The stability of phycocyanin extracted from *Arthrospira platensis* against osmotic, acid, and temperature stress conditions

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Abstract

The main problem of using natural blue pigment of phycocyanin produced by *Arthrospira platensis* (spirulina) is its instability in the food matrix because of environmental stress. This study aimed to investigate the stability of phycocyanin under simulated conditions in food formulations against osmotic, acid, and temperature stress conditions. Thermal degradation constant (Dc) and half-life ($t_{\frac{1}{2}}$) of phycocyanin extracted from *Arthrospira platensis* were analyzed using response surface methodology followed by a first-order kinetic reaction. The stability of phycocyanin was assessed under various temperature (50–98°C), NaCl (0–2% w/w), and pH (4–7) values. Results showed that the stability of phycocyanin extracted from *Arthrospira platensis* is high at neutral pH and concentration of 1% (w/w) NaCl. The stability decreased with increase in temperature at ≥75°C. The highest stability of phycocyanin (the lowest Dc and the maximum $t_{\frac{1}{2}}$ were 0.011 min^{-1} and 54.03 min, respectively) was achieved at 66.89°C, pH = 6.6, and NaCl of 0.40% w/w. According to processing conditions, content of phycocyanin required for a food matrix is successfully calculated by the response surface method. This research showed that phycocyanin is stable at thermal shock in a neutral pH medium and low content of NaCl (0.40% w/w).

Keywords: arthrospira platensis; phycocyanin; response surface methodology; spirulina; stability

Introduction

Microalgae *Arthrospira* (*A.*) *platensis* (spirulina) has been considered as one of the important targets of biotechnological research because of its economic, ecological, and nutritional significance. *A. platensis* algae is of particular importance in the food industry (Beheshtipour *et al.*, 2012, 2013; Hoseini *et al.*, 2013a; Massoud *et al.*, 2015; Mazinani *et al.*, 2016), medical sector (anti-inflammatory and anticancer with several kidney and liver protective properties; Hoseini *et al.*, 2013b; Soheili *et al.*, 2011), and aquaculture industry because of its digestibility and high nutritional value (50–70% w/w protein) and also having essential amino acids, vitamins, mineral elements, and essential fatty acids (Fernández-Rojas *et al.*, 2014). *A. platensis* produces high amounts of phycobili proteins (Antelo *et al.*, 2008) and significant amounts of natural pigments chlorophyll, carotenoid, and phycocyanin (Banayan *et al.*, 2020; Ghaeni *et al.*, 2014; Santiago-Morales *et al.*, 2018).

Phycocyanin is a blue pigment and light receptor with antioxidant and fluorescent properties in cyanobacteria. This pigment is a water-soluble compound with high antioxidant properties. The blue powder of phycocyanin is nontoxic, odorless, and slightly sweet. Phycocyanin and other phycobiliproteins (PBPs) are used in chewing gum, chocolate, jellies, beverages, cosmetic health industries, and disease diagnosis (Ansarifard *et al.*, 2017).

The photoautotrophic production of phycocyanin by *A. platensis* is carried out in photobioreactors (PBRs; Panutai *et al.*, 2020) and/or open large ponds or pools in tropical or subtropical areas at the edges of oceans. Biomass production efficiency is modified by supply of light. *A. platensis* grows in mixotrophic cultures; therefore, the conditions for growth and production of phycocyanin are optimized by providing suitable level of light and organic carbon sources (Banayan *et al.*, 2020; Soheili *et al.*, 2013).

Many researchers have optimized cultivation to increase production efficiency and reduce production costs of this pigment. The effect of carbon and nitrogen sources as well as exposure period and intensity of light on the production of phycocyanin is investigated (Banayan *et al.*, 2020; Chen and Zhang, 1997). The extraction efficiency, purity, and concentration of phycocyanin depend on the process of cell disruption. Various methods of extraction, such as the use of lysozyme, ethylenediaminetetraacetic acid (EDTA), and phosphate buffer, are used to extract phycocyanin. Lysozyme affects the cell wall more, and EDTA and phosphate buffer release phycocyanin by chelating magnesium ions and destroying the cytoplasmic membrane. Ultrasound, homogenization, freezing, and thawing techniques, as well as organic and inorganic solvents, are applied for the extraction of phycocyanin (Safari *et al.*, 2018).

