which the temperature was controlled at 25±1 ^{o}C. The solution was then titrated with 20 M sodium hydroxide solution. The solution was well mixed using a magnetic stirrer and continuously pumped from the vessel through a UV flow-through cell using a peristaltic pump (Ismatec, Switzerland) and returned to the vessel. The UV flow-through cell was placed in a Shimadzu UV-spectrophotometer Model 1601 (Japan). A pH meter (model 420 A, Orion, MA, USA) was used to monitor pH change of the solution in the vessel until the pH reached approximately 10. Both pH and UV spectrum were taken after each increment of sodium hydroxide solution.

A solution series of the colorant was prepared at different concentrations (1.0×10^{-4} g/mL – 6.0×10^{-4} g/mL) in the buffered solutions at pH 4 (succinate buffer), 7 (phosphate buffer) and 10 (carbonate buffer) in the absence or presence of micelles. Total buffer concentration was kept constant at 0.1 M. Surfactant concentrations were 7.29×10^{-4}, 5.77×10^{-2} and 2.62×10^{-4} g/mL for CTAB, SLS and Tween 80, respectively, which are higher than the critical micelle concentrations [7]. Spectra of the samples were collected and plots of absorbance versus concentration were constructed. Absorptivities were then determined from the slopes.

Stability of *Clitoria ternatea* color extract at various pH

The colorant was prepared at 2.5 mg/mL concentration in 0.1 M succinate buffer solution (pH 4), phosphate buffer solution (pH 7) or carbonate buffer solution (pH 10). Each solution contained 5×10^{-4} g/mL methylparaben and 5×10^{-5} g/mL propylparaben as preservatives [13]. The solutions were placed in amber glass air-tight vials and incubated in a controlled temperature chamber (30±1 ^{o}C). At appropriate time intervals, UV spectra, absorbances at the analytical wavelenghts and pH of the samples were collected up to two half-lives or 60 days whichever came first. Each experiment was done in triplicate. At the maximum absorption wavelength (λ_{max}), % color remaining was obtained from the following (equation 1).

$$\%\ \text{color remaining} = \frac{\text{absorbance at time } t}{\text{absorbance at initial}} \times 100 \quad \text{equation 1}$$

Along with UV spectrophotometry, a digital imaging method was modified and employed in order to monitor color change using CIE Lab system (Commission internationale de l'éclairage or International Commission on Illumination) [14]. The sample was placed in a clear 10-mL vial and pictures of the sample were captured by a digital camera (3 mega-pixel) under proper controlled lighting. The sample was placed at 15 cm in front of the camera and three pictures were captured. The pictures were transferred to a computer set loaded with Adobe® Photoshop® CS version 8.0. The software was set for determining Lab color mode (L*a*c*) in Info Palette. Five readings were taken from each picture. The Lab color mode reports a lightness component (Luminance, L*) that can range from 0 to 100 (black-white). In this palette, the a* component (green-red axis) and the b* component (blue-yellow axis) can range from -120 to +120. Chroma (C*), hue (H*) and CIE total color difference between two samples (ΔE^*_{ab}) were obtained from the following equations (equation 2-4) [15, 16].

$$C^* = (a^{*2} + b^{*2})^{1/2} \quad \text{equation 2}$$

$$H^* = \tan^{-1}(b^*/a^*) \quad \text{equation 3}$$

$$\Delta E^*_{ab} = (\Delta L^{*2} + \Delta a^{*2} + \Delta b^{*2})^{1/2} \quad \text{equation 4}$$

C*represents color intensity; H value in degrees from 0 to 360^{o}, where 0^{o}, 90^{o}, 180^{o}, 270^{o} and 360^{o} are red, yellow, green and blue colors, respectively; ΔE^*_{ab} indicates color difference between two samples or between two time points.

Stability of *Clitoria ternatea* color extract in micellar solutions at various pH

CTAB, SLS and Tween 80 solutions were prepared at concentrations of 7.29×10^{-4}, 5.77×10^{-2} and 2.62×10^{-4} g/mL, respectively. The micellar solutions were prepared using the same buffer systems as mentioned above. The freeze dried colorant was dissolved in the micellar buffered solutions to give 2.5 mg/mL concentration. The solutions were kept and monitored in the same manner as mentioned above. This experiment was done in triplicate.

