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Comparative Analysis of Solvent Systems and Extraction Techniques for Phycocyanin Recovery from *Spirulina sp*. Powder

Spirulina sp. Tozundan Fikosiyanin Elde Edilmesinde Çözücü Sistemleri ve Ekstraksiyon Tekniklerinin Karşılaştırmalı Analizi

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ABSTRACT: Phycocyanin (PC), a high-value blue phycobiliprotein derived from *Spirulina sp*., has attracted considerable industrial interest due to its broad applications in food, pharmaceutical, cosmetic, and biotechnological sectors. In this study, conventional and green extraction approaches, including freeze-thawing , solvent-based extraction, ultrasound-assisted extraction, microwave-assisted extraction, and supercritical carbon dioxide (SC-CO_2) extraction, were comparatively evaluated for PC recovery from dried *Spirulina sp*. powder. Different solvent systems and four consecutive extraction cycles were assessed in terms of phycocyanin content, purity, yield, total protein concentration, visual appearance, and CIE Lab* color characteristics. Significant differences among extraction methods, solvent systems, and extraction cycles were observed ($p < 0.05$). Ultrasound-assisted extraction using sodium phosphate buffer produced the highest phycocyanin yield (480 mg/g), whereas freeze-thawing extraction provided comparatively lower yields but higher pigment purity and stability. Solvent polarity strongly influenced extraction performance, with buffered aqueous systems generally exhibiting superior pigment retention and chromatic stability compared with organic solvents. Microwave-assisted extraction promoted pigment degradation due to thermal stress, while SC-CO_2 extraction showed limited phycocyanin selectivity under the tested conditions. Overall, extraction efficiency and pigment stability were highly dependent on both solvent composition and extraction strategy, highlighting the importance of optimizing extraction conditions for industrial-scale phycocyanin recovery.

Keywords: *Spirulina*, phycocyanin, extraction methods, conventional methods, green methods

ÖZ: Spirulina türlerinden elde edilen yüksek değerli mavi bir fikobiliprotein olan fikosiyanin (PC), gıda, ilaç, kozmetik ve biyoteknoloji sektörlerindeki geniş uygulama alanları nedeniyle endüstride büyük ilgi görmektedir. Bu çalışmada, kurutulmuş *Spirulina sp*. tozundan PC geri kazanımı için dondurma-çözme, çözücü bazlı ekstraksiyon, ultrason destekli ekstraksiyon, mikrodalga destekli ekstraksiyon ve süperkritik karbondioksit (SC-CO_2) ekstraksiyonu dahil olmak üzere geleneksel ve yeşil ekstraksiyon yaklaşımları karşılaştırmalı olarak değerlendirilmiştir. Farklı çözücü sistemleri ve dört ardışık ekstraksiyon döngüsü, fikosiyanin içeriği, saflık, verim, toplam protein konsantrasyonu, görsel görünüm ve CIE Lab* renk özellikleri açısından değerlendirilmiştir. Ekstraksiyon yöntemleri, çözücü sistemleri ve ekstraksiyon döngüleri arasında anlamlı farklılıklar gözlemlenmiştir ($p < 0,05$). Sodyum fosfat tamponu kullanılarak yapılan ultrason destekli ekstraksiyon en yüksek fikosiyanin verimini (480 mg/g) sağlarken, dondurma-çözme ekstraksiyonu nispeten daha düşük verim sağlamış; ancak daha yüksek pigment saflığı ve stabilitesi sağlamıştır. Çözücü polaritesi ekstraksiyon performansını güçlü bir şekilde etkilemiştir; tamponlu sulu sistemler, organik çözücülere kıyasla genel olarak daha iyi pigment tutma ve kromatik stabilite göstermiştir. Mikrodalga destekli ekstraksiyon, termal stres nedeniyle pigment bozulmasını teşvik ederken, SC-CO_2 ekstraksiyonu test edilen koşullar altında sınırlı fikosiyanin seçiciliği göstermiştir. Sonuç olarak, bu çalışmada ekstraksiyon verimliliği ve pigment stabilitesinin hem çözücü bileşimine hem de ekstraksiyon stratejisine büyük ölçüde bağlı olduğu gösterilmiştir. Bu sonuç endüstriyel ölçekte fikosiyanin geri kazanımı için ekstraksiyon koşullarının optimize edilmesinin önemini ortaya koymaktadır.