The degradation of phycocyanin depends on its protein structure, which is affected by many factors, such as light, pH, temperature, and protein concentration. Hence, stabilizing substances are used to maintain the protein structure (Sarada *et al.*, 1999). Research has shown that phycocyanin is more stable in cold temperature and acidic pH 5–5.4. Increase in temperature to more than 70°C leads to fast denaturation of phycocyanin (Safari *et al.*, 2018). The proteinaceous nature of phycocyanin protects this pigment to be less affected by contamination and microbial decomposition at low temperatures and to denature at high temperatures. Phycocyanin extract is more stable at 50–55°C (Doke, 2005).

Many researchers have focused on thermal stability and prediction of the degradation rate of natural pigments used in food matrices. Thermal analysis of sweet potato purple anthocyanins at 90°C and pH of 3–7 showed that content of anthocyanin decreased with increase in heating time at all pH values and especially in neutral conditions (pH 7; Li *et al.*, 2013). The anthocyanins obtained from red radish extract are much more resistant at pH 5 than at pH 3 (Wang *et al.*, 2017). Modeling of thermal degradation constant (Dc) and half-life ($t_{\frac{1}{2}}$) of *Monascos poroporeus* was successfully carried out to predict its degradation rate and survival in food processes (Abdollahi *et al.*, 2021). To the best of our knowledge, there is no report about modeling and predicting the behavior of phycocyanin stability against osmotic, temperature, and acid stress conditions in food matrices.

This study was designed to investigate the effect of temperature, pH, and salt on the stability of phycocyanin in simulated food conditions. Modeling of the effect of temperature (50–98°C), pH (4–7), and salt (0–2% w/w) on Dc and $t_{\frac{1}{2}}$ of phycocyanin was carried out by the response surface method (RSM). By controlling pigment degradation in thermal processes, similar conditions are predicted and the desired pigment content in food is calculated.

Material and Methods

Microalgae source and culture condition

The microalgae strain (*A. platensis*, APP1) was provided by the Microalgae Culture Collection of Tarbiat Modares University, Tehran, Iran. *A. platensis* was cultivated using Zarrouk media (pH 9.8) in 250-mL glass flasks containing 150 mL of cell suspension under sterile conditions. Growth and maintenance of the culture were carried out using illuminated (150 μmol m^2 s^{-1}) phytotron at 30±1°C under a 12–12-h light–dark cycle with mild agitation (100 rpm) for 12 days (Banayan *et al.*, 2020; Gami *et al.*, 2011; Zeng *et al.* 2012).

Phycocyanin extraction

To extract and measure phycocyanin pigment, freezed biomass and 0.15-M potassium phosphate buffer (pH = 7) were mixed at a ratio of 1:50 v/v. Then, the cell suspension was centrifuged (Universal 320R made in Iran) at 1800 g for 10 min at 25°C. Pellets were removed and the supernatant was collected. Absorbance of the extract was measured with an ultraviolet-visible (UV-VIS) spectrophotometer (2100 model; UNICO, China) at 615 nm and 652 nm, and the concentration of phycocyanin was calculated according to Equation (1) (Banayan *et al.*, 2020; Ferreira-Santos *et al.*, 2020):

$$\text{Phycocyanin (g / L)} = \frac{\text{OD}_{615} - 0.474(\text{OD}_{652})}{5.34}. \quad (1)$$

Calculation of reaction kinetics

Effects of temperature (50–98°C), NaCl (0–2% w/w), and pH (4.3–7.7) on Dc and $t_{½}$ of phycocyanin extracted from *A. platensis* were assessed using a water bath. Phycocyanin solutions were heated for 30 min in a water bath (50–98°C), and samples were collected after 5 min. Degradation constant obtained in the first-order kinetic model was expressed according to Equation (2). Regression lines were obtained by plotting changes in the degradation of phycocyanin logarithmically as a function of heat treatment time (Vendruscolo *et al.*, 2013):

$$\frac{d_A}{d_t} = -D_c.t, \qquad (2)$$

$$\ln\left(\frac{A}{A_0}\right) = -D_c.t, \qquad (3)$$

where A is the amount of phycocyanin (g/L), A_0 is the amount of phycocyanin at t = 0, t is the time (minute), and Dc is the color instability constant because of heat (min^{-1}). Equation (2) had a logarithmic graph in Equation (3). To convert it into a linear graph, boundary conditions must be used for each parameter. The $t_{½}$ was obtained from the Dc parameter given in Equation (4), where $t_{½}$

means the time when the amount of phycocyanin was twice the amount of initial phycocyanin:

$$t_{1/2} = \left(\frac{Ln^2}{D_C}\right). \qquad (4)$$

Statistical analysis

In this study, temperature (50–98°C), pH (4–7), and NaCl content (0–2% w/w) were analyzed as independent variables using central composite design (CCD) of response surface methodology (RSM) and the Expert Design Software v.7.0.0. Levels of real variables in CCD are shown in Table 1. Effects of significant independent variables were assessed in terms of D_C (Y1) and $t_{½}$ (Y2) of phycocyanin. The research was designed by applying RSM with α = 1.7. Data were analyzed by the Design 7.0.0 Expert software at 95% confidence level (95% CI).

