Effect of Sucrose on Thermal and pH Stability of *Clitoria ternatea* Extract

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Abstract: The aim of this work was to investigate the effect of sucrose on the stability of *Clitoria ternate* extract against thermal and pH degradations. Lyophilised extract of the flower (1 mg/ml) was added into a series of sucrose solutions with concentrations ranging from 0.1% to 20% at pH7. The thermal stability of the extract in the solutions at 60°C was monitored using a UV-VIS spectrophotometer over 24 days. High temperature (60°C) accelerated degradation of the anthocyanin-rich extract but the presence of sucrose appeared to have slowed down the degradation process. However, sucrose asserted no protective effect against pH even at a concentration of 20%. It was thought that sucrose enhanced the thermal stability of anthocyanins by reducing water activity, partially preventing nucleophilic attack at the pyrylium ring of anthocyanins by water molecules. The present work provides some useful information for evaluating the potential of *C. ternatea* extract on food applications.

Keywords: Anthocyanins, *Clitoria ternatea*, Food colorants, Sucrose, Stability.

INTRODUCTION

Colour is a major factor contributing towards the acceptability, quality and organoleptic characteristics of food products. As stable colour is vital in the marketability of food, food manufacturers add food colourings to improve, simulate or replace colour loss caused by preparation, processing and storage conditions [1, 2], or to correct natural variation in colour [3]. However, some synthetic colorants have shown to be harmful to human health, a study conducted by McCann et al. [4] discovered the increase of Attention Deficit Hyperactivity Disorder (ADHD) in children after the consumption of beverages containing benzoic acid and azo dyes. The Food Standard Agency has recommended that UK food manufactures exclude these colorants, identified as the Southampton Six, in their products. Although still permitted under EU legislation, since July 2010 the European Commission Directive 1333/2008 (EC) specifies that any food products supplied to the EU containing the Southampton Six must be labelled with warning notices [5]. Such studies have raised public awareness and concerns over the safety of synthetic colorants, providing a rising demand for natural alternatives.

Among the natural food colourings, blue colorants are relatively uncommon and they are sensitive to pH change and thermal treatments; therefore a growing interest in the search, use and stabilisation of natural blue colorants especially for the confectionary and drink industries. This work was initiated to investigate the potential of anthocyanins extracted from *Clitoria ternatea* flower as a natural blue colorant. *C. ternatea*, also known as butterfly pea, is native to equatorial Asia and belongs to the Fabaceae family. The plantis herbaceous, slender and tall with climbing vines; with flowers (blooming within six weeks) ranging from white to blue with a yellow centre [6, 7]. Anthocyanins extracted from *C. ternatea* flower are relatively more stable when compared to those of other sources in domestic cooking and is used commonly to colour rice cakes in Malaysia [7, 8]. Terahara and co-workers [9] isolated six main anthocyanins ternatins from *C. ternatea* with structures characterising as malonylationdelphinidin, obtaining 3'5-side chains with alternating D-glucose and four p-coumaric acid with one molecule of malonic acid. Delphinidin glucoside is the main anthocyanin responsible for the distinctive blue colour of the flower [6, 9].

Many attempts have been taken to stabilise anthocyanins of different sources including the use of sugars. However, controversial results have been reported on the impact of sugars on the anthocyanins stability, ranging from having disruptive or stabilising effects to having no influence. Wrolstad [10] revealed that the stability of the anthocyaninsin strawberries during storage increased when the concentration of sucrose increased by 20%. In contrary, Dyrby *et al.* [11] showed that low concentrations of sucrose increased the degradation rate of these compounds in

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blackcurrant, red cabbage and elderberry extracts, whereas a stabilising effectfor grape extract was found [11, 12]. Nevertheless, Duhard *et al.* [13] found no changes in the thermal stability of commercial anthocyanins in non-sugar and sugar drink models. Rosso and Mercadante [14] reported that the use of salt and sugars had decreased anthocyanin stability although this was strongly dependant on the structure of the anthocyanins. This work aims to investigate the effect of sucrose on the stability of *C. ternatea* aqueous extract at different pHs and accelerated storage temperature (60°C) over a period of 24 days. The results from this work could be helpful in evaluating the potential of *C. ternatea* as a natural blue colorant for the confectionary and drink industries.