Anahtar Kelimeler: *Spirulina*, fikosiyanin, ekstraksiyon yöntemleri, konvansiyonel yöntemler, yeşil metotlar

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1. INTRODUCTION

Spirulina platensis, also known as *Arthrospira platensis*, is a cyanobacterium (blue-green algae) among the most ancient and primitive photosynthetic representatives of prokaryotic organisms. *S. platensis* is characteristically blue-green due to the presence of pigments such as chlorophyll, carotenoids, phycocyanin, and phycoerythrin. *S. platensis* is naturally grown in saline or alkaline waters and humid climates; on the other hand, the optimal controlled growth conditions of *S. platensis* are pH 8.8-11 and 25-37°C temperature (Athiyappan et al., 2024; Soni et al., 2019). *S. platensis*'s nutritional value forms the foundation for its modern applications as a globally popular bioresource in the health, food, and biotechnology industries (Bortolini et al., 2022). Spirulina powder is recognized as a significant source of phytochemicals, such as chlorophyll a, diatoxanthin, xanthophyll, zeaxanthin, echinenone, 3'-hydroxy echinenone, beta-carotene, canthaxanthin, beta-cryptoxanthin, myxoxanthophyll, phycobiliproteins, oxyloxanthin, and allophycocyanin (Gaur et al., 2024). The bioactive components of *Spirulina sp.* offer significant potential for advancement in health, medicine, biotechnology, food, pharmaceuticals, and cosmetics due to the promising role of cyanobacterial metabolites as valuable raw materials.

Phycocyanin (PC), the predominant blue pigment in *Spirulina sp*., is a heterodimer composed of α and β subunits with molecular weights of 14 and 20 kDa, respectively. It also forms part of a supramolecular protein complex with phycoerythrin and allophycocyanin (Patel et al., 2005). Phycocyanin is crucial in capturing sunlight and transferring energy to the photosystem II reaction center. This process drives the light-dependent reactions of photosynthesis, including water splitting and the generation of high-energy electrons, with a maximum absorbance wavelength of around 620 nm (Gómez-Lojero et al., 1997). *Spirulina sp.* is renowned for its high phycocyanin content, constituting 20-25% of its total biomass, making it a valuable source of this pigment for various applications (Athiyappan et al., 2024). The global phycocyanin market is projected to grow significantly, with estimates reaching $245.5 million by 2027 and $279.6 million by 2030 (Cavallini et al., 2024). These projections highlight the increasing commercial interest in phycocyanin, driven by its diverse applications and potential benefits. The growing demand for natural, sustainable, and multifunctional bioactive compounds in food, pharmaceuticals, and cosmetics underscores the importance of developing efficient and scalable phycocyanin extraction methods.

The isolation of phycocyanin involves several key steps: cell lysis, primary extraction, and purification. Effective disruption of Spirulina cells is essential for efficient phycocyanin extraction. Various techniques are employed during the primary extraction phase, including freeze-thawing, solvent-based mixing and homogenization, ultrasound-assisted extraction, and combined techniques. Pre-treatment, such as pulsed electric fields, microwave applications, high-pressure techniques, and supercritical fluid extraction, has been shown to enhance phycocyanin content from *Spirulina sp.* during extraction. These methods help disrupt the cell walls, improving the release of intracellular compounds like phycocyanin. Pulsed electric fields and microwave applications facilitate cell membrane permeability. At the same time, high-pressure techniques and supercritical fluid extraction provide controlled environments that preserve the integrity of bioactive compounds, thereby increasing extraction efficiency and phycocyanin yield. The efficiency of these methods depends on several factors, including biomass structure, cell disruption efficiency, pretreatment of the *Spirulina sp*. biomass, choice of solvent, and process parameters such as temperature, pressure, and extraction duration (Pez Jaeschke et al., 2021). Dried Spirulina biomass presents greater cell disruption challenges than wet biomass because dehydration promotes structural compaction and increased cellular rigidity, which can restrict solvent penetration and reduce the release efficiency of intracellular compounds such as phycocyanin, thereby reducing extraction efficiency compared with fresh or wet biomass. Consequently, more intensive or optimized disruption strategies are often required to achieve efficient pigment recovery from dried biomass (Safi et al., 2014; Chen et al., 2022).