Results and Discussion

Table 1 shows the effect of independent variables of temperature (50–98°C), pH (4–7), and NaCl (0–2% w/w) on the constant variables of phycocyanin pigment instability, Dc and $t_{½}$. Figure 1 shows changes in phycocyanin

Table 1. Degradation constant (Dc) and half-life ($t_{½}$) for various heat treatments, pH, and salt proportions of phycocyanin produced by *Arthrospira platensis* (spirulina).

Run No.	Independent variables			Responses	
	Temperature (ºC) (A)	NaCl (%) (B)	pH (C)	Degradation constant, Dc (min^{-1})	Half-life, $t_{½}$ (min)
1.	59.7	0.44	4.6	0.0169	40.01
2.	88.3	0.44	4.6	0.0198	35.01
3.	59.7	0.44	6.4	0.0118	58.74
4.	88.3	0.44	6.4	0.0152	45.60
5.	59.7	1.6	4.6	0.0177	39.16
6.	88.3	1.6	4.6	0.0122	56.82
7.	59.7	1.6	6.4	0.0199	34.83
8.	88.3	1.6	6.4	0.0167	41.51
9.	50	1.0	5.5	0.0169	41.01
10.	98	1.0	5.5	0.0211	32.85
11.	74	1.0	4	0.0192	36.10
12.	74	1.0	7	0.0169	41.01
13.	74	0	5.5	0.0138	50.23
14.	74	2.0	5.5	0.0128	54.15
15.	74	1.0	5.5	0.0133	52.12
16.	74	1.0	5.5	0.0123	56.45
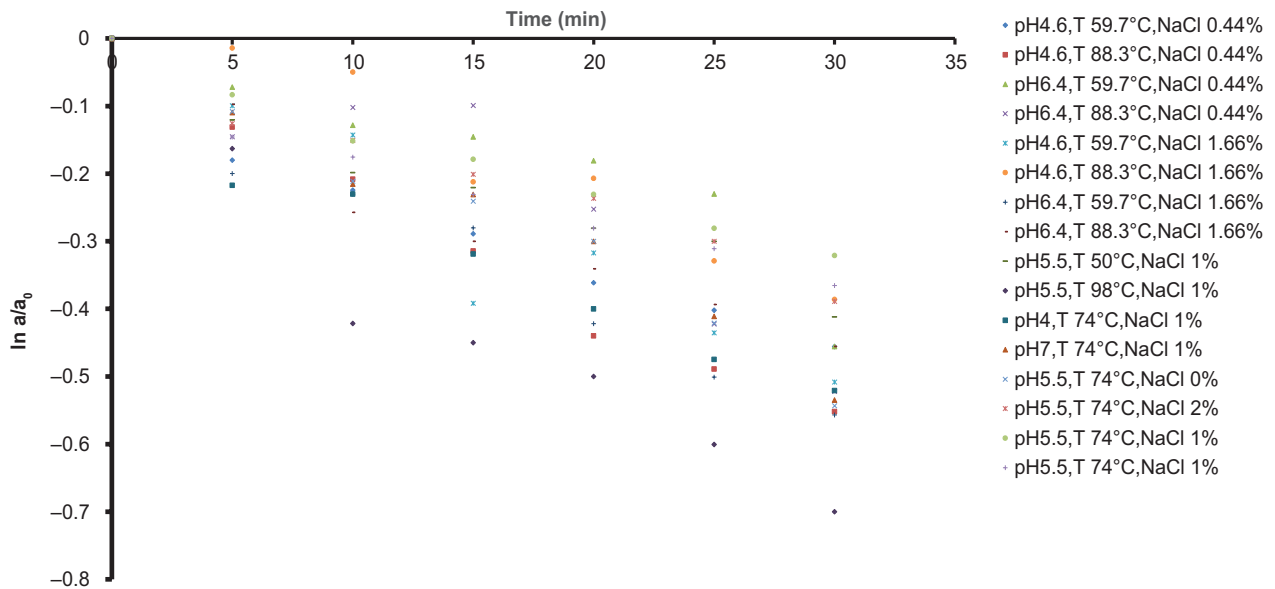
Figure 1. Degradation constant (Dc) of the phycocyanin produced by *Arthrospira platensis* (spirulina) in aqueous solution versus various conditions (temperature: 50–98°C; pH 4–7, and salt content: 0–2%).

