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Development and validation of an HPLC-PDA method for the analysis of phycocyanobilin in *Arthrospira maxima*

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Abstract An high-performance liquid chromatography (HPLC)-photodiode array (PDA) method was developed and validated for quantifying phycocyanobilin (PCB) in *Arthrospira maxima* extracts. While previous methods for PCB analysis lacked comprehensive validation and struggled with coelution issues, this approach demonstrated high specificity, accuracy, sensitivity, and reproducibility. Effective separation was achieved, with a retention time of 18.7 min. The calibration curve exhibited excellent linearity (r^2 = 1.0000) across a concentration range of 3.125-50 μg/mL, with a limit of detection and limit of quantification of 0.22 and 0.67 μg/mL, respectively. Recovery rates ranged from 97.75 to 103.36%, with relative standard deviations (RSD) between 0.50 and 2.14%, confirming the accuracy and reliability of the method. The intra- and inter-day precisions yielded mean contents of 0.122 (RSD: 1.61%) and 0.128 mg/g (RSD: 0.71%), respectively. Notably, this study is the first to validate an HPLC-PDA method for direct quantification of PCB in *A. maxima* with full validation parameters. By offering a standard for PCB analysis, this study contributes to quality control in research and industrial processes, paving the way for advancements in pharmacology and biotechnology.

Keywords *Arthrospira maxima* · Bioactive compound · Method validation · Reproducibility

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Introduction

Arthrospira maxima, a species of cyanobacteria commonly known as spirulina, is rich in protein and thrives in alkaline lakes [1]. It contains phycocyanobilin (PCB), a blue pigment with significant biological activities [2]. PCB is an open-chain tetrapyrrole chromophore that is covalently bound to the polypeptide chains of C-phycocyanin [3]. However, it is distinct from the full protein complex itself. The biological relevance of PCB is well-documented, showcasing known antioxidant, anti-inflammatory, and anticancer properties [4-6]. Recent studies have identified PCB role as a potent legumain inhibitor, further enhancing its therapeutic potential [7]. These findings highlight the importance of accurately quantifying PCB in natural products and supplements. Additionally, the interaction of PCB with thiol groups and its potential role in platelet inhibition suggest promising applications in cardiovascular health [7]. Both phycocyanin and its derivative PCB have demonstrated significant antioxidant and anti-inflammatory effects [8], offering protective benefits against various conditions such as diabetic nephropathy [8], colitis [9], and neurodegenerative diseases, including multiple sclerosis and cerebral ischemia. It interacts with human serum albumin (HSA), enhancing HSA's thermal stability and protecting it from proteolytic degradation [10]. These effects are achieved through mechanisms that mitigate oxidative stress and promote cellular health [11].

PCB has demonstrated remarkable stability under harsh conditions, retaining its structure during high-pressure and heat processing, unlike phycobiliproteins and phycocyanin [12,13]. However, the stability of PCB is influenced by environmental factors such as pH. It shows low solubility and aggregation under acidic pH conditions, while oxidation occurs under neutral

to alkaline conditions, with the rate increasing as pH rises [12]. Despite these promising characteristics and its potential applications in pharmaceuticals, cosmetics, and food industry, research on PCB remains limited, and standardized methods, such as high-performance liquid chromatography (HPLC) analysis for its quantification and characterization have not yet been fully established. Additionally, PCB is typically extracted from *A. maxima* using methanol. These properties highlight the significance of PCB in biotechnology and health-related fields, supporting its continued exploration for pharmaceutical and nutraceutical applications [14].

The detection and quantification of compounds related to PCB, such as C-phycocyanin, have been subjected to method studies [15]. However, previous studies on PCB quantification did not include a fully validated HPLC method. In this context, this study is the first to establish and validate an HPLC-photodiode array (PDA) method for the direct quantification of PCB in *A. maxima*, addressing this gap in the literature [16,17]. The validated HPLC method presented in this study offers a reliable tool for future research and quality control processes involving PCB extracts.