MATERIALS AND METHODS

Materials

Sundried *C. ternatea* flowers were purchased from a local marketplace in Penang, Malaysia. Potassium chloride, potassium hydrogen phthalate, potassium phosphate monobasic, Borax, hydrochloric acid and sodium hydroxide were used to prepare a series of buffer solutions with pH ranged from pH 1 to 13. Sodium azide (0.02%) was used to prevent microbial growth in all sample solutions. All chemicals used were purchased from Sigma-Aldrich (Dorset, UK) and were of analytical grade.

Extraction of Anthocyanins

Ground dry flower (*ca*. 2.45 g) was weighed into an extraction thimble, which was then inserted into a Soxhlet extractor with a vessel containing 180ml of distilled water as the solvent. Extraction occurred at boiling temperature and was refluxed for 4 hrs. Once completed the extract was collected and freeze-dried using an Edwards Micro Modulyofreeze-dryer (Bristol, UK). The extract was ground into powder and frozen (-20 °C) until required.

Preparation of Sample Solutions

Buffer solutions (0.05 M) at pH 1, 3, 5, 7, 9, 11 and 13 were prepared with deionised water. A *C. ternatea* anthocyanin-rich stock solution was prepared by dissolving 1.98 g of the lyophilised extract in 10 ml deionised water. Appropriate amount of the stock solution was pipetted into the buffer solutions so that the final concentration of the extract was 1 mg/ml. A series of sucrose solutions at concentrations of 0.1%, 1%, 10% and 20%, containing 1 mg/ml extract and 0.02% sodium azide were prepared in order to study the effect of sucrose on thermal degradation at pH 7.The sample solutions were kept at 60^{o}C in an oven to accelerate the ageing for 24 days and withdrawn at predetermined time intervals (Days 0, 1, 2, 3, 6, 7, 10, 14, 17, 21 and 24) for spectrophotometric analysis. For comparison purposes, another set of samples were prepared and kept in the dark at room temperature for 24 days. In order to investigate the effect of pH on anthocyanins degradation, sucrose solutions (20%) containing 1 mg/ml of extract at pH 3 and 9 were prepared. Change in visible light absorbance was scanned spectrophotometrically. Control samples were prepared without the presence of sucrose at the respective pH values.

Spectrophotometric Analysis

Samples were scanned through the light spectrum from 350 to 800 nm using a UV-VIS spectrophotometer (Model 6 Genesys, Thermo Fisher Scientific, Waltham, USA). The wavelength of maximum absorbance within the visible spectrum (λ_{max}) of the anthocyanin samples was determined from the spectra and the absorbance was recorded. The Colour Stability Index (CSI) of the samples was calculated from the absorbance at λ_{max} for the storage experiment using the equation below:

$$\text{Colour Stability Index} = \frac{Absorbance\ at\ \lambda_{\max}\ on\ Day\ 0}{Absorbance\ at\ \lambda_{\max}\ on\ Day\ t} \quad (1)$$

The CSI measured the colour shift of *C. ternatea*, providing a representation of how test conditions affect the stability of the anthocyanins.

Statistical Analysis

All experiments were triplicated and results were the average of three measurements. The SAS software package (SAS Institute Inc., Cary, NC, United States) was used to analyse the data and significant differences ($P<0.05$) between means were determined using Duncan's multiple range test.

RESULTS

Effect of Sucrose

Figure **1** shows the effect of sucrose on the stability of *C.ternatea* extract at pH 7 kept at accelerated storage temperature of 60^{o}C (Figure **1A**) and room temperature (Figure **1B**) in the dark for 24 days. The stability was measured by monitoring the change in

