Effects of storage conditions on total anthocyanin content of Butterfly pea flower (Clitoria ternatea L.)

T N Pham,1, *, X T Le2, P T N Nguyen1, T H Tran1,3, T P Dao1, D H Nguyen4, V T Danh5, H L T Anh6

1NTT Hi-Tech Institute, Nguyen Tat Thanh University, Ho Chi Minh City, Vietnam
2Department of Chemical Engineering, HCMC University of Technology, VNU-HCM, Ho Chi Minh City, Vietnam
3Center of Excellence for Biochemistry and Natural Products, Nguyen Tat Thanh University, Ho Chi Minh City, Vietnam
4Institute of Applied Materials Science, Vietnam Academy of Science and Technology, Ho Chi Minh city, Vietnam
5BKU Institute of Advanced Applied Science and Technology (BKIST), Ho Chi Minh City, Vietnam
6Mientrung Institute for Scientific Research, Vietnam Academy of Science and Technology, Vietnam
*Corresponding author: phamtrinhut96@gmail.com, labasm2013@gmail.com

Abstract. The aim of this research is to evaluate the stability of anthocyanins in extracts obtained from the flower of butterfly pea flower (Clitoria ternatea L.). Different combinations of solvents were created by mixing ethanol 50%, distilled water and different amounts of acids (ethanol 50 %, ethanol 50 % with 1.5N HCL, ethanol 50 % with 1.5N CH₃COOH, distilled water, distilled water with 1.5N HCL, distilled water with 1.5N CH₃COOH). To evaluate the stability of extracted pigment, it was kept for 3 months in oven at 45°C, ambient 25°C and refrigerated conditions at 10 °C. The temperature exerted dramatic effects on the total anthocyanins properties of the butterfly pea flower when stored in these conditions. The retained anthocyanin content of all treated samples was all higher than 60%, with distilled water 50% of 1.5N HCl (61.93%) and distilled water with 1.5 N CH₃COOH (65.59%).

1. Introduction
The pigments of natural origin have received a great deal of public attention because people are aware of their safety in food, cosmetics, and pharmaceuticals. [1–9]. Besides, people also realize the toxic effect of synthetic dyes existing in manufactured fabrics on health as well as to the environment. Therefore, the natural coloring matter extracted from nature has been researched and developed. Butterfly Pea flower is one of the richest sources of natural pigments. Clitoria ternatea L., also called butterfly pea flower is a plant that is native in subtropical regions. The plant is geographically distributed and cultivated in various areas and continents in the world. It is easy to identify butterfly pea flower by the green color of the petals. Butterfly pea is recognized as an abundance source of dietary fiber, vitamins, minerals and bioactive compounds such as organic acids, phytosterols and polyphenols. Moreover, fifteen of ternatins comprising...
mainly malonylated delphinidin 3,3',5'-triglucosides [10,11] with 3',5'-side chains with alternative D-glucose and p-coumaric acid units and delphinidin 3-O-(2"-O-D-rhamnosy1-6'-O-malonyl-D-glucoside have been found in butterfly pea [12,13]. Flavonoids in the butterfly pea flowers could act as an anti-infection agent in the upper respiratory tract [14]. Chemical composition of butterfly pea was also revealed in another study where five main structures of anthocyanins, which are high-acylated delphinidin derivatives, were discovered using HPLC [15].

Health benefits of anthocyanin have been recently reviewed and evaluated. It found that the benefits conferred by intrinsically consuming anthocyanin might vary depending on species, environmental and agronomic factors. For example, thermally treated foods may significantly contain anthocyanins at a much lower quantity in comparison with untreated food [16–18]. Seemingly, the processing temperature is not the sole determinant of anthocyanin stability, but rather, belongs to a group various process parameters including pH, light, storage temperature, presence of enzymes, chemical structure [19]. The purpose of the study is to investigate the effects of storage conditions on the stability of anthocyanin.

2. Material and methods

2.1 Materials

The present investigation was carried out at Center of Excellence for Green Energy and Environmental Nanomaterials, Nguyen Tat Thanh University, Ho Chi Minh city, Vietnam. The raw material required for the experiment were grown in Vinh Long province, Viet Nam. Ethanol was purchased from Sigma Aldrich (US). Whereas, hydrochloric acid, acetic acid and acid nitric were purchased from Xilong Chemical Industry Co. Ltd. (Chengdu, China).

