P47: EXTRACTION, PURIFICATION AND EVALUATION OF FOOD-GRADE PHYCOCYANIN FROM SPIRULINA PLATENSIS

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Abstract – Phycocyanin (C-PC) is the major phycobiliprotein in *Spirulina (Arthrospira) platensis (Sp.)*, which is a cyanobacterium with a massive commercial value representing a nutrient-dense food source. This study describes a simple protocol for purifying C-phycocyanin from *Spirulina Sp.*. The cell lysis of blue-green algae has been carried out by sonication and repeated cycles of freezing and thawing. Among the various buffers used for phycocyanin yield efficiency, phosphate buffer (pH 7, 0.1 M) was found to be the most suitable for highest yield. The purification process involves a multistep treatment of the crude extract, of purity 1.41, by fractional precipitation with ammonium sulfate followed by dialysis and ion-exchange chromatography on DEAE-Sepharose Fast Flow column obtaining food-grade phycocyanin. The purity of phycocyanobilin chromophore has been tested by UV-Vis spectrophotometry by monitoring the absorption after each stage of purification. Also, separation and identification of the purified protein has been achieved by High Performance Liquid Chromatography with Photodiode-array Detection.

Keywords*:* C-phycocyanin; Spirulina; Arthospira Platensis; Protein Purification; Ion exchange chromatography; HPLC; Photodiode array detection; Blue confectionery

1. INTRODUCTION

C-phycocyanin (C-PC) is the major protein, especially phycobiliprotein, in Spirulina namely *Arthospira Platensis* [1]. Phycobiliproteins consist of covalently linked tetrapyrole groups that play a biological role in collecting sunlight and, through fluorescence resonance energy transfer,

conveying it to a special pair of chlorophyll molecules located in the photosynthetic reaction center. A solution of C-PC appears dark blue color with a maximum absorption at 615-620 nm [2].

The chemical structure of C-PC is shown in Figure 1. C-PC has multiple applications in food industry as an additive in nutraceutical products or as natural colorant in chewing gums, candies, ice-cream, dairy products, beverages, confectionery, and cosmetics. Also, it is used in many molecular and pharmaceutical diagnostic applications due to its fluorescence properties, and its antioxidant, neuroprotective agent, anti-inflammatory and anti-cancer activities [3-5].

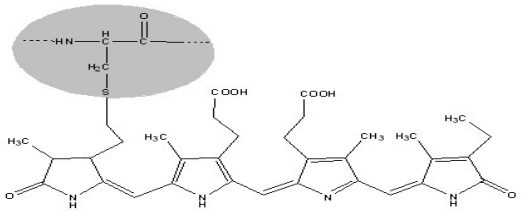

Fig. 1. Chemical structure of C-phycocyanin.

Isolation of C-PC has been achieved with many cell disruption techniques, such as osmotic shock [6], high pressure homogenizer [7], repeating freezing and thawing cycles [8] or lysozyme digestion [9]. Ammonium sulfate precipitation combined with ion exchange chromatography [10] and gel filtration [11] have been performed to obtain C-PC of all grades, food, cosmetic, reagent, analytical.

The aim of the current study was at first to develop a simple and effective protocol for the extraction, isolation and purification of C-PC food-grade from *Spirulina*. The next step was to develop and validate an HPLC-DAD method for the determination and identification of C-phycocyanin (food grade, A_{620}/A_{280} ~ 2.5). The method has been applied to blue pigment extracts from commercial confectionery as well as to C-PC isolated in the

present study.

2. EXPERIMENTAL

2.1. Extraction, isolation and purification of C-phycocyanin from Spirulina Platensis

Spirulina Platensis dried powder was suspended in 0.1 M sodium phosphate buffer pH 7 in a ratio 1:15 w/v. The suspension was homogenized using sonication for 10 min and three repeated cycles of freezing and thawing (RFT cycles) were followed. The suspension was frozen for 3 hours at -18°C and thawed for 1 hour at 25°C. The blue crude extract containing the C-phycocyanin was obtained after centrifugation at *4 000* × *g* for 30 min.

The absorption spectra of phycocyanobilin chromophore were measured on a UV-Vis Spectrophotometer (Jasco) at wavelengths 280, 620 and 650 nm. The ratio of A_{620} to A_{280} gives the purity of C-phycocyanin, wherein A_{620} is the maximum absorbance of C-PC, A_{650} is the maximum absorbance of allophycocyanin and A_{280} is the absorbance of total proteins.

The C-phycocyanin concentration (C-PC) in mg mL^{-1} was calculated, using the following equations as described by Bennett and Bogorad [2]:

$$C-PC\ (mg\,ml^{-1}) = \frac{A_{620}-0.474(A_{650})}{5.34} \quad (1)$$

The extraction yield was calculated using Equation 2 [5]:

$$Y(mg \times g^{-1}) = \frac{(C-PC)\times V}{DB} \quad (2)$$

where, Y is the extraction yield of phycocyanin in mg of C-phycocyanin/dry biomass (g), *V* is the solvent volume (mL) and *DB* is the dry biomass (g).

