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Stability of phycocyanin extracted from *Arthrospira platensis* under different conditions

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Phycocyanin is a pigment-protein complex widely used in the cosmetic, pharmaceutical, and food industries. A green method was used to extract phycocyanin from *Spirulina* (*Arthrospira platensis*) with the best yield and purity at an ultrasonic frequency of 40 kHz, extraction time of 1 hour, and temperature of 40ºC. The present study aimed to observe phycocyanin's thermal and chemical stability over 1 h. Model buffered solutions of phycocyanin with pH 4.8, 5.8, 6.0, and 7.4 were tested at temperatures 50ºC, 60ºC, and 70ºC. Phycocyanin quantification was performed every 10 minutes. The results showed a first-order kinetic model. For all temperatures, phycocyanin remained stable at pH=4.8 and pH=5.8. At a temperature of 70ºC, its concentration significantly decreased. A strong correlation between the color parameters of the solutions and their concentrations was found. Based on the results, the phycocyanin half-life $t_{50\%}$ in the model systems used was determined.

Keywords: stability of phycocyanin, green extraction methods, *Arthrospira platensis*

INTRODUCTION

Natural pigments from plants, algae, and animals are a desirable alternative to dyes in the pharmaceutical, cosmetic, and food industries. Their application in these industries aims to provide healthier lifestyles to consumers [1]. The phycobiliproteins contained in the cyanobacterium *Arthrospira platensis* and other green algae are divided into four spectroscopic classes by their absorption in the visible range of the spectrum: phycoerythrocyanins, phycoerythrin, phycocyanins, and allophycocyanins [2]. They are natural blue dyes for chewing gums, dairy products, jellies [3], and lipsticks. They are also biomarkers in medicine because of the high relative fluorescence intensity [4]. Phycocyanin is found in *Arthrospira platensis* in the highest concentrations, reaching up to 20% of the dry mass, with a relative molecular weight between 20 and 50 kD [5], containing a protein and a non-protein component [6]. It can be included in medicinal preparations to alleviate and treat conditions associated with inflammation, weakened immunity, or tumor cell development due to oxidative stress [7]. The valuable properties of this pigment lead to its frequent use as an additive in pharmaceutical, cosmetic, and food products. It is water-soluble and can be easily extracted from *Arthrospira platensis* as a protein-pigment complex. The main problem with this pigment is its protein nature. Like all proteins, phycocyanin is sensitive to certain temperatures and pH levels. The presence of a chromophore in its structure also makes it photosensitive [8].

A number of authors indicate that phycocyanin is insoluble in acidic environments with pH levels below 3.0 and highly soluble in neutral environments with pH levels around 7.0 [9]. It is unstable in the pH range of 4 to 4.5, located near the isoelectric point of the indicated protein (at pH = 3.4) [10]. Due to the reduced electrostatic repulsion around that point, aggregation and precipitation are enhanced.

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A team of scientists from the Central Food Science and Technology Research Institute, Mysore, India, investigated the stability of phycocyanin at pH ranging from 2.5 to 13 using different buffers at both room temperature (25±2°C) and low temperature (9±1°C). Temperature stability was investigated at 10, 30, 45, and 55°C by incubating the samples in a water bath while maintaining the appropriate temperature. Stability studies at different pH levels over a period of 4 weeks showed that phycocyanin was stable in the pH range 5-7.5 at ambient temperature (25±2°C) and low temperature (9±1°C). The effect of temperature on the stability of phycocyanin shows that it is precarious at 45°C and above [11].

These studies show that phycocyanin is unstable at the acidic pH of the stomach, and enteric-coated dosage forms need to be prepared to increase drug release and its bioavailability.

The present study aimed to investigate the effects of different temperatures and pH levels on the chemical stability of phycocyanin with respect to its potential inclusion in oral dosage forms and nutritional supplements.

MATERIALS AND METHODS

Materials

The present study used *Arthrospira platensis* grown in a bioreactor in Bulgaria's Varvara region to extract phycocyanin. All chemicals and reagents (acetic acid and its sodium acetate salt, hydroxymethylaminomethane hydrochloride, hydroxymethylaminomethane, and sodium chloride) required for the preparation of the buffers were purchased from Merck, Germany.