2.2 Degradation Studies

The thermal degradation of CLs extract was investigated at refrigerated (10 °C), temperature room (25 °C), and oven (45 °C) for 90 days under different conditions of extract. Dried petals of butterfly pea were ground using a commercial grinder (sunhouse SHD5322) and were weighed. Then, the material was put in the two necks round bottom flask and was extracted by sequentially changing the solvents. Different types of solvents which was combined between ethanol, distilled water and acid were used for extraction of anthocyanin from *Clitoria ternatea L*. Solvents used are pure ethanol 50 %, pure distilled water and ethanol, distilled water mixed with different acid (hydrochloric acid, acetic acid and acid nitric). Conduct sampling determine anthocyanins content after 0, 20, 40, 60 and 90 days respectively.

2.3. Determination of total monomeric anthocyanin content

The pH-differential method with two buffer systems: potassium chloride buffer, pH 1.0 (0.025 M), and sodium acetate buffer, pH 4.5 (0.4 M) was used to determine total anthocyanin content (TAC). For this analysis, 051 mL aliquot of the extract was diluted to a 9.5 mL with corresponding buffer and the absorbance was measured at 510 and 700 nm. Total anthocyanins were calculated as cyanidin-3-glucoside according to the following equation: Total anthocyanins (mg/L) = A × MW × DF × 1000 / (ℇ × 1); where: A = (A_{λvis max} - A_{700nm})_{PH1.0} - (A_{λvis max} - A_{700nm})_{PH4.5}; MW(molecular weight) = 449.2 g/mol for cyanidin-3-glucoside; DF = dilution factor; 1 = pathlength in cm; ℇ = 26,900 msolar extinction coefficient in L/mol/cm for cyanidin-3-glucoside; 1000 = conversion from g to mg [20,21].

2.4. Statistical analysis

Results are expressed as mean ± standard deviation. ANOVA was also adopted a statistical significance was recognized as p<0.05
3. Results and discussion

Table 1 represents the significant difference among the treatments for the anthocyanin content present in extracted pigment which are stored in temperature room (25 °C) condition. The highest anthocyanin was found in the treatment Ethanol 50 % with 1.5N HCL (25.54±0.95 mg L−1) after 90 days which was decreased from 46.34±0.89 mg L−1 at time zero (T₀). The lowest was found in the T₁- Ethanol 50 % (15.22±0.54 mg L−1) at 90 days which was reduced from 47.59±0.59 mg L−1 at T₀. The accelerated degradation of anthocyanin under high temperature could be due to hydrolyzation of 3-Glycoside structure, which plays a vital role in protecting unstable anthocyanin [22,23].

Results presented Table 2 and 3 reveal that after 90 days, the maximum and minimum anthocyanin content in treatment ethanol 50 % with 1.5N HCL (T₂) were 31.27±0.65 mg L−1 and 4.84±0.43 mg L−1 obtained at refrigerated (10 °C) and oven (45 °C), respectively. This result show that the storage temperature increase in pH, prolonged storage time, which had a significant effect on the stability of anthocyanin. The previous study shows the extraction method and the stability of the color extracted from the strawberries also showed similarities. In terms of storage capacity, ethanol 50% with 1.5N HCl (67.47%) and ethanol 50% with 1.5N CH₃COOH (60.84%) is similar to distilled water with 1.5N CH₃COOH (65.59%) and distilled water with 1.5N HCL (61.93%) at refrigerated (10 °C). This suggests that pH is one of the factors that affect the color fastness and color of anthocyanins. For the combined solvent with water as the main factor, the significant difference between the results is shown in Table 2 and Table 3. It is clearly show that elevated temperature is associated with declining TAC. Moreover, TAC degradation was also accelerated with higher temperature. Therefore, high temperatures in an extended period should be avoided during the processing and storage of anthocyanin color from pea flowers and their products. This result is in line with a previous study that proved that the TAC content in blueberry was found to be declining with storage decreased through storage time.