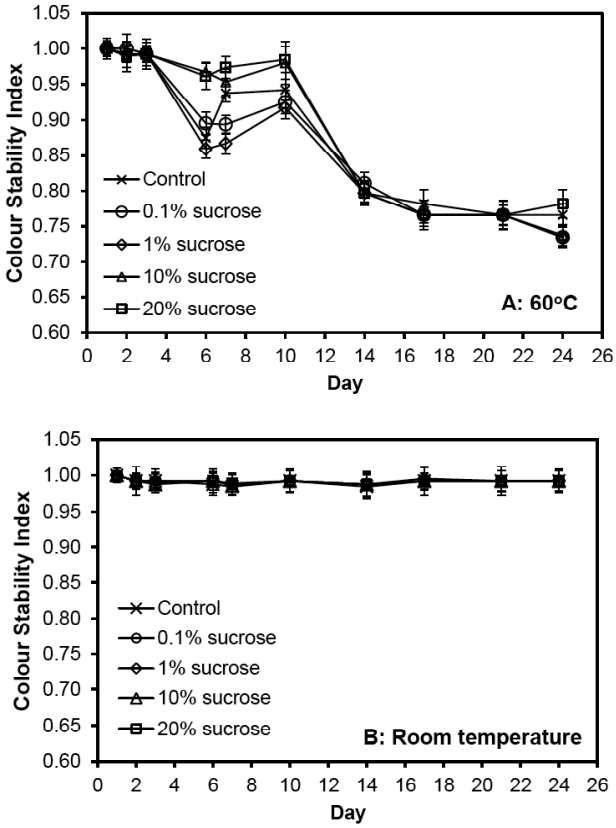

Figure 1: Change in colour stability index of *C. ternatea* extract as function of sucrose concentration stored at (**A**) 60°C and (**B**) room temperature during 24 days at pH 7.

CSI over the storage period. For samples kept at room temperature, there was no significant change (P>0.05) in CSI values during the storage period. High storage temperature degraded the colour of the extracts as indicated by the steep decrease in CSI of the samples kept at 60°C, in accordance with previous findings [15, 16]. However, the presence of sucrose appeared to have slowed down the degradation process: the higher the sucrose concentration in the solution, the slower the change in CSI. At 20% sucrose, the colour index remained stable for up to six days (no significant changes in CSI, P>0.05) as compared to sample solutions with a sucrose concentration <10% where their CSI values started to significantly (P<0.05) deteriorate after Day 3 (Figure **1**). The change in the visible light absorbance spectra of the samples provided more information on the impact of sucrose in stabilising the anthocyanin-rich extract (Figure **2**). At the beginning of the experiment (Day 0), all samples had a similar absorbance spectra with a λ_{max} of *ca.* 630nm within the visible light spectrum. The absorbance at this wavelength decreased over time (Days 2 and 7) until it eventually reached similar absorbance as other concentrations and the control on Day 14. However, in the control experiments (without the presence of sucrose) a dramatic decrease in colour intensity was observed. It should also be pointed out that solutions containing higher sucrose concentrations had a slower decrease in their absorbency. The results demonstrate that sucrose asserted a protective effect against thermal degradation on the extract which increased with concentration.

Effect of pH

The *C.ternatea* aqueous extract exhibited different colour in regards to pH change. Table **1** lists the λ_{max} ranged from 410 nm to 630 nm at different pH which was reflected in a wide colour spectrum, varying from pinkish red and purple to different shades of blue, green and yellow. At neutral pH, the extract was deep blue and had a maximum absorbance at 630nm, indicating that the blue neutral quinoidal base was the predominant form of anthocyanins in the solution. It is of interest to see if sucrose was capable of stabilising the quinoidal form of anthocyanins at an acidic or alkaline condition, therefore *C. ternatea* extract at different sucrose concentrations were subjected to pH change (pH 3 and 9). Figure **3** shows that the absorbance spectra of the samples were altered instantaneously in accordance to the pH regardless of sucrose concentration, indicating that sucrose had no protective effect on the anthocyanins against pH change.