Materials and Methods

Equipment and reagents

A Waters Alliance e2695 system (Milford, MA, USA) equipped with a pump, autosampler, and Waters 2998 PDA detector (Milford, MA, USA) was used for the analysis. HPLC-grade methanol (MeOH), distilled water, and acetonitrile (ACN) were purchased from Honeywell (Burdick and Jackson, Muskegon, MI, USA). HPLC-grade trifluoroacetic acid (TFA) was supplied by Sigma-Aldrich (St. Louis, MO, USA). PCB standard (purity ≥99.0%) was purchased from SiChem GmbH (SC-1800, Bremen, Germany) (Fig. 1).

Preparation of PCB

Fig. 2 shows the *A. maxima* grown in the aquaculture tanks of Jeju Bio Research Center, Korea Institute of Ocean Science and Technology (Jeju, Republic of Korea), while Fig. 3 presents its characteristic spiral morphology under magnification. The extraction of PCB from this marine cyanobacterium was carried out by air-drying the fresh samples and homogenizing it into fine powder. The powder (1 g) was mixed with 0.1 M sodium phosphate buffer (100 mL) with a pH of 7.0 at a ratio of 1 : 100 (*w/v*). The mixture was sonicated for 15 min, followed by three freeze-thaw cycles to enhance cell disruption. Centrifugation was carried out at 2322 × *g* for 20 min to collect the crude extract. Afterward, phycocyanin was purified according to the methodologies described in prior studies [3,18]. Specifically, by single-step precipitation using 65% $(NH_4)_2SO_4$ at 4 ºC overnight. The mixture was centrifuged at 4000 × *g* and 4 ºC for 20 min to collect the pellets and resuspended in 10 mL of the extraction buffer. The extract was dialyzed by placing 10 mL of the extract into 1000 mL of the same buffer, repeating the process twice while keeping it in the dark at 4 ºC for 24 h. Once the dialysis was completed, the extract was removed from the membrane and filtered through a 0.45 µm syringe filter.

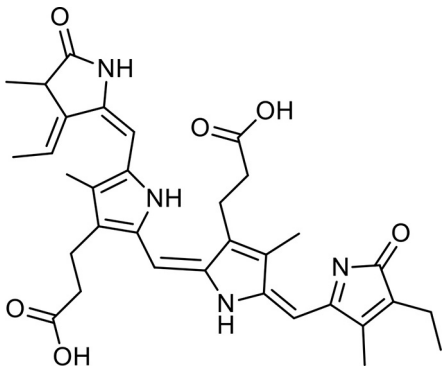
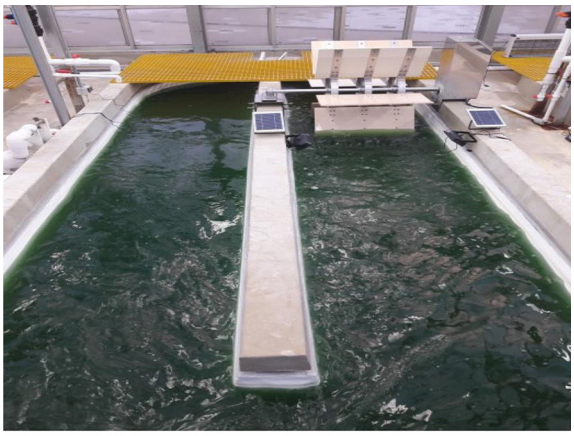


Fig. 1 Chemical structure of PCB



(A)

(B)

Fig. 2 Cultivation of *A. maxima* in liquid medium in the aquaculture tanks (A). Harvesting of biomass by filtration and drying prior to phycocyanobilin extraction (B)

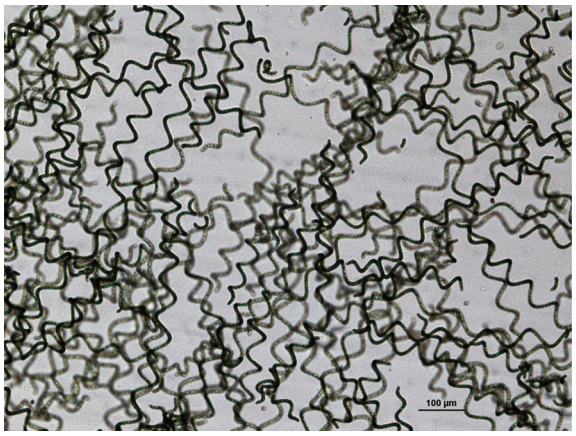


Fig. 3 Microscopic image of the spiral trichome structure of *A. maxima*. The characteristic helical morphology of *A. maxima* filaments observed under a microscope at ×100 magnification

To obtain PCB, 1.5 L of ethanol was combined with the recovered phycocyanin powder in 1 : 50 (*w/v*) ratio in the dark at 70 ºC for 15 h followed by filtration with 0.45 µm syringe filter [19]. Finally, the solvent was evaporated followed by evaporation to achieve a powdered PCB. The powder was stored at –20 °C in the dark until further use.