| Table 1. Effect of extraction methods on anthocyanin content (milligram cyd-3-glc L−1) during temperature room (25 °C) at different types of solvent | T₀       | 30 days   | 60 days   | 90 days   | Retention (%) |
|---------------------------------------------------------------|---------|-----------|-----------|-----------|---------------|
| T₁ Ethanol 50 %                                                | 47.59±0.59 | 32.32±0.64 | 24.22±0.35 | 15.22±0.54 | 31.98         |
| T₂ Ethanol 50 % with 1.5N HCL                                   | 46.34±0.89 | 37.19±0.25 | 29.22±0.48 | 25.54±0.95 | 55.11         |
| T₃ Ethanol 50 % with 1.5N CH₃COOH                              | 36.57±0.67 | 29.23±0.22 | 17.79±0.35 | 18.22±0.74 | 49.82         |
| T₄ Distilled water                                             | 52.35±0.30 | 43.25±0.54 | 32.22±0.61 | 21.64±0.25 | 41.33         |
| T₅ Distilled water with 1.5N HCL                               | 56.61±0.65 | 51.77±0.24 | 44.32±0.35 | 27.56±0.48 | 48.68         |
| T₆ Distilled water with 1.5N CH₃COOH                           | 58.36±0.47 | 49.63±0.23 | 38.09±0.51 | 24.98±0.46 | 42.80         |
The pH of the extracted pigment was increased and decreased respectively at temperature room (25 °C) and refrigerated (10 °C) storage conditions compare to initial (T0) towards 90 days of (Table 4,5 and 6). The highest pH 4.23 and 4.21 was recorded during 90 days in treatment T1- ethanol 50 % at temperature room (25 °C) and oven (45 °C) storage condition respectively at table 4,5,6. In parallel with the increase in pH, the anthocyanin content also increased. It is concluded that higher pH is associated with significant destruction of anthocyanin in samples. The pigment is unstable and could bond to water, forming chromenol, a colorless compound. Based on the results, the pH between 3 and 4 induced more significant anthocyanin degradation than pH in the range of 0 and 2d. This is in good with another study, which also showed that increasing pH, temperature, or exposure to light could make anthocyanin molecules less stable [25].

Table 2. Effect of extraction methods on anthocyanin content (milligram cyd-3-glc/L) during refrigerated (10 °C) at different types of solvent

| Treatments                        | T0     | 30 days | 60 days | 90 days | Retention (%) |
|-----------------------------------|--------|---------|---------|---------|---------------|
| T1 Ethanol 50 %                   | 47.59±0.59 | 44.57±0.21 | 39.15±0.24 | 25.12±0.32 | 52.78         |
| T2 Ethanol 50 % with 1.5N HCL     | 46.34±0.89 | 43.21±0.32 | 38.76±0.44 | 31.27±0.65 | 67.47         |
| T3 Ethanol 50 % with 1.5N CH3COOH| 36.57±0.67 | 31.51±0.37 | 27.53±0.29 | 22.25±0.18 | 60.84         |
| T4 Distilled water                | 52.35±0.30 | 49.18±0.33 | 44.89±0.71 | 30.54±0.31 | 58.33         |
| T5 Distilled water with 1.5N HCL | 56.61±0.65 | 53.27±0.42 | 48.20±0.25 | 35.06±0.68 | 61.93         |
| T6 Distilled water with 1.5N CH3COOH | 58.36±0.47 | 53.26±0.63 | 47.74±0.29 | 38.28±0.72 | 65.59         |

Table 3. Effect of extraction methods on anthocyanin content (milligram cyd-3-glc/L) during oven (45 °C) at different types of solvent

| Treatments                        | T0     | 30 days | 60 days | 90 days | Retention (%) |
|-----------------------------------|--------|---------|---------|---------|---------------|
| T1 Ethanol 50 %                   | 47.59±0.59 | 34.03±0.25 | 21.12±0.15 | 0.78±0.44 | 1.63          |
| T2 Ethanol 50 % with 1.5N HCL     | 46.34±0.89 | 32.35±0.59 | 17.08±0.58 | 4.84±0.43 | 12.60         |
| T3 Ethanol 50 % with 1.5N CH3COOH| 36.57±0.67 | 22.12±0.30 | 11.31±0.48 | 3.32±0.37 | 9.07          |
| T4 Distilled water                | 52.35±0.30 | 38.41±0.21 | 13.62±0.35 | 1.14±0.35 | 2.17          |
| T5 Distilled water with 1.5N HCL | 56.61±0.65 | 21.24±0.35 | 14.24±0.61 | 3.86±0.68 | 6.81          |
| T6 Distilled water with 1.5N CH3COOH | 58.36±0.47 | 24.12±0.31 | 18.07±0.40 | 3.25±0.49 | 5.56          |
Table 4. Effect of extraction methods on pH during temperature room (25 °C) storage condition

| Treatments                   | T₀       | 30 days | 60 days | 90 days |
|------------------------------|----------|---------|---------|---------|
| T₁ Ethanol 50 %              | 4.05     | 4.15    | 4.18    | 4.23    |
| T₂ Ethanol 50 % with 1.5N HCL| 1.48     | 1.63    | 1.71    | 1.79    |
| T₃ Ethanol 50 % with 1.5N CH₃COOH | 1.79  | 1.85    | 1.92    | 2.03    |
| T₄ Distilled water           | 3.88     | 3.93    | 3.97    | 4.04    |
| T₅ Distilled water with 1.5N HCL | 2.15  | 2.19    | 2.21    | 2.27    |
| T₆ Distilled water with 1.5N CH₃COOH | 1.97  | 2.01    | 2.06    | 2.11    |