DISCUSSION

The maximum absorbance within the visible spectrum of the extract is highly pH-dependant (Table **1**), because of the change in chemical structure of anthocyanins at different pH (Figure **4**). In an aqueous media four anthocyanin structures exist in equilibrium *i.e*. flavylium cation, carbinolpseudobase, quinonoidal base and chalcone [17] and the colourdepends on the relative concentration of these species. At pH 1 and 3, the predominant form of anthocyanins was red-coloured flavylium cation with a λmax of 550-570nm. Non-acylatedanthocyanins form colourless carbinolpseudo base at pH 4-5 [17], however, *C. ternatea* anthocyanins are mainly polyacylated and polyglycosylateddelphinidin [9]. The flavylium cation of *C. ternatea* anthocyanins might appear in the form of intramolecular complex, preventing the hydration of the pyrylium ring, and thus the formation of carbinol pseudobase. However, the maximum visible light absorbance at pH 5 suggested that some molecules of the red flavylium cation were converted into blue

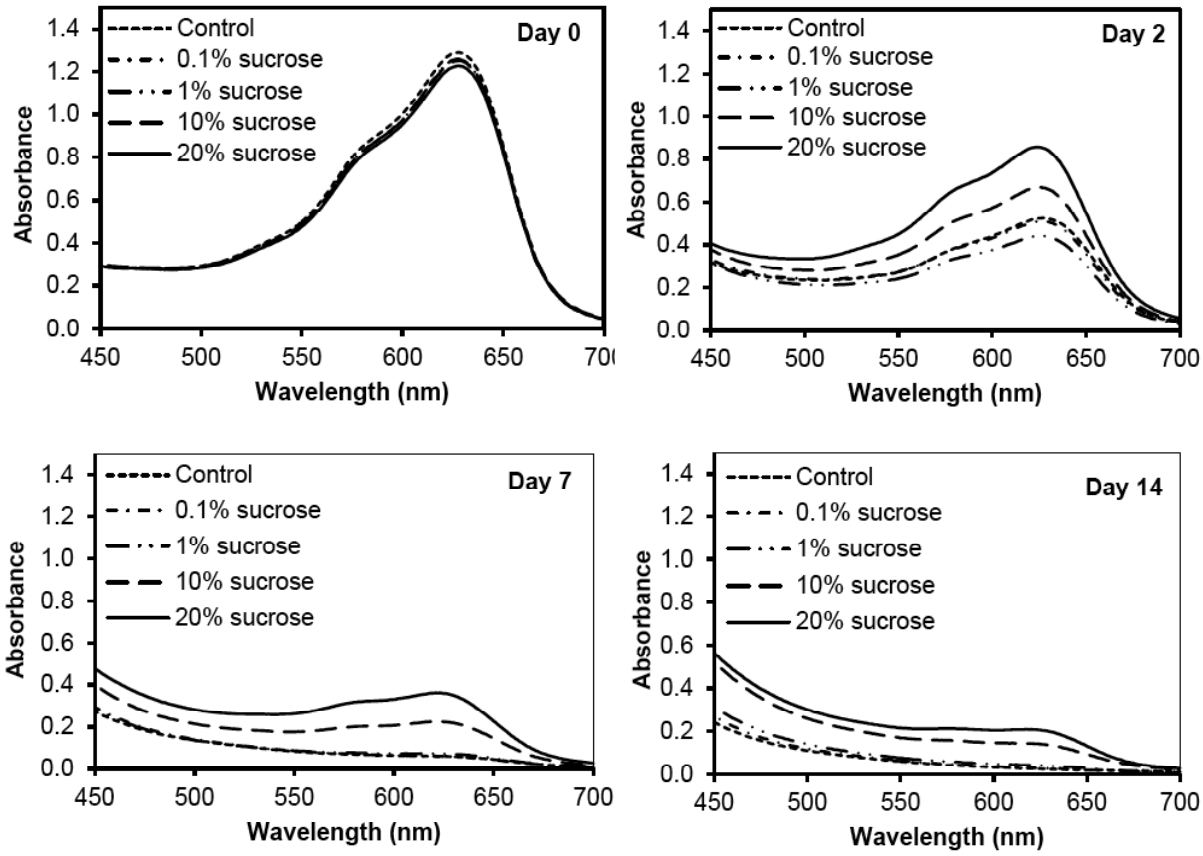

Figure 2: Change in absorbance profile in visible light spectrum of *C. ternatea* extract as function of sucrose concentration at 60°C on Days 0, 2, 7 and 14 at pH 7.