Preparation of samples and standard solutions for HPLC-PDA

A stock solution of PCB was prepared by accurately weighing 5.0 mg of PCB and dissolving it in 10 mL of 20% methanol, resulting in a concentration of 0.5 mg/mL. The solution was sonicated until completely dissolved and then filtered using a 0.2 µm polyvinylidene fluoride (PVDF) syringe filter. To prepare the test solutions, 2.0 g of PCB extract was accurately weighed and dissolved in 10 mL of 20% methanol, yielding a concentration of 200 mg/mL. This solution was vortexed thoroughly, centrifuged at 10,416 × *g* 4 ºC for 10 min, and filtered through a 0.2 µm PVDF syringe filter. The filtrate was further diluted to a concentration of 100 mg/mL for subsequent HPLC analysis.

HPLC-PDA conditions

HPLC analysis was conducted using a YMC-Pack Pro C18 column (4.6 × 250 mm, 5 µm). The mobile phase consisted of solvents A (0.1% TFA in water) and B (0.1% TFA in ACN). Gradient elution was performed at a flow rate of 1.0 mL/min. The mobile phase started at with 72% solvent A and 28% solvent B for 7 min, increased to 38% solvent B by 13 min, and remained constant until 21 min. Solvent B reached 100% at 25 min and was maintained until 30 min, then returned to the initial ratio by 32 min, holding until 45 min. The column temperature was maintained at 26 ºC, and the injection volume was 10 µL. The PDA detector was set to monitor at 375 nm, with the autosampler kept at 12 ºC HPLC analysis of PCB was performed according to the method described in previous studies [20,21].

Method validation

The HPLC-PDA method was validated for specificity, linearity, accuracy, precision, limit of detection (LOD), and limit of quantification (LOQ) in accordance with ICH guidelines, with reference to previous studies [22-24]. Specificity was assessed by comparing the retention times and UV spectra of the PCB standard and PCB extract. No interference peaks were observed. Linearity was evaluated by preparing five concentrations of the PCB standard (3.125 to 50 µg/mL) and plotting peak areas against concentrations (n = 3). The correlation coefficient (r^2) was calculated to confirm linearity. Accuracy was determined through recovery tests by spiking known amounts of the standard into the extract at three different concentrations, with each concentration measured in triplicate, and calculating the recovery percentage. Precision was assessed by intra- and inter-day variability, with the results expressed as relative standard deviation (RSD; %). LOD and LOQ were calculated using the formulas LOD = 3.3 (σ/S) and LOQ = 10 (σ/S), where σ is the standard deviation of the y-intercept and S is the slope of the calibration curve.

Calibration curve

The concentrations of the PCB standard solutions were plotted against their corresponding peak areas to construct the calibration curve. Linearity was determined by calculating the correlation coefficient (r^2), which confirmed the validity of the calibration. The calibration curve was constructed to determine the PCB concentration in the PCB extract samples. Calibration functions were established using the peak area (Y), the standard concentration (X, µg/mL), and the mean ± standard deviation (n = 3) (Table 1).

Table 1 Calibration curve for the PCB and related statistics

Compound	t_R [b]	Range (µg/mL)	Calibration equation[c]	r^2 [d]	LOD (µg/mL)	LOQ (µg/mL)
PCB[a]	18.7	3.125 – 50.0	Y = 21669X + 4149.4	1.0000	0.22	0.67

[a]Pycocyanobilin
[b]Retention time
[c]Y: peak area, X: concentration of the standard (µg/mL)
[d]Correlation coefficient for the five data points in the calibration curve (n = 3)