To date, various conventional and green extraction methods have been employed to obtain phycocyanin from *Spirulina sp*. powder. However, only a limited number of these methods have been reported with comprehensive experimental data, while the majority lack essential information required to assess method reliability and reproducibility. Given the high economic value of phycocyanin and its extensive application potential across multiple industries, there is a growing need to systematically evaluate, compare, and analyze Spirulina-based phycocyanin extraction techniques in terms of extraction efficiency, reproducibility, optimization potential, cost-effectiveness, and practical applicability. Therefore, this study aimed to conduct a comparative analysis of the effects of solvents used in the phycocyanin extraction methods reported in the literature for *Spirulina sp*. powder on phycocyanin content, yield, and purity, and to evaluate four consecutive extraction cycles for each buffer tested. The solvent-to-mass ratio was set to the optimal value reported in the literature. Additionally, while the literature generally reports a maximum of two consecutive extractions, four consecutive extractions for each method were performed in this study.

2. MATERIALS AND METHODS

2.1. Materials, Chemicals, Tools, and Devices

Spirulina sp. powder used in all extraction methods evaluated in this study was obtained from Algolina

Spirulina Company (Istanbul, Türkiye). Organic Blue Spirulina (phycocyanin), used as the positive control (+), and Organic Chlorella Powder, used as the negative control (−), were purchased from Micro Ingredients (Montclair, California, USA) and Naturiga (Istanbul, Türkiye), respectively. Distilled water was supplied by the Center for Scientific and Technological Application and Research of Mehmet Akif Ersoy University (Burdur, Türkiye). Ethanol (EtOH) and calcium chloride ($CaCl_2$) were obtained from Tekkim Chemical Limited Company (Bursa, Türkiye). Tris–Cl buffer (pH 6.8), sodium acetate, cyclohexane, and sodium phosphate were purchased from Sigma-Aldrich® (St. Louis, MO, USA).

A fixed-speed mini vortex mixer (Ohaus VXMNFS, 2500 rpm, China) was used to ensure homogeneous mixing of the samples. Centrifugation of samples for separation of liquid and solid phases was performed using a centrifuge (DLAB DM0412, PRC, China). Sample weights were measured using a precision analytical balance (Shimadzu ATX224R, Japan). Microwave-assisted treatments and microwave extraction procedures were carried out using a microwave oven (Simfer 4605/4606 Retro Series (20L), Türkiye). Continuous and uniform mixing of samples was achieved using a magnetic stirrer (Ohaus Guardian 5000, China). Ultrasonic treatments were performed using a sonic bath (Kudos-SK3300HP, Korea) to facilitate homogenization and cell disruption. A UV–VIS spectrophotometer (Shimadzu UV-1280, Japan) was used to determine absorbance values for the analysis of phycocyanin concentration, purity, and content. Temperature-controlled treatments were conducted using a thermal cycler (Bio-Rad T100, USA). Supercritical CO_2 extraction was performed using a Superex SC-1000 supercritical extraction system (Konya, Türkiye). Samples were frozen and stored at −20°C using a deep freezer (Altus AL 708 NE 7, Türkiye). Color characteristics of all extracts were determined using a color measurement instrument (Konica Minolta CR-400, Singapore).

2.2. Literature Search for Phycocyanin Extraction Methods

Previously reported phycocyanin extraction studies in the literature using freeze-thawing, solvent-based, microwave-assisted, ultrasound-assisted, and supercritical carbon dioxide extractions were identified through a literature search using various indexing databases such as ScienceDirect, PubMed, Web of Science, Scopus, MDPI, JSTOR, SpringerLink, and TÜBİTAK ULAKBİM TR Dizin. A total of 93 articles on existing extraction methods were selected, and from among these, methods with the highest phycocyanin yield were listed in Table 1.

2.3. Freeze and Thawing Extraction Methods

The solvents used in this experiment included distilled water and 20 mM sodium acetate buffer (pH 5.1). The mass and solvent were mixed at a 1:30 (w/v) ratio. For each solvent, 0.5 g of *Spirulina sp*. powder was weighed using a precision balance and added to a 50 mL screw-cap centrifuge tube. To prevent the sedimentation of the sample powder, the mixed samples were vortexed for 15 minutes. Homogeneous mixed samples were used for a four-cycle freeze-thawing extraction process. Initially, the mixture samples were frozen at -20 °C for 12 hours and subsequently thawed at room temperature (25 °C ± 2) for 6 hours. At the end of the first cycle, the samples were centrifuged at 4000 rpm for 15 minutes at room temperature (RT) to remove residual cell debris and to obtain the extract (supernatant). At the end of each centrifugation cycle, the remaining cell debris was used for the subsequent extraction cycle. After all cycles were applied, the remaining pellet was discarded. The supernatants obtained from each cycle were collected separately and stored at -20 °C and in the dark until further analyses.