To increase the purity and yield of phycocyanin without the need for further purification, ultrasonic extraction was performed. 2 g of *Arthrospira platensis* was weighed into 50 mL test tubes with caps, and the extraction was carried out in distilled water (1:25 w/v) using a VWR ultrasonic bath (Malaysia) at 45 kHz, power 30W (UAE 45 kHz). The extracts were filtered through a paper filter and then prepared for analysis.

Preparation of buffer solutions

The thermal stability of phycocyanin was tested by preparing buffer solutions of different acidity and of the indicated substance (1 mg/mL). Acetate buffers with pH levels of 4 - 7 were prepared from weak acetic acid and sodium acetate salt. Buffer systems with pH levels of 4.8, 5.8, and 6.0 were prepared. The buffer solution with physiological pH level of 7.4 was prepared by using hydroxymethylaminomethane hydrochloride (0.2313 g), hydroxymethylaminomethane (0.0282 g), and sodium chloride (0.7540 g) for volume 100 mL. Acetic acid and sodium acetate salt concentrations are 0.1 mol/L. The mixing ratio of salt to acid is as follows: pH=4.8 (1:1); pH=5.8 (10:1); pH=6.0 (15.5:1).

Spectrophotometric evaluation of phycocyanin content

The phycocyanin content of the solutions was determined spectrophotometrically (Thermo Science, USA) according to the formulas:

$$Cphycocyanin\left(\frac{mg}{mL}\right) = \frac{A_{620nm} - 0.474.A_{652nm}}{5.34}, \quad (1)$$

$$Alophycocyanin\ \left(\frac{mg}{mL}\right) = \frac{A_{652} - 0.208 * A_{620}}{5.09} \quad (2)$$

A_{620nm} is the optical density of the sample at the wavelength characterizing the absorption maximum of phycocyanin, and A_{652nm} is the optical density of the sample characterized as the absorption length for phycoerythrin.

The concentration of phycocyanin was determined as the sum of the values from formulas (1) and (2) [12, 13].

Determination of phycocyanin degradation kinetics

The degradation kinetics of phycocyanin were studied under "stress" conditions at 50°C, 60°C, and 70°C and pH levels of 4.8, 5.8, 6.0, and 7.4. After spectrophotometric determination of the initial concentration of phycocyanin in the respective buffer solution (pH 4.8, 5.8, 6.0, and 7.4) at 25°C, 6 equal parts of it were placed in closed flasks which were tempered in a water bath (50°C, 60°C and 70°C) for 1 hour. The chemical degradation of phycocyanin in the buffer solutions was studied spectrophotometrically by reading the absorbance at the indicated wavelengths in formulas (1) and (2) every 10 minutes at the respective temperatures for an hour.

Determination of degradation rate constant

That constant can be determined from the integral form of the following differential equation describing a first-order reaction:

$$\frac{dC}{C} = -k.dt\ , \quad (3)$$

where: C is phycocyanin concentration (mg/mL); k is the constant of degradation rate (min^{-1}); t is time (min).

The half-life of phycocyanin under various conditions was determined from equation (4) [14, 15].

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

The relative concentration (C_R, %) of phycocyanin at each combination of external factors (pH, temperature, and time) was determined. C_R is the remaining phycocyanin concentration as a percentage of its initial concentration in the respective buffer.

$$C_R, \% = \frac{C}{C_0} \times 100 \quad (5)$$

Colorimetric measurements

Using VISIONlite ColorCalc software, the color parameters of the samples were determined using a spectrophotometer (Thermo Fisher, USA). The samples were in a buffer solution of concentration 0.01 mg/ mL .

Statistical analysis

All results were obtained by three parallel analyses. Table 1 showя the mean results ± the standard deviation of the measurement using Excel and Anova.

RESULTS AND DISCUSSION

Phycocyanin degradation kinetics and determination of degradation rate constant

The pH levels of the solvents used are one of the most important factors related to the stability of phycocyanin. In the present study, the stability of the substance in acetate buffer was determined by spectroscopy in the visible range, and the optimal pH range for its stability was found.