RESULTS AND DISCUSSION

UV characteristics of *Clitoria ternatea* color extract at various pH

UV spectra of the sample solutions consisted of changes in color and color intensity at various pH values (Figure 2). The observed difference in color depends mainly on whether the flavylium cation

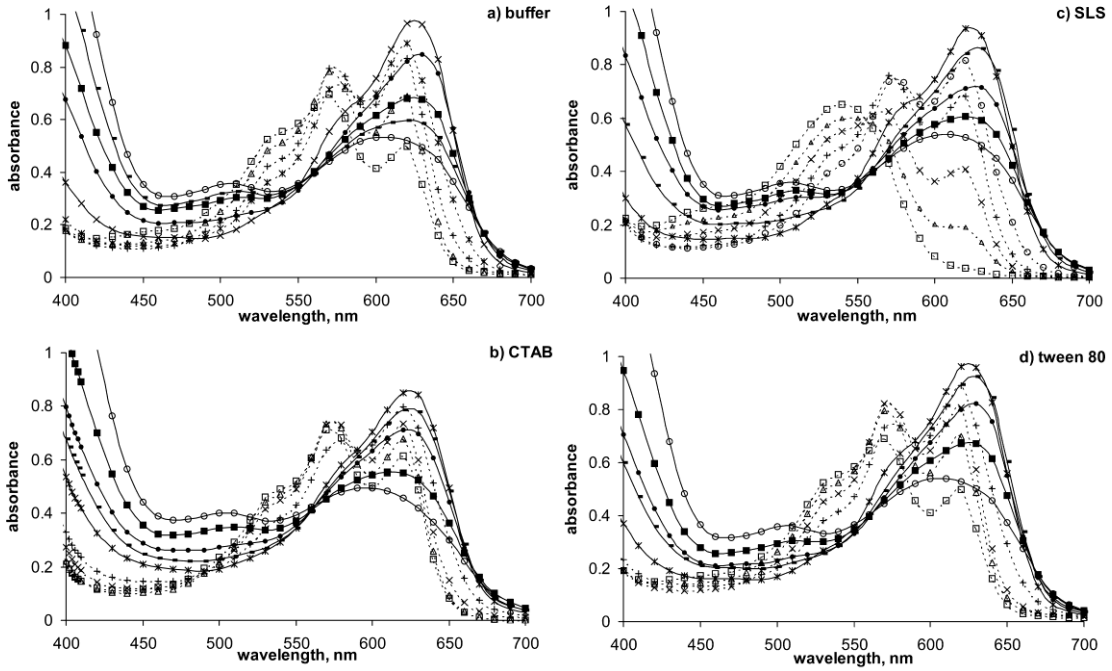

Figure 2 UV spectra of *Clitoria ternatea* extract in various pH solutions without and with micelles. a) buffer: - -(□)- - 3.17, - -(Δ)- - 3.87, - -(+)- - 5.48, - -(*)- - 6.12, —(X)— 6.99, —(•)— 8.05, —(■)— 8.49, —(-)— 8.99, —(o)— 10.12; b) CTAB: - -(□)- - 3.16, - -(Δ)- - 3.87, - -(X)- - 5.04, - -(+)- - 6.01, —(*)— 7.09, —(-)— 7.48, —(•)— 7.82, —(■)— 8.52, —(o)— 10.12; c) SLS: - -(□)- - 3.16, - -(Δ)- - 3.87, - -(X)- - 4.37, - -(+)- - 5.11, - -(o)- - 6.00, —(*)— 7.00, —(-)— 7.98, —(•)— 8.47, —(■)— 9.30, —(o)— 10.05; d) Tween 80: - -(□)- - 3.18, - -(Δ)- - 3.88, - -(X)- - 5.03, - -(+)- - 6.06, —(*)— 6.96, —(-)— 7.77, — (•)— 8.16, —(■)— 8.61, —(o)— 10.00

Table 1 Peaks and absorptivites of *Clitoria ternatea* color extract in wavelength range 400 - 700 nm in various buffered solvents

system	pH					
	4		7		10	
	peak, (nm)	absorptivity, (cm^2/g)	peak, (nm)	absorptivity, (cm^2/g)	peak, (nm)	absorptivity, (cm^2/g)
buffer	574	1487.12	624	2023.83	606	1216.04
	618	1342.04				
CTAB	574	1518.38	624	2214.3	596	1087.87
	618	1360.06				
SLS	566	1120.96	624	1949.89	606	1255.31
	618	619.28				
Tween 80	574	1526.41	624	2022.91	606	1215.85
	618	1376.21				

(AH^+) or quinonoidal base (A) form of anthocyanin is a major component in the system [17]). In acidic solutions, the sample turned red-purple with two UV absorption maxima and as solution pH was increased, the sample turned purple-blue with a UV absorption maximum (Table 1). The observed color change is explained by the acid-base equilibrium of anthocyanins in solution as depicted in Scheme 3. In addition, as pH increases from pH 7 to 10, deprotonation of the phenolic groups in the quinonoidal base yields a mixture of quinonoidal anions (A^-) [3, 6].