The crude extract was fractionally precipitated by solid ammonium sulfate ($(NH_4)_2SO_4$), first at 25% and then at 70% (w/v). Solid $(NH_4)_2SO_4$ was added to 25% (w/v) to the crude extract with gentle stirring for 1 h at 4 °C. The green precipitate from the 25% (w/v) $(NH_4)_2SO_4$ saturation was discarded after centrifugation at *4000* × *g* for 30 min. The blue supernatant was subjected to 70 % (w/v) $(NH_4)_2SO_4$ saturation in a similar way as 25% (w/v) saturation. The green-yellow supernatant after centrifugation was excluded too. The blue precipitate was dissolved in a small volume of the extraction buffer (5 mL) and dialyzed against 500 mL of the extraction buffer using dialysis membrane for 24 h changing the buffer 4 times.

DEAE-Sepharose Fast Flow (FF) was used for anion exchange chromatography. The dialyzed sample (5 mL) was loaded on a glass column packed with DEAE-Sepharose FF, which had been pre-equilibrated with 10 bed volumes of 0.1 M sodium phosphate buffer pH 7.8. After the unbounded proteins have been removed by washing the column with the above buffer, the C-PC was eluted with NaCl solution of a linear increasing ionic concentration from 0 to 0.35 M NaCl at a flow rate of 0.8 mL min^{-1}. C-PC gets eluted at NaCl concentration between 0.25 and 0.30M was collected in 5mL fractions.

2.2. High performance liquid chromatography

Chromatographic analysis was performed on a Shimadzu HPLC system (Kyoto, Japan) equipped with a SPD-M6A Photodiode Array UV-Vis detector, combined with data acquisition *software Class M10A*. The HPLC system was consisted of a LC-$9AD_{vp}$ pump (Shimadzu), a helium DGU-10B degassing unit and an auto sampler injector SIL-9A (Shimadzu). The chromatographic separations were achieved using an Orbit 100 C_4, 5 µm, 250 × 4.0 mm (MZ-Analysentechnik, Mainz, Germany). The composition of the mobile phase was: 0.1% v/v TFA in water (solvent A) and CH_3CN containing 0.1% v/v TFA (solvent B). The following gradient program, at a constant flow rate of 0.8 mL min^{-1} was used: from 0 to 10 min, the composition was increased from 45 to 100% B. The injection volume was 100 µL. Column effluent was monitored using a photodiode array detector, set at 620 nm.

2.3. Sample preparation

A variety of blue candies and sweets were purchased from a local supermarket in Thessaloniki, Greece. The foodstuff was dissolved in a small amount of phosphate buffer (0.1 M, pH 7) until all color was removed. Then the sample was centrifuged for 10 minutes and the supernatant was injected in the HPLC column.

2.4. Validation of the analytical procedure

To validate the identification method of phycocyanin as natural color additive in confectionery, the following performance parameters were evaluated in terms of ICH [12]: selectivity, linearity, precision, accuracy, limit of detection (LOD) and limit of quantification (LOQ) using standard solutions and blank samples (blue pigment extract of candy with no C-PC).

3. RESULTS AND DISCUSSION

3.1. Optimization of the extraction and isolation process of C-phycocyanin

Optimization of extraction procedure of *Spirulina Sp.* was performed by testing the extraction solution, freeze time and solid-liquid ratio in the freeze-thaw cell digintegration technique. Results are shown in Tables 1-3. Among the various extraction solvents, the highest phycocyanin yield were obtained by sodium phosphate buffer (0.1 M, pH 7) (Table 1).

Table 1. Yield and purity of the crude extract of phycocyanin from *Spirulina Platensis* by the repeated cycles of freezing and thawing combined with sonication method with different extraction solvents.

Extraction solution	Yield (mg/g)	Purity (A_{620}/A_{280})
Distilled water	58.84	0.63
Sodium acetate buffer 20 mM (pH 5)	64.03	0.75
Sodium citrate buffer 0.1 M (pH 6)	63.20	0.72
Sodium phosphate buffer 0.1 M (pH 7)	66.44	0.95
Tris-HCl buffer 20 mM (pH 8.1)	64.89	0.87

The highest C-phycocyanin purity (1.41) was obtained by 3 h freezing in every cycle. The type of sonication didn't have a significant impact on the purity of crude extract (Table 2). Sonication bath was chosen, as a more convenient technique for the operator.

Table 2. Comparison of the purity of the crude extract of C-phycocyanin at different freezing times and using different sonication devices in the presence of phosphoric acid buffer (0.1 M, pH 7).

	Purity C-PC (A_{620}/A_{280})			
	Freeze time (h) at -18 °C			
	1	*2*	*3*	*4*
Sonication bath	0.95	1.13	1.41	1.42
Probe-type sonication	0.96	1.15	1.42	1.42

According to Table 3, the solid-liquid ratio 1:15 w/v was found optimal, since the highest extraction yield and purity were achieved in this ratio.