The relative protein concentration was determined for each of the three temperatures at four different pH levels for 60 min. The results are presented in Figure 1.

After applying linear regression models using the Origin Pro 2015 software product, phycocyanin degradation was found to be of first-order. All coefficients of determination are above 0.87. The half-life and rate constant results at different temperatures and pH levels were calculated according to correlations (3) and (4) and are presented in Table 1.

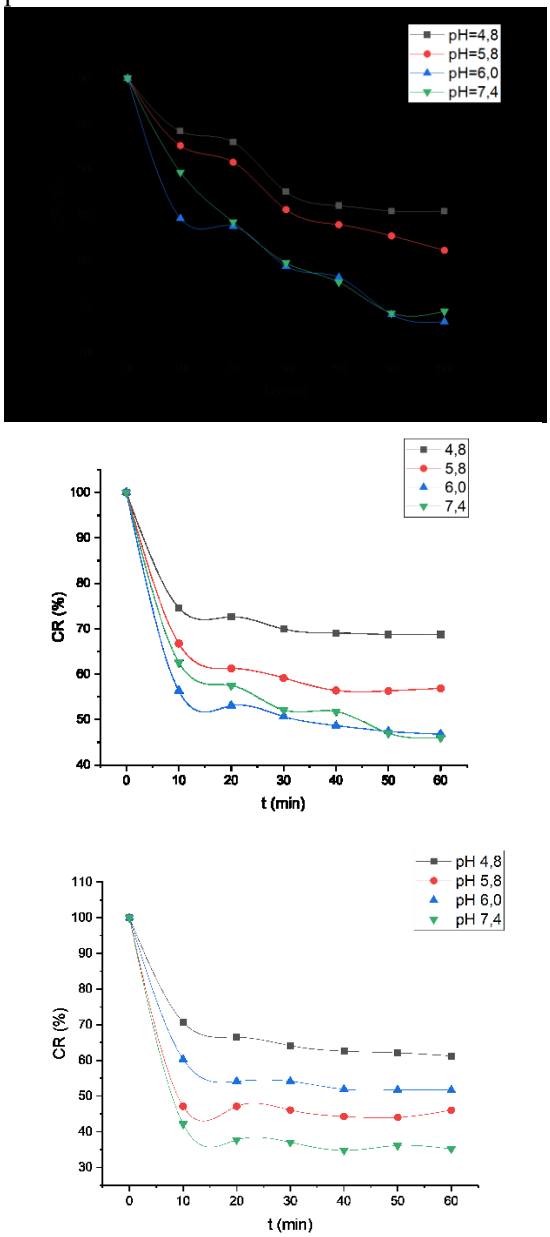


Figure 1. Effect of pH on the stability of phycocyanin in acetate buffer at A: 50°C, B: 60°C, C: 70°C.

Table 1. Degradation rate and half-life values of phycocyanin at 50 ºC, 60 ºC, 70 ºC and pH 4.8, 5.8, 6.0, 7.4.

T,^{0}C	pH=4.8		pH=5.8		pH=6.0		pH=7.4	
	k ± SD, min^{-1}	$\tau_{1/2}$ ± SD, min	k ± SD, min^{-1}	$\tau_{1/2}$ ± SD, min	k ± SD, min^{-1}	$\tau_{1/2}$ ± SD, min	k ± SD, min^{-1}	$\tau_{1/2}$ ± SD, min
50	0.0029 ± 3.10^{-4}	239.02 ± 3.2	0.00583 ± 4.10^{-4}	119.51 ± 2.7	0.004 ± $2.2.10^{-4}$	173.2 ± 5	0.0059 ± 1.10^{-4}	117.48 ± 4.4
60	0.0083 ± 7.10^{-4}	83.51 ± 4.7	0.0123 ± 2.10^{-4}	55.90 ± 1.9	0.0165± $7,9.10^{-4}$	42.01 ± 2.3	0.0160 ± 2.10^{-4}	43.32 ± 2.1
70	0.0101 ± 10.10^{-4}	68.63 ± 1.4	0.0182 ± 9.10^{-4}	38.09 ± 3.2	0.0235± 8.10^{-4}	46.83 ± 2.3	0.0235 ± 5.10^{-4}	29.5 ± 1.9