Color and UV spectra of *Clitoria ternatea* extract in micellar solutions were different from that in the absence of micelles at the same pH values (Figure 2 and Table 1). In SLS micellar solutions at pH 3.16, UV spectrum of the sample showed only one absorbance peak at 540 nm when compared to two absorption peaks at 574 and 618 nm in solutions without micelles. The shift of UV maxima in the presence of SLS micelles at low pH is likely due to ionic interactions between the negatively charged SLS micelles and the flavylium cations (AH^+) of anthocyanins in the colorant. SLS micelles stabilize AH^+ and decrease the deprotonation rate constant in scheme 3. In the presence of CTAB micelles in alkali pH, the absorption maxima of sample shifted from 606 to 596 nm, which is likely due to ionic

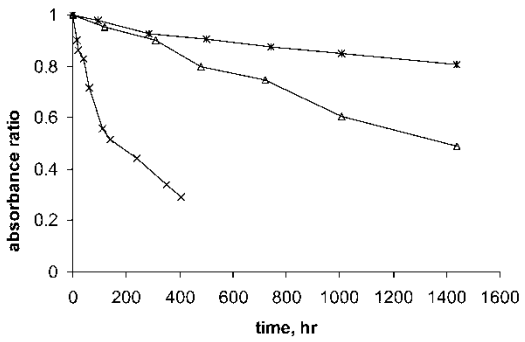

Figure 3 Effect of pH on the degradation of anthocyanins over storage time: (*) pH 4, (Δ) pH 7, (X) pH 10

interactions between the cationic micelles and the quinonoidal anions (A^-) of anthocyanins. In the presence of non-ionic micelles, the UV spectra were almost identical to that in the absence of micelles.

Stability of *Clitoria ternatea* color extract in solutions at various pH

At pH 10, color remaining was less than 30% after 17 days while 50% and 80% of color remained after 60 days in pH 7 and 4 solutions, respectively (Figure 3). Loss of color in the solutions can be explained by anthocyanin stability. Anthocyanins in the samples at pH 4 were more stable than that at pH 7 and 10.

The mechanism could be neucleophilic attack of water on anthocyanins to produce the colorless carbinol pseudobases and off-yellow chalcones [5]. Hydration of AH+ under acidic conditions giving rise to the colorless carbinol pseudophase (B) is unfavorable (scheme 1). In neutral and alkaline media, the unstable quinonoidal base (A, blue) is the predominant species and AH+ also favorably undergoes hydration and gives the colorless B and C (scheme 1 and 2) providing pale yellow color.

Stability of *Clitoria ternatea* color extract in micellar solutions at various pH

Both pH and presence of micelles affect the color remaining in solutions (Figure 4). In the absence and presence of micelles, the color fading rate at various pH values was in the order: pH 10> pH 7> pH 4. CTAB micelles dramatically accelerated color instability at all pH values. Only SLS micelles at pH 4 slightly stabilize the colorant since color remaining after 60 days (85%) was significantly greater than that in buffer solution (80%), student t-test analysis at α=0.05.

The micellar pseudophase approach was used for explaining the above results. The microheterogeneous nature of micelles significantly alters the reactivity of anthocyanins through two distinct factors, 1) electrostatic and 2) micellar medium effects [18]. The electrostatic interaction between the micellar surface charge and the positively-charged species (AH+) is stronger than the interaction with the uncharged species, B and C. As a result, if the micelles are negatively charged, the electrostatic effect reduces the probability of neucleophilic attack of water on AH+ species. Additionally, the electrostatic interaction shifts the ionic dissociation equilibrium of anthocyanins so that the AH^+ species is the preferred species causing a shift in the apparent pK_a of AH^+. If the micelles are positively charged, the opposite of the aforementioned takes place. The micellar medium effect is described by low dielectric environment at the palisade layer and in the core of micelles compared to the high dielectric environment of water [19]. The low dielectric environment of the micelles is not suitable to accommodate the colored AH^+ species but proper to house the A, B and C species. The AH^+ species in the continuous phase undergoes hydration and forms B and C.

As a result, the color stability of anthocyanins was worsened in the positively charged CTAB micelles due to both factors. Apparently, the electrostatic effect of anionic SLS micelles predominates under acidic condition, where AH^+ is the predominant species. Under neutral and alkaline conditions in the presence of CTAB, SLS or Tween 80, H^+ concentration is very low; therefore, the reaction scheme was preferably shifted towards the formation of B (scheme 1). In other words, only the micellar medium effect exists. The different extent of anthocyanin degradation in micellar systems is likely due to the different affinities in each system.