2.4. Solvent-Based Extraction Methods

Six different solvents, including distilled water, 1.5% calcium chloride ($CaCl_2$), 100% ethanol (EtOH), Tris-Cl buffer (pH 6.8), cyclohexane (C_6H_{14}), and sodium phosphate, were used. 0.5 g of *Spirulina sp*. powder was mixed with each buffer at a 1:20 (w/v) ratio in a 50 mL screw-capped centrifuge tube. The mixed samples were vortexed for 15 minutes. After homogenization, the mixed samples were centrifuged at 4000 rpm for 15 minutes (at RT) to separate the cell debris from the supernatant. This process was sequentially repeated in four cycles for each sample, and the extracts obtained in each cycle were collected separately. At the end of each centrifugation cycle, the remaining cell debris was used for the subsequent extraction cycle. After all cycles were applied, the remaining pellet was discarded. The resulting supernatants were stored at -20 °C and in the dark until further analyses.

2.5. Microwave-Assisted Extraction Methods

Two buffers, including distilled water and 1.5% calcium chloride ($CaCl_2$), were used. 5 g of *Spirulina sp*. powder was transferred to a 250 mL glass Erlenmeyer flask and mixed with 125 mL of buffer at a 1:25 (w/v) ratio. The mixture was homogenized using a magnetic stirrer for 15 minutes to ensure complete homogenization. The extraction process was conducted in a microwave at 2450 megahertz (MHz) with a maximum input power of 1000 watts for 15 minutes. This procedure was sequentially repeated in four cycles. After each microwave application, the mixed samples were centrifuged at 4000 rpm for 15 minutes (at RT) to separate the cell debris from the supernatant. At the end of each centrifugation cycle, the remaining cell debris was used for the subsequent extraction cycle. After all cycles were applied, the remaining pellet was discarded. The resulting supernatants were stored at -20 °C and in the dark until further analyses.

2.6. Ultrasound-Assisted Extraction Methods

Two buffers, including distilled water and 0.1 M sodium

phosphate buffer (pH 7, Na_3PO_4) and 1.5% calcium chloride ($CaCl_2$), were used. 0.5 g of Spirulina sp. powder was mixed with each buffer at a 1:100 (w/v) ratio in a 100 mL flask. The mixture was homogenized using a magnetic stirrer for 15 minutes to ensure complete homogenization. The extraction process was conducted using a sonic bath (30 × 15 × 15 cm) at full power for 35 minutes at a frequency of 53 kilohertz (kHz). Following the ultrasonic treatment, each sample was centrifuged at 4000 rpm for 15 minutes at RT to separate the cell debris from the supernatant. At the end of each centrifugation cycle, the remaining cell debris was used for the subsequent extraction cycle. After all cycles were applied, the remaining pellet was discarded. The resulting supernatants were stored at - 20°C and in the dark until further analyses.

2.7. Supercritical Carbon Dioxide Extraction Methods

The Superex SC-1000 series supercritical extraction device (Pard Engineering, Konya, Turkey) was employed for the extraction process. Two buffers, including distilled water and EtOH, were used. Four different trials were tested as follows:

Trial 1: 10 grams of *Spirulina sp*. powder were subjected to extraction at a pressure of 310 bar and a temperature of 45°C for a duration of 90 minutes. During this process, the ethanol (100%) flow rate was set to 2.5 mL/min, while the carbon dioxide flow rate was set to 4 mL/min.

Trial 2: 10 grams of *Spirulina sp*. powder were subjected to extraction at a pressure of 310 bar and a temperature of 45°C for a duration of 45 minutes. During this process, the ethanol (100%) flow rate was set to 2.5 mL/min, while the carbon dioxide flow rate was set to 25 mL/min.

Trial 3: 5 grams of *Spirulina sp*. powder were first subjected to extraction at a pressure of 310 bar and a temperature of 45°C for a duration of 30 minutes. During this process, the d-water (100%) flow rate was set to 2.5 mL/min, while the carbon dioxide flow rate was at 25 mL/min. The first extract obtained was stored at -20 °C and in the dark. Following the first extraction, a 10% ethanol solution was applied to the remaining pellet for an additional 30 minutes, with an ethanol flow rate of 2.5 mL/min, and the carbon dioxide flow rate was maintained under the same pressure and temperature. The second extract obtained was stored at -20 °C and in the dark. Following the second extraction, 100% d-water was applied to the remaining pellet for an additional 30 minutes with a d-water flow rate of 2.5 mL/min, and the carbon dioxide flow rate was maintained under the same pressure and temperature.