Table 1: The Wavelength of Maximal Absorbance (λ_{max}) of *Clitoria ternatea* Extract at Different pH

	pH						
	1	3	5	7	9	11	13
λmax, nm	550.3 ±1.5a	569.6 ±2.1b	589.7 ±0.9c	630.1 ±1.2d	642.4 ±3.3e	410.3 ±0.9f	409.7 ±1.1f

Each value in the table represents the mean ± standard deviations of triplicated results. Means with different letter are significantly different at $P<0.05$.

neutral quinonoidal bases, presumably through proton transfer between hydroxyl groups [17]. The appearance of a purplish blue colour in the solution is explained by the co-existence of red flavylium cationand blue neutral quinoidal base purplish blue [18]. At pH 7, the *C. ternatae* extract was deep blue and the λ_{max} was observed at 630nm, indicating that the anthocyanins existed primarily in the form of neutral quinoidal base. Increasing the pH to 9 and 11 changed the colour of the solution to bluish green and green, respectively. This was due to the relative amount of blue quinoidal base and yellow chalcone that co-existed in the solution, whereby the former was predominant at pH 9 and the latter was at pH 11. At pH 13 the colour of the solution turned yellow and the maximal visible light absorbance was at 410nm. The yellow colour appearance was caused by the hydrolysis of acyl bonds and the loss of the blue quinoidal base, leading to the production of chalcone, which was then converted to benzoic acid and benzaldehyde derivatives [19]. The colour of the solution did not turn brown at any point, even when pH reached 13; most likely due to the lack of dihydroxy phenolic compounds and/or polyphenol oxidase in the *C. ternatea* extract [20].

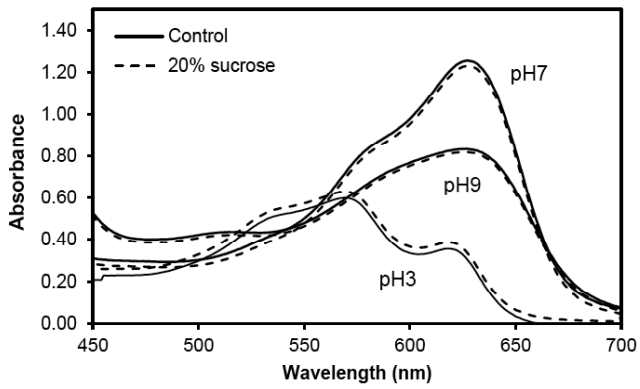

Figure 3: Change in absorbance profile in visible light spectrum of *C. ternatea* extract at pH 3, 7 and 9 with and without the presence of 20% sucrose.

It is well documented in the literature that many anthocyanins are sensitive to high temperatures[18, 21], thus it was expected for the *C.ternatea* extract to display a colour loss due to thermal degradation during storage at 60°C(Figures **1** and **2**). In this work, the colour of all solutions began as blue, changing to green and finally pale yellow at different rates depending on the sucrose concentration. It was thought that the high storage temperature (60°C) resulted in the loss of glycosyl moieties of the anthocyanins, most likely through the hydrolysis of the 3-glycoside structure, causing the aglycones to become less stable [18, 22, 23]. Prolonged thermal treatment also caused the opening of the pyrylium ring of the anthocyanin structure, resulting in the production of chalcone, which was accountable for colour loss [21]. Concentrated sucrose solutions seemed to have decreased the thermal degradation process of anthocyanins at pH 7(Figures **1** and **2**), although we could not exclude the possibility that Maillard reaction products contributed to the colour change especially towards to the end of the experiment. It is thought that the reduced water activity caused by sucrose was a contributing factor towards the enhanced stability of anthocyanins against thermal degradation. Syamaladevi *et al*. [24] suggested that water hydrated the glycosidic bonds of anthocyanins, which generated unstable anthocyanidins, creating chalcones through the subsequent opening of the pyrylium ring. Low water activity was also thought to protect the pyrylium ring from the nucleophilic attack of water [25]. The water molecule interacted with the pyrylium ring where the positive charge appeared. The reaction then proceeded through a thermally allowed electrocyclic ring opening [26], leading to the formation of colour less chalcones [27]. In the presence of sucrose, the availability of free water for the nucleophilic attack was reduced, and thus partially