Table 5. Effect of extraction methods on pH during refrigerated (10 °C) storage condition

| Treatments                   | T₀       | 30 days | 60 days | 90 days |
|------------------------------|----------|---------|---------|---------|
| Ethanol 50 %                 | 4.05     | 4.10    | 4.13    | 4.17    |
| Ethanol 50 % with 1.5N HCL   | 1.48     | 1.57    | 1.60    | 1.62    |
| Ethanol 50 % with 1.5N CH₃COOH | 1.79   | 1.82    | 1.84    | 1.87    |
| Distilled water              | 3.88     | 3.90    | 3.92    | 3.95    |
| Distilled water with 1.5N HCL | 2.15  | 2.17    | 2.19    | 2.21    |
| Distilled water with 1.5N CH₃COOH | 1.97  | 1.98    | 2.01    | 2.03    |

Table 6. Effect of extraction methods on pH during oven (45 °C) storage condition

| Treatments                   | T₀       | 30 days | 60 days | 90 days |
|------------------------------|----------|---------|---------|---------|
| T₁ Ethanol 50 %              | 4.05     | 4.12    | 4.15    | 4.21    |
| T₂ Ethanol 50 % with 1.5N HCL| 1.48     | 1.59    | 1.67    | 1.71    |
| T₃ Ethanol 50 % with 1.5N CH₃COOH | 1.79  | 1.82    | 1.90    | 1.95    |
| T₄ Distilled water           | 3.88     | 3.91    | 3.94    | 4.01    |
| T₅ Distilled water with 1.5N HCL | 2.15  | 2.17    | 2.21    | 2.25    |
| T₆ Distilled water with 1.5N CH₃COOH | 1.97  | 2.02    | 2.07    | 2.12    |

4. Conclusion
The extracted pigment displayed fairly good storage stability in the refrigerated (10 °C) condition compared to temperature room (25 °C) and oven (45 °C) condition. In general, the temperature has significantly affected the total anthocyanins properties of the butterfly pea flower. By using a refrigerator to preserve, we can prevent the impact of external factors such as temperature, pH increase in the decomposition process of anthocyanin, and increase the retention of content anthocyanin for a long time. In this study, the treatment of ethanol 50 % Ethanol with 1.5 N HCl (67.47%) was found to be the best condition with highest anthocyanin retention compare to all other treatments which can used for large scale extraction of anthocyanin pigment from Clitoria Tenatea.

Acknowledgment
This study was funded by the Nguyen Tat Thanh University, Ho Chi Minh City, Vietnam.

References
[1] Tran T H, Nguyen P T N, Pham T N, Nguyen D C, Dao T P, Nguyen T D, Nguyen D H, Vo D V N, Le X T, Le N T H and Bach L G 2019 Green technology to optimize the extraction process of turmeric (Curcuma longa L.) oils IOP Conf. Ser. Mater. Sci. Eng. 479 012002
[2] Nguyen P M, Bach L G, Mac H C, Luu Y L, Vo T B T 2019 Production of Dried Tea from Okra (Abelmoschus Esculentus) J. Pharm. Sci. Res. 11 279–83
[3] Tran T H, Nguyen P T N, Ho V T T, Le T H N, Bach L G and Nguyen T D 2019 Using soft computing
approaches for orange (Citrus nobilis Lour. var. nobilis) oils extraction process IOP Conf. Ser. Mater. Sci. Eng. 479 012015

[4] Pham T N, Tran B P, Tran T H, Nguyen D C, Nguyen T N P, Nguyen T Q, Vo D V N, Le N T H, Le X T, Nguyen T D and Bach L G 2019 Response surface modeling and optimizing conditions for anthocyanins extraction from purple sweet potato (Ipomoea batatas (L.) Lam) grown in Lam Dong province, Vietnam IOP Conf. Ser. Mater. Sci. Eng. 479 012012

[5] Dao T P, Vo D-V N, Ha L K, Nhan L T H, Bach L G, Nguyen T D, Tran Q T, Nguyen D C, Nguyen D H and Tran T H 2019 The Study on Extraction Process and Analysis of Components in Essential Oils of Black Pepper (Piper nigrum L.) Seeds Harvested in Gia Lai Province, Vietnam Processes 7 56