The degradation of phycocyanin at 50°C is very slow. Its half-life at 50°C and pH 4.8 is 239.02 min. At 60°C, the degradation rate increases dramatically, and the half-life decreases up to 3 times for different pH levels. With the increase in temperature, the half-life decreases. According to the data in Table 1, the half-life of phycocyanin is most extended at 50°C and pH 4.8 – 239.02 min and shortest at 70°C and pH 7.4 – 29.5 min. Other authors have reported similar results. Chaiklahan *et al.* stated that at pH 5.0, 6.0, 7.0, and temperature 51°C, the half-lives were 62 min, 167 min, and 108 min, respectively [3]. Our results at pH 6.0 and 7.4 are close to those shown, but at pH 4.8, they are about 4 times higher. That might have happened because:

✓ In the above study the information was obtained using citrate-phosphate buffer, whereas in the present study acetate buffer was used;

✓ the stability of phycocyanin depends very much on the strain of *Spirulina* from which it is extracted [16].

At 70°C, protein denaturation is accelerated. A similar result was reported in [17, 16]. Accordingly, phycocyanin was more stable at pH levels of 5.0 to 6.0 since the hexameric form of phycocyanin predominates in this pH range [18]. Significantly longer half-lives in the present study were found at a temperature of 70°C relative to other sources. At the indicated temperature and pH 4.8, 6.0, and 7.0, the half-life was 68.6 min, 46.8 min, and 29.5 min, respectively. Carle and Schweiggert, 2016 at the same temperature and pH of 5.0, 6.0, and 7.0, indicated half-lives of 7.5 min, 14.5 min, and 6.0 min, respectively [19].

The following can be concluded from the study of temperature-dependent phycocyanin degradation.

✔At 50°C, degradation occurs slowly, and the relative amount of undegraded phycocyanin decreases in an average of 30 minutes at different pH levels from 10% to 13% relative to the original quantity.

✔At 60°C, the relative amount of undegraded phycocyanin decreases in 30 minutes at different pH levels from 40% to 50% relative to the original quantity.

✔At 70°C, the reduction is 40% to 70% from the original quantity under the abovementioned conditions.

In addition to temperature, protein denaturation depends on the pH of the environment. Phycocyanin was most stable at a pH of 5.0-6.0 and a temperature of 50°C (the decrease in relative concentration in 60 minutes was from 13% to 19%), and at a temperature of 60°C (the decrease in the relative amount of undegraded phycocyanin in 60 minutes was from 30% to 60%). The protein is unstable at pH 7.4 and temperatures of 60°C and 70°C. Therefore, pH values significantly control phycocyanin's aggregation and dissociation processes to monomers, trimers, hexamers, and oligomers. The hexameric structure of the protein is known to be protected from denaturation.

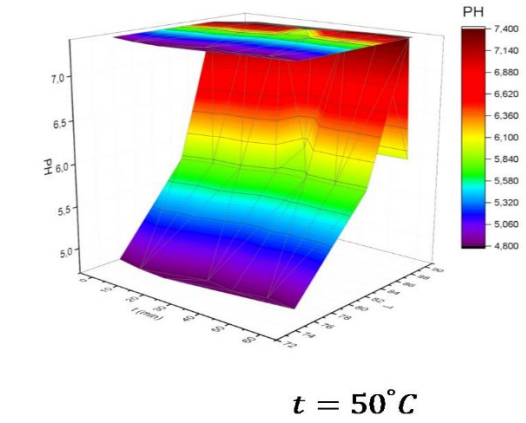
Table 2. Estimated values of k and $t_{1/2}$ at 25°C and 37°C.