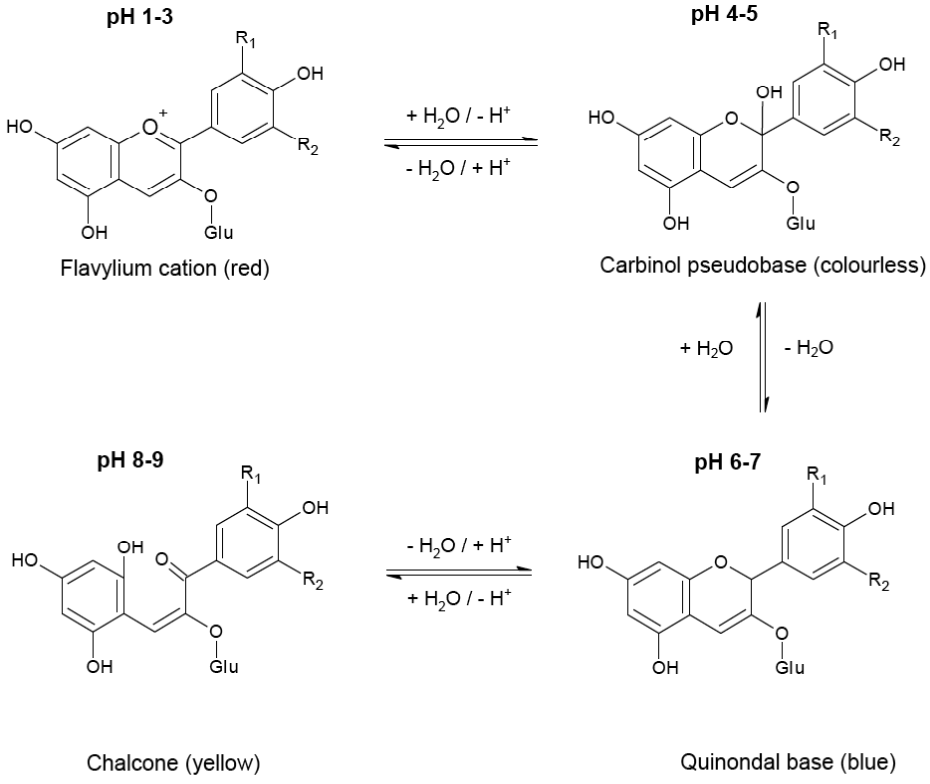

Figure 4: The four mainforms of anthocyanins at different pH.

preventing the degradation of the anthocyanins. The protective effect was more pronounced in samples with higher sucrose concentration or lower water activity (Figures **1** and **2**).

Unfortunately sucrose holds no protective properties against pH (Figure **3**).The colour of *C.ternatea* extract was blue at neutral pH, indicating the quinonoidal blue form prevailed. As the pH value shifted towards acidic (pH 3), the flavylium cation form of the anthocyanins dominated; and at pH 9 the colour changed to green, indicating the existence of chalcone. Hydrogen (H^+) and hydroxide (OH^-) ions are strong electrophile and nucleophile, and theyprotonated or hydrated the quinonoidal base of the anthocyanins to flavylium cation or chalconeform, respectively. Lowering the water activity of the solutions by addition of sucrose,in this case, was insufficient to prevent the degradation caused by pH, as there was still free water available to allow the acid and base molecules to dissociate. Co-pigmentation, indeed, could stabilise anthocyanins and induce hypechromatic effect (more intense colour) [27], and that acylated anthocyanins are more stable than non-acylated ones against mild acidic pH. However, at extreme pH the colour of the anthocyanins changes regardless, as demonstrated by the *C.ternatea* anthocyanins at pH3 and 9 (Figure **3**).

CONCLUSION

This study has demonstrated that *C. ternate* extract was affected by pH and temperature during storage. The presence of sucrose in the solution slowed down thermal degradation of the anthocyanins most likely due to reduced water activity which at least partially prevented nucleophilic attack of water against the pyrylium ring. Nevertheless, sucrose did not demonstrate any protective effect at pH 3 and 9. The results from this work provide some insights into the influence of sucrose on *C.ternatea* extract stability which could be useful particular for the confectionary and drink industries.

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