color degradation after exposure to 17 treatment combinations. Since the graph is logarithmic, $\ln(A/A_0)$ was used to linearize the same. The weakening of phycocyanin pigment during the heating period of 5 min is shown in Figure 1, where the slope of the line is the Dc of the pigment. As shown in Figure 1, placed for 30 min, the highest degradation was related to treatment 10 at a temperature of 98°C, pH = 5.5, and salt concentration of 1% w/w, and the lowest degradation was related to treatment 3, which was at a temperature of 59.7°C, pH = 6.4, and salt concentration of 0.44% w/w.

The results of the final analysis of variance presented in Table 2 show that the model was significant ($p < 0.0006$) and the lack of fit was not significant ($p = 0.99$). The distribution of statistical data in samples was suitable (CV = 8.22). The value of regression coefficient, R^2, was 0.9060 and showed significant relationships between experimental and predicted values according to Equation (5):

$$Dc = 0.0126 + 0.0003\ A - 0.0005\ B + 0.0001\ C - 0.0019\ AC + 0.0021\ BC + 0.0021\ A^2 + 0.0018\ B^2 \qquad (5)$$

In which A, B, and C are Temperature (°C), NaCl (%), and pH. Also, AC and BC are interaction effects and A^2 & B^2 are quadratic effect of main factors A, B, C.

The $t_{1/2}$ indicates the stability of phycocyanin under processing conditions. In other words, the $t_{1/2}$ is the time taken by the pigment to reach half of its initial amount. The $t_{1/2}$ of a pigment has an inverse relationship with its thermal instability (Dc) (Abdollahi *et al.*, 2021). As the results of the analysis

of variance showed, the model was significant ($p < 0.0013$), error in the lack of fit was not significant ($p = 0.6183$), and the value of regression coefficient, R^2, was 0.8869.

The experimentally obtained data for $t_{1/2}$ and coefficient of variables led to an objective function as shown in Equation (6):

$$t_{1/2} = 55.05 + (0.625\ A) + (1.24\ B) - (0.106\ C) + (5.34\ AC) - (5.9\ BC) - (6.09\ A^2) - (5.51\ B^2) \qquad (6)$$

The linear effects of temperature, pH, and NaCl content and interaction of temperature–NaCl and pH–NaCl were significant on Dc and $t_{1/2}$ of phycocyanin (Table 2). Furthermore, the square effect of temperature and pH was significant ($p < 0.05$). With increasing temperature, stability of phycocyanin decreased to a minimum level of about 5.5. Figures 2B and 2D show that the interaction of salt and pH was significantly effective on both Dc and $t_{1/2}$ of phycocyanin ($p \leq 0.05$). At pH = 6.4, Dc increased with increasing salt, while at pH = 4.6, changes in salt had no effect on it.

The results of the present study showed that at pH > 4.6, Dc of phycocyanin increased with increase in salt content. At low temperatures, addition of salt maintained the stability of the pigment. Also, at a temperature of 59.7°C, pH = 6.4, and a salt content of 0.44% w/w, the lowest Dc (0.011) and maximum $t_{1/2}$ (58.74 min) were observed. The Dc of phycocyanin increased and $t_{1/2}$ of phycocyanin decreased with increasing temperature (Figures 2A and 2C). The $t_{1/2}$ of phycocyanin increased if preservatives were used (Martelli *et al.*, 2014).

Table 2. Effects of (A) temperature, (B) pH, and (C) salt and their interactions on Dc and $t_{1/2}$ of phycocyanin produced by *Arthrospira platensis* (spirulina) using response surface method (RSM).