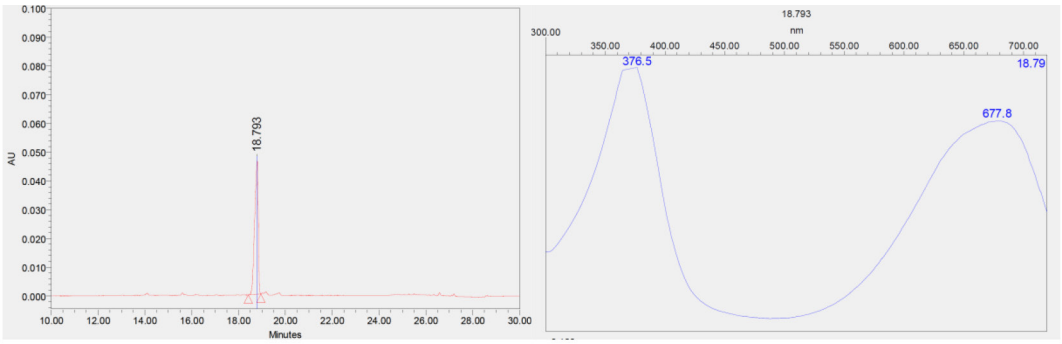
Results and Discussion

The development and validation of the proposed HPLC-PDA method for PCB quantification yielded reliable results. The specificity of this method was confirmed through UV spectral analysis by comparing the absorption profiles of the PCB standard, samples, ensuring no interfering peaks were present. The chromatograms exhibited consistent retention times at 18.7 min, and no interference was observed from other components, ensuring effective separation throughout the 45-min analytical process. The HPLC chromatograms demonstrated effective detection of PCB (Fig. 4). Additionally, the UV spectra exhibited distinct absorption characteristics, further confirming its identification. The high accuracy of the validated method was further demonstrated by the absence of overlapping peaks and the successful identification of PCB in test extracts via recovery rate testing.

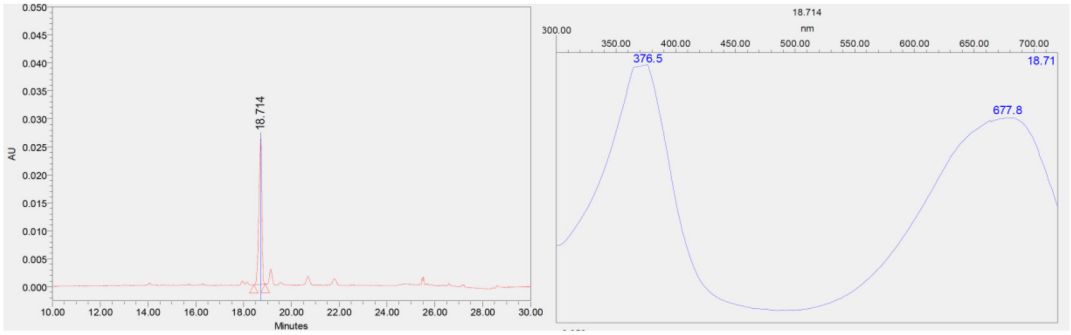
A calibration curve was constructed using PCB standard solutions in the concentration range of 3.125-50 µg/mL. The calibration equation was determined as Y=21669X+4149.4, where Y denotes the peak area and X represents the PCB concentration (µg/mL). The coefficient of determination (r^2=1.0000) indicated excellent linearity over the tested range (Table 1). The LOD and LOQ for PCB were estimated from the calibration curve. The LOD was determined to be 0.22 µg/mL, while the LOQ was 0.67 µg/mL, indicating the high sensitivity of the method (Table 1).

Accuracy was evaluated using spiking experiments at three concentrations (low, medium, and high). The recovery rates ranged from 97.75 to 103.36%, with RSD values between 0.50 and 2.14%. This confirms the reliability of the method for quantifying PCB extracts (Table 2). Both intra- and inter-day precision analyses demonstrated the reproducibility of the method. Intra-day precision, assessed by five replicate analyses of a 100 mg/mL PCB extract, yielded a mean content of 0.122 mg/g with an RSD of 1.61%. Inter-day precision, determined by analyzing the same extract across three consecutive days, resulted in a mean content of 0.128 mg/g with an RSD of 0.71%, both of which met the validation criteria (Table 3).