[6] Mai H, Nguyen T, Le T, Nguyen D and Bach L 2019 Evaluation of Conditions Affecting Properties of Gac (Momordica Cocochnensis Spreng) Oil-Loaded Solid Lipid Nanoparticles (SLNs) Synthesized Using High-Speed Homogenization Process Processes 7 90

[7] Tran Q T, Le T T T, Pham M Q, Do T L, Vu M H, Nguyen D C, Bach L G, Bui L M and Pham Q L 2019 Fatty acid, lipid classes and phospholipid molecular species composition of the marine clam meretrix lyrata (Sowerby 1851) from Cua Lo Beach, Nghe An Province, Vietnam Molecules 24 895

[8] Dao T P, Nguyen D C, Nguyen D T, Tran T H, Nguyen P T N, Le N T H, Le X T, Nguyen D H, Vo D V N, Bach L G 2019 Extraction Process of Essential Oil from Plectranthus amboinicus Using Microwave-Assisted Hydrodistillation and Evaluation of It’s Antibacterial Activity Asian J. Chem. 31 977–81

[9] Minh N P, Trang T H P, Trang N T T, and Bach L G 2019 Effect of different drying methods on antioxidant of sacha inchi (Plukenetia volubilis L.) nut, Research on Crops 20 180-6

[10] Terahara N 1998 Eight New Anthocyanins, Ternatins C1− C5 and Preternatins A3 and C4 from Young Clitoria ternatae Flowers J. Nat. Prod. 61 1361–7

[11] Kirc A, Özkan M and Cemeroğlu B 2006 Effects of temperature, solid content and pH on the stability of black carrot anthocyanins Food Chem. 101 212–8

[12] Kazuma K, Noda N and Suzuki M 2003 Flavonoid composition related to petal color in different lines of Clitoria ternatea Phytochemistry 64 1133–9

[13] Rosmawati Abdullah P M L and Hungee K 2010 Multiple Color and pH Stability of Floral Anthocyanin Extract: Clitoria Ternatea International Conference on Science and Social Research 254–8

[14] Zakaria N N A, Okello E J, Howes M- J, Birch- Machin M A 2018 In vitro protective effects of an aqueous extract of Clitoria ternatea L. flower against hydrogen peroxide- induced cytotoxicity and UV- induced mtDNA damage in human keratinocytes Phyther. Res. 1–9

[15] Terahara N and Oda M 1996 Five New Anthocyanins, Ternatins A3, B4, B3, B2, and D2, from Clitoria ternatea Flowers Society 59 139–44

[16] AyşegülKirc A 2003 Degradation kinetics of anthocyanins in blood orange juice and concentrate Food Chem. 81 583–7

[17] Clifford M N 2000 Review Anthocyanins – nature, occurrence and dietary burden J. Sci. Food Agric. 80 1063–72

[18] Garry G, Duthie S J D and J A M K 2000 Plant polyphenols in cancer and heart disease: implications as nutritional antioxidants Nutr. Res. Rev. 13 79–106

[19] Maarit R 2005 Copigmentation reactions and color stability of berry anthocyanins

[20] Wrolstad R E, Durst R W and Lee J 2005 Tracking color and pigment changes in anthocyanin products Trends Food Sci. Technol. 16 423–8

[21] Lee J, Durst R W and Wrolstad R E 2005 Determination of total monomeric anthocyanin pigment content of fruit juices, beverages, natural colorants, and wines by the pH differential method:
Collaborative study *J. AOAC Int.* **88** 1269–78

[22] Gradinaru G, Biliaderis C G, Kallithraka S, Kefalas P and Garcia-Viguera C 2003 Thermal stability of Hibiscus sabdariffa L. anthocyanins in solution and in solid state: Effects of copigmentation and glass transition *Food Chem.* **83** 423–36

[23] Mourtzinos I, Makris D P, Yannakopoulo K, Kalogeropoulos N, Michali I and Karathanos V T 2008 Thermal stability of anthocyanin extract of Hibiscus sabdariffa L. in the presence of β-cyclodextrin *J. Agric. Food Chem.* **56** 10303–10

[24] Ya L, Yongxiao L, Cui T, Mei L, Yue P, Zhaolin L 2018 Effect of temperature and pH on stability of anthocyanin obtained from blueberry *J. Food Meas. Charact.* **12** 1744-53

[25] Laleh G H, Frydoonfar H, Heidary R and S Z 2006 The Effect of Light, Temperature, pH and Species on Stability of Anthocyanin *Pakistan J. Nutr.* **5** 90–2