Source	Degradation constant (Dc)				Half-life ($t_{\frac{1}{2}}$)		
	Degree of freedom	Sum of squares	*F* value	*p* value	Sum of squares	*F* value	*p* value
Model	7	0.0001	12.40	0.006	1,229.68	10.08	0.001
Temperature (A)	1	1.592E-06	0.94	0.35	5.35	0.3037	0.593
pH (B)	1	3.454E-06	2.06	0.18	21.03	1.21	0.30
NaCl (C)	1	9.156E-08	0.0546	0.82	0.1544	0.0089	0.92
AC	1	0.0000	16.77	0.002	236.26	13.56	0.005
BC	1	0.0000	20.04	0.001	287.52	16.50	0.002
A²	1	0.0001	33.54	0.00	457.72	26.27	0.0006
B²	1	0.0000	23.82	0.009	375.37	21.54	0.001
Residual error	9	0.0000			156.81		
Lack of fit	7	0.0000	10.2	0.99	119.08	0.9019	0.61
Pure error	2	1.807E-06			37.72		
Total	16	0.0002			1,386.48		
R²: 0.9096					R²: 0.88		
CV: 8.22%					CV: 9.13%		
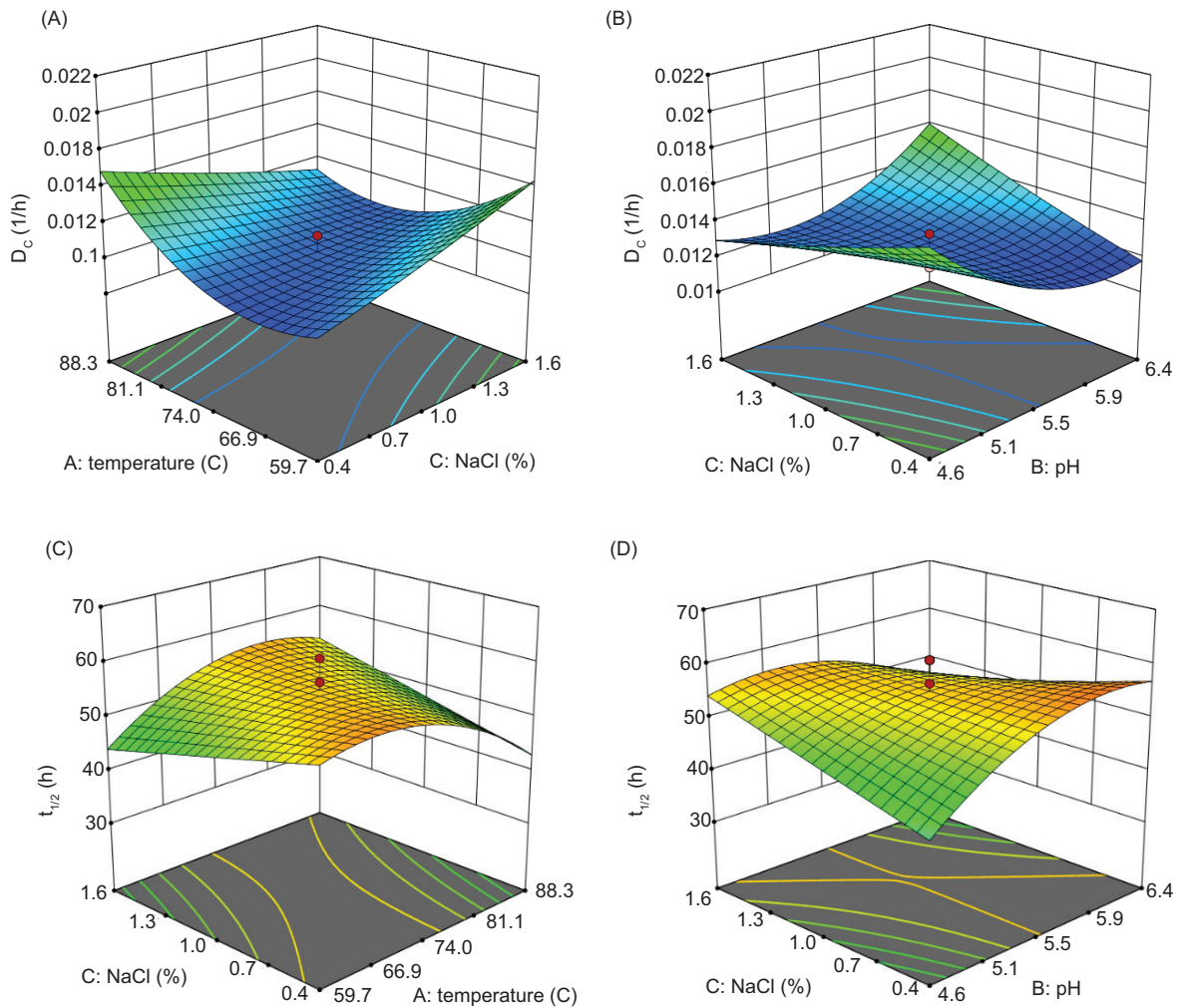
Figure 2. The 3D response surface plot demonstrating effects of: (A) temperature and pH and (B) NaCl and pH on the thermal degradation constant (Dc); and (C) temperature and pH and (D) NaCl and pH on half-life ($t_{1/2}$) of phycocyanin.