Recent studies have employed HPLC-based techniques to analyze phycobilin compounds in cyanobacterial samples, using systems with UV-Vis or PDA detectors and standard columns [19,25]. However, these approaches did not include systematic method validation parameters such as linearity, precision, recovery, and sensitivity. This validated HPLC-PDA approach presented in this study was strong, dependable, and appropriate for measuring PCB in extracts made from *A. maxima*. The absence of interference peaks and the accurate exact recovery rates, which demonstrate specificity and accuracy, confirm that this approach can identify PCB in intricate biological matrices. The linearity achieved across a broad concentration range (r^2=1.0000) highlights the precision of the method and its potential adaptability for various experimental requirements. Additionally, the low LOD (0.22 µg/mL) and LOQ



(A)



(B)

Fig. 4 HPLC-PDA chromatograms measured at 375 nm and UV spectra of PCB (A) and *A. maxima* extract (B)

Table 2 Accuracy analysis statistics for PCB determination

Compound	Spike amount		Recovery (%)			Average (%)	RSD[b] (%)
	Sample (mg/mL)	PCB (μg/mL)[a]	1st	2nd	3rd		
PCB [a]	100.0	6.25	99.05	101.49	103.36	101.30	2.14
		12.50	100.59	100.22	99.41	100.07	0.60
		25.00	97.75	98.67	97.91	98.11	0.50

[a]Phycocyanobilin
[b]Relative standard deviation

Table 3 Intra- and inter-day precision for PCB determination

Sample concentration (mg/mL)	PCB[a] content (mg/g)	
	Intra-day (n = 5)	Inter-day (n = 5)
100.0	0.124	0.129
	0.121	0.127
	0.122	0.128
	0.123	0.127
	0.119	0.127
Average content (mg/g)	0.122	0.128
Standard deviation	0.002	0.001
RSD[b] (%)	1.61	0.71
Average content (mg/g)	0.125	
Standard deviation	0.004	
RSD[b] (%)	3.44	

[a]Phycocyanobilin
[b]Relative standard deviation

(0.67 μg/mL) values indicate its high sensitivity, which is crucial for detecting PCB even at trace levels. This is particularly significant for biological and pharmaceutical applications where accurate quantification of bioactive compounds is critical [26].

This study is the first to validate an HPLC-PDA method with full validation parameters for the direct quantification of PCB in *A. maxima*, thereby addressing a notable gap in the literature [16,17]. In addition to improving analytical capabilities for PCB, this notable study contributes to a better understanding of the properties and potential applications of PCB across a range of biological and pharmacological domains. Application-wise, this verified approach ensures reproducibility and consistency, making it possible to integrate it into industry and research quality control procedures. The discovery and accurate measurement of PCB can facilitate future pharmacological research into its anti-inflammatory, antioxidant, and therapeutic properties. Its potential utility is further expanded because the validation protocols and methodological framework developed herein establish a standard for analyzing other phycobilin-based substances.

Future directions for this approach include extending its use in bioengineering contexts and incorporating it into research that uses PCB as a cofactor in optogenetic applications [27,28]. As previous studies have shown, precise PCB quantification could facilitate sophisticated applications such as CreLite systems and PhiReX2.0 [29,30], highlighting the significance of validated techniques in ensuring accuracy and reproducibility in contemporary research and industrial processes [31].

In summary, the HPLC-PDA technique developed and validated in this study provides a strong, dependable, and effective method to measure PCB in extracts from *A. maxima*. The high specificity, precision, sensitivity, and reproducibility of this method ensure precise PCB quantification even at trace levels, making it suitable for a wide range of biological and pharmaceutical applications. This study lays the groundwork for PCB measurement from *A. maxima* using HPLC-PDA, in contrast to previous technique validations that have focused on compounds such as C-phycocyanin. Notably, the efficacy of this method for measuring PCB represents a significant advancement in the analytical field. This study provides an important foundation for investigating the medicinal properties of PCB, such as its anti-inflammatory and antioxidant activities. Additionally, the validated approach offers a vital tool for quality control procedures in the synthesis of bioactive compounds, promoting research-based and industrial developments in pharmacology and biotechnology. Therefore, this study emphasizes the importance of developing accurate and validated procedures for the efficient utilization of bioactive substances in innovative applications.

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