t = 25 °C	pH = 4.8	pH = 5.8	pH = 6.0	pH = 7.4
k	0.0012	0.0005	0.0004	0.0009
$\tau_{1/2}$, min	599.54	1275.90	1907.47	788.79
t = 37 °C	pH = 4.8	pH = 5.8	pH = 6.0	pH = 7.4
k	0.0027	0.001343	0.001312	0.0024
$\tau_{1/2}$, min	260.25	516.0	528.46	290.10

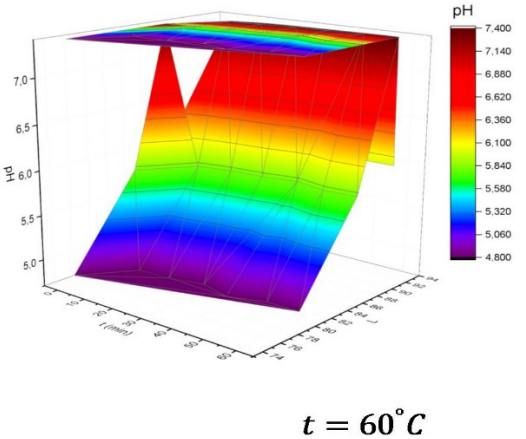
Colorimetric measurements

A linear regression dependence was demonstrated with correlation coefficients above 0.9 between luminosity and relative phycocyanin concentration. Figure 2 shows 3D graphs of luminosity's dependence on pH and time for the three temperatures [20]. The latter better explains the stability at pH 4.8 and 6.0 at 50°C and 60°C. A similar fact was reported by Adams *et al*., who found that at pH 6.0, the trimeric form is about 23% of the protein, and the remainder is hexameric [21]. At pH 6.0, 50°C, and 70°C, phycocyanin is more stable than at pH 5.8. But at 60°C, it is more stable at pH 5.8 compared to pH 6.0. This fact is also confirmed by Antelo *et al.* [9]. Several other authors confirm our study's findings [3, 22]. The stability of phycocyanin is due to the hexameric structure resulting from linking the monomers into a ring trimer [23]. The monomers consist of two polypeptides: alpha (α) – linked to one phycocyanobilin, and beta (β) – related to two phycocyanobilins [23]. At pH levels around 2, the protein does not dissolve even after homogenization. The fact is explained by the cyclic conformation and folding of the chromophore [24, 25]. At pH 3.0, phycocyanin precipitates and denatures. At pH 4.0, the solution with the indicated protein is turbid.

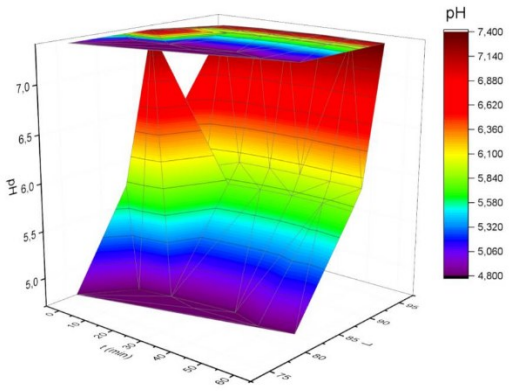
The degradation of phycocyanin under different factors is associated with the loss of color of the solutions. Color is one of the primary sensory characteristics that consumers evaluate when choosing food and food supplements. To assess the color degradation, transmission and absorption spectra measurements were made, and color parameters such as lightness (L), color coordinates (a), and (b) were determined in the CIELab small color difference colorimetric system.



$t = 50^{\circ}C$



$t = 60^{\circ}C$



$t = 70^{\circ}C$

Figure. 2. Dependence of luminosity of phycocyanin solutions on pH and time at different temperatures.

A linear regression relationship with correlation coefficients above 0.9 between lightness and relative phycocyanin concentration is demonstrated. Figure 2 shows 3D plots of the dependence of luminosity on pH and time for each of the three temperatures. The temperature-increasing degradation of phycocyanin appears after 40 minutes as decreasing color density at pH 6.0 and 7.4.

CONCLUSION

The optimal conditions for incorporating phycocyanin into food products and food additives under which it remains stable are in the temperature range from room temperature up to about 50ºC and at pH 4.5 to 5.5. There is a linear correlation between luminosity and relative concentration of phycocyanin.

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