Phycocyanin is a heat-sensitive protein. Increase in temperature destroys the protein structure of phycocyanin because at high temperatures, structure of the protein is affected by heating, breaking down of bonds between and within the chain. Therefore, the stability of phycocyanin decreases (Chaiklahan *et al.*, 2012). This change is irreversible (Doke, 2005). Decreased color stability because of high temperatures is expected in most natural colors (Priatni, 2015).

Under thermal processing, minimum changes in phycocyanin pigment occur at a temperature of 59.7°C, and maximum degradation occurs at a temperature of more than 88.3°C. Degradation of the pigment is slow at a temperature of 26–43°C, but the rate of degradation is faster at a temperature of 47–64°C and the pigment is destroyed at a temperature of more than 64°C (Chaiklahan *et al.*, 2012; Doke, 2005).

Salt stabilizes phycocyanin by covering the surface of phycocyanin and changing its structure (Chaiklahan *et al.*, 2012; Hadiyanto *et al.*, 2019; Martelli *et al.*, 2014). Hence, the addition of NaCl at a concentration of more than 1% w/w prevents phycocyanin degradation by more than 50–70% (Chaiklahan *et al.*, 2012). Use of preservatives decreases Dc but increases $t_{1/2}$ of phycocyanin (Figure 2).

One of the effective parameters in the degradation of phycocyanin pigment under different thermal processing conditions is pH. The results showed that the lowest degradation was at pH = 5.5, hence under these conditions, the lowest Dc and the highest $t_{1/2}$ of phycocyanin were observed (Figures 2B and 2D). Phycocyanin is much more stable at pH = 5 than 6 but shows more instability at pH = 7 (Antelo *et al.*, 2008). Because of the proteinaceous nature of phycocyanin, pH depends on temperature and other environmental conditions (Chaiklahan *et al.*, 2012; Priatni, 2015). By investigating the addition of preservatives to food formulations, degradation of the protein part of the pigment is delayed. Therefore, the Dc and $t_{1/2}$ of phycocyanin pigment decreases and increases, respectively. The use of different preservatives, such as NaCl, amino acids, and sorbitol, increases the stability of phycocyanin at different temperatures and pH conditions (Barbiroli *et al.* 2017; Hadiyanto *et al.*, 2019).

Conclusions

The results of the current study demonstrated the stability of phycocyanin produced by *A. platensis* under the stress conditions of temperature, NaCl, and pH shocks. Kinetic model for the thermal degradation of aqueous phycocyanin produced by *A. platensis* was validated as the first-order model. In general, Dc increased and $t_{1/2}$

of the pigment decreased with increase in temperature. Instability of the pigment decreased with increase in pH from 4 to 5.5 at high temperatures. In addition, salt stress played a protective role regarding stability of the pigment. Modeling and predicting the degradation of phycocyanin in food products by the response surface method provided the possibility of calculating and designing the exact amount of this pigment to create a desired color in a food product. Data showed that the stability of this pigment was suitable during thermal processing, pasteurization, baking, and in food matrix at a near neutral pH of 6.6 and a low NaCl content of 0.40% w/w.

Author Contributions

Conceptualization: Mahshid Jahadi; Methodology: Niloofar Shafieiyoun, Mahshid Jahadi, and Kianoush Khosravi-Darani; software: Mahshid Jahadi; validation: Kianoush Khosravi-Darani; Analysis: Mahshid Jahadi and Kianoush Khosravi-Darani; investigation: Niloofar Shafieiyoun; resources: Mahshid Jahadi; data curation: Niloofar Shafieiyoun, Mahshid Jahadi, and Kianoush Khosravi-Darani; preparation and writing of original draft: Niloofar Shafieiyoun, Mahshid Jahadi, and Kianoush Khosravi-Darani; review and editing, Niloofar Shafieiyoun, Mahshid Jahadi, and Kianoush Khosravi-Darani; supervision: Mahshid Jahadi; and project administration: Niloofar Shafieiyoun, Mahshid Jahadi, and Kianoush Khosravi-Darani.

Conflict of Interest

The authors stated that they had no conflict of interest to declare.

Data Availability

The raw/processed data required to reproduce these findings cannot be shared at this time as the data also form part of an ongoing study.

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