CIE Lab parameters are summarized in Table 2. A decline of a^* and an increase in b^* values was observed over time. The increase in L^* is also an indication of formation of colorless compounds and the increase in ΔE^*_{ab} represents overall color change of samples. CIELab technique also confirmed that the presence of CTAB micelles facilitated color fading at all pH and yielded the highest ΔE^*_{ab} values. At pH 4, a high a^* value, representing red color, was observed in the SLS micellar system while the lowest value was achieved in the CTAB micellar system. This finding was consistent with the result from the UV spectrophotometric technique. The greatest change in b^* values was observed in samples at pH 7 and 10 where the b^* values were negative at the beginning and reached positive values at the end. This implies that the colored substrates turned to the colorless compound B. Furthermore at pH 10, B further degraded to C through the aforementioned base catalyzed reaction. Therefore, the b^* values were positive resulting in an increase in the C^*values over time at pH 10. In the high pH range (7-10) with and without micelles, the significant change of H* value represented a shift in color of samples upon storage. However, this change in the H* value was not observed at pH4 since, firstly, the degradation rate was slow and, secondly, the colorless B species was a major degradation product. Thus at pH 4, H* value of solutions was considerably constant while C^*value decreased.

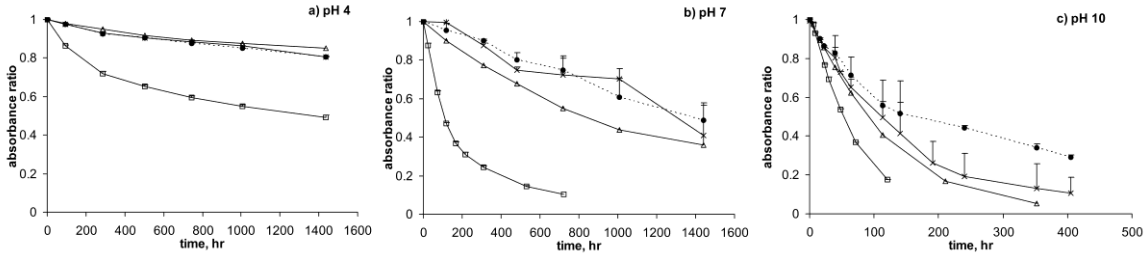

Figure 4 Effect of micellar solutions on degradation of anthocyanins over storage time:- -(●)- - buffer, —(□)— CTAB, —(Δ)— SLS, —(X)— Tween 80

Table 2 Calculated CIE Lab parameters comparing the values before and after storage time

system	parameter	time	buffer	CTAB	SLS	Tween 80
pH 4	ΔE±SD	60 days	14±1	28±1	13±1	14±2
	C*±SD	initial	55±2	48±1	49±2	55±2
		60 days	42±1	23±1	45±1	41±2
	H*±SD	initial	292±1	287±0	317±1	291±1
		60 days	292±1	284±0	330±0	291±1
pH 7	ΔE±SD	30 days	13±1	41±2	22±1	18±2
		60 days	25±1	-[a]	37±1	36±1
	C*± SD	initial	35±2	26±1	42±1	36±1
		30 days	24±1	15±2	20±1	22±2
		60 days	13±1	-[a]	7±1	10±1
	H*± SD	initial	259±1	241±2	268±3	261±1
		30 days	247±3	113±1	246±2	238±2
		60 days	224±2	-[a]	208±5	181±1
pH 10	ΔE± SD	5 days	22±1	45±2	24±1	22±2
		17 days	34±1	-[a]	44±1	39±1
	C*± SD	initial	12±2	14±2	12±1	12±1
		5 days	18±1	29±1	19±1	16±1
		17 days	25±1	-[a]	25±1	27±1
	H*± SD	initial	200±3	170±2	197±6	202±3
		5 days	125±3	111±2	124±3	125±3
		17 days	109±1	-[a]	110±0	109±0

[a] = not available since samples past two half-lives before the date

CONCLUSIONS

Both UV spectrophotometry and CIE Lab methods were successfully utilized to study the stability of color extract from *Clitoria ternatea* petal. Shade of the color from *Clitoria ternatea* petal as well as stability depends on pH and surface charge of micelles in the system. The colorant is most stable at pH 4 in the presence of SLS micelles. The cationic micelles destabilized the colorant at all pH.

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