The results of ammonium precipitation study are shown in Table 4. The two-step precipitation of C-PC with 25%-70% (w/v) $(NH_4)_2SO_4$ saturation is suitable for the isolation of C-PC.

Table 3. Effect of solid-liquid ratio on yield and purity of C-PC on crude extract.

Ratio Spirulina powder: extraction solvent (g/mL)	Yield (mg/g)	Purity C-PC (A_{620}/A_{280})
1:10	38.56	1.32
1:15	66.44	1.41
1:20	55.32	1.13
1:25	43.27	1.08

Table 4. Purity of C-phycocyanin at various ammonium sulphate saturation rates.

Ammonium sulfate precipitation (w/v)	Purity (A_{620}/A_{280})
50% saturation	1.28
70% saturation	1.36
25%-50% saturation	1.48
25%-70% saturation	1.81
30%-65% saturation	1.56

After the ammonium sulfate precipitation steps, the purity of C-phycocyanin was increased to 1.81. An ion exchange chromatography column was used to obtain food-grade C-phycocyanin with purity ratio 2.5. The concentration of C-PC solution was calculated through Eq.1 and was found to be 356.3 ng/μL.

3.2. Method validation

The calibration curve was calculated by least-squares linear regression analysis and the linear regression equation obtained for C-PC was:

Y= (15118.05 ± 338.82)X+(16700.89 ± 15555.45),

with coefficient of determination (r^2) of 0.9982 proving the satisfactory linearity of the developed method within the range analyzed (2-100 ng/μL). The limit of quantitation (LOQ) was calculated by the S/N=10 ratio, where S is the signal and N is the noise. While the detection limit (LOD) was calculated as LOD=LOQ/3.3. LOQ was 2 ng/μL and LOD was 0.67 ng/μL.

As for selectivity, no interferents were observed in corresponding retention time of C-PC by comparing chromatograms of spiked samples and blank samples. Figure 2 represents the chromatograms of blank sample and of a spiked sample with C-PC at a concentration of 25 ng/μL monitored at 620 nm.

Table 5 provides the RSD and the mean recovery percentages used to evaluate accuracy and precision in the same day and in five different days. The results obtained at each concentration were expressed as percent recoveries using the equation:

$$R = \frac{C_1 - C_2}{C_3} \times 100 \quad (Eq. 3)$$

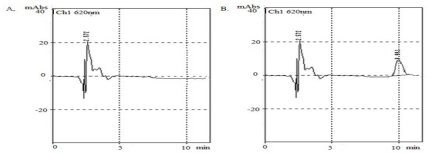

Fig.2 Chromatograms of (A) blank sample, a blue pigment extract of a candy with no C-phycocyanin and (B) spiked sample of blue pigment with the addition of 25 ng/μL standard solution of C-phycocyanin.

Table 5. Intra-day and inter-day accuracy and precision of the developed HPLC-DAD method for the determination of C-phycocyanin in spiked sample of blue pigment.

Added concentration (ng/μL)		Intra-day			Inter-day		
		SD	RSD (%)	R (%)	SD	RSD (%)	R (%)
C-PC	10	1.6	5.6	98.1	1.2	4.0	96.8
	25	1.0	2.4	93.4	0.8	2.1	95.5
	50	0.8	1.5	91.2	1.7	3.2	95.0

The developed and validated method was applied for the identification and quantification of C-PC in blue candies. Five types of blue candies were analyzed and the presence of C-PC was found only in one sample at a concentration of 32.4 ng/μL. Its chromatogram is shown at Fig. 3.

Also, the purified protein derived from the above purification procedure has been quantified through HPLC method after 1:5 (v/v) dilution and its concentration was found to be 70.9 ng/μL. Its chromatogram is shown at Fig. 3. This value was similar with the expected value of C-PC concentration calculated by Eq. 1.

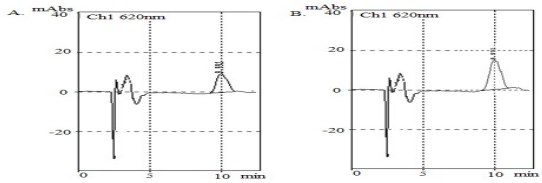

Fig.3. Chromatograms of (A) blue pigment extract of a purchased candy, where C-Phycocyanin was identified, (B) C-phycocyanin solution obtained after isolation and purification from *Spirulina Platensis*, monitored at 620 nm.

4. CONCLUSIONS

The extraction, isolation and purification of food-grade C-PC from *Spirulina Sp.* were achieved using an effective protocol with low energy and economic cost. Also, the developed HPLC method the method was selective with low detection and quantification limits. Satisfactory repeatability and accuracy were obtained, when applied to a blue dye extract of a candy, which did not contain C-phycocyanin. The method had a practical application to foodstuff. Sample preparation is simple and non time-consuming, requires low consumption of solvent ensuring high purity of the samples and removing any obstructions from the matrix.

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