Trial 4: 5 grams of *Spirulina sp*. powder were subjected to extraction at a pressure of 345 bar (the highest pressure) and a temperature of 70°C for a duration of 60 minutes. During this process, the ethanol (100%) flow rate was set to 5 mL/min, while the carbon dioxide flow rate was set to 100 mL/min.

2.8. Colorimetric Analysis of Phycocyanin Extracts

Phycocyanin extracts obtained from *Spirulina sp*. powder through various extraction methods in this study were analyzed by the CIE-L*a*b* System (Kinoca Minolta CR400, Singapore). The measurements were conducted under D65 illumination and a 10° observer angle. The "L*" value indicates the lightness of the color, where a value of '100' represents pure white and a value of '0' represents pure black. The "+a*" value indicates redness, while the "-a*" value indicates greenness. Additionally, the "+b*" value signifies yellowness, whereas the "-b*" value denotes blueness.

2.9. Total Protein Content

The total protein concentration was measured using the Bio-Rad Bradford protein assay for extracts from each of the extraction methods applied. Bovine serum albumin (BSA) was used to develop a standard curve to calculate total protein concentrations. To prepare the BSA stock solution, 2 mg of BSA was weighed using a precision balance and dissolved in 1 mL of distilled water. The resulting stock BSA solution was used to prepare standard solutions with known concentrations. These standard solutions were prepared at concentrations of 0.0025, 0.005, 0.01, 0.02, 0.04, and 0.08 mg/mL. 1000 µL of each BSA standard solution was added to 900 µL (1X) Bradford buffer contained in a 1.5 mL Eppendorf tube. The mixture was gently and thoroughly mixed via pipetting and incubated for 5 min at room temperature. The absorbance values of the mixtures were recorded at 595 nm using the UV-VIS spectrophotometer. The measurements were analyzed using Microsoft 365 Excel Worksheet (Version 2405) to create a BSA standard curve. The total protein concentration of the samples was calculated by applying the same procedure and using the absorbance values and the BSA standard curve.

2.10. Phycocyanin Content, Purity, and Yield

The phycocyanin concentration, purity, and yield for each extract were determined by measuring the absorption spectra at 620, 652, and 280 nm using a UV-Vis spectrophotometer and calculated using the following equations (1), (2), and (3).

1) The phycocyanin (PC) content of the extract was estimated spectrophotometrically according to the equation of Bennett and Bogorad (Bennett & Bogorad, 1973) as follows:

$$PC\ (mg/mL) = [OD_{620} - 0.474 \times OD_{652}] / 5.34 \quad (1)$$

where the 0.474 constant was used as a correction factor to account for the spectral overlap arising from allophycocyanin, the value of 5.34 represents the specific molar extinction coefficient of phycocyanin under the

defined conditions. The absorbance at A_{620} nm corresponds to phycocyanin. The absorbance at A_{652} nm is attributed to allophycocyanin.

2) The purity of phycocyanin was determined by using the following absorption ratio:

$$\text{Purity} = OD_{620} / OD_{280} \quad (2)$$

Where OD_{620} nm and OD_{280} nm are absorbances of phycocyanin and total proteins, respectively

3) The yield of phycocyanin was calculated by the equation described by Silveira et al. (2007) as follows:

$$PC_{Yield} = C_{PC} \times V / DWB \quad (3)$$

where PC_{Yield} is the extraction yield of phycocyanin, expressed in mg g^{-1} dry biomass, C_{PC} is the phycocyanin concentration in the liquid extract, expressed in mg mL^{-1}per gram of dry biomass, V is the total volume of the extraction solvent used, expressed in (mL), and DWB is the dried weight of the initial Spirulina biomass, expressed in (g).

2.11. Statistical Analysis

All measurements were conducted in triplicate, and the results were expressed as mean ± standard deviation (SD). To evaluate the effects of the extraction method, solvent system, and subsequent extraction cycle on phycocyanin recovery parameters, a three-way analysis of variance (ANOVA) was performed to simultaneously assess the main effects and interactions of the extraction method, solvent type, and extraction cycle. In addition, one-way analysis of variance (ANOVA) was applied separately to compare extraction cycles within each method–solvent combination, solvent systems within each extraction method, and extraction methods for overall comparisons. For the supercritical CO_2 (SC-CO_2) extraction method, independent trials rather than sequential extraction cycles were statistically compared. Whenever significant differences were identified, Tukey's honestly significant difference (HSD) post hoc test was employed for multiple comparisons. Statistical significance was determined at $p < 0.05$. All statistical analyses were performed using Python (Python Software Foundation, Wilmington, DE, USA) with the SciPy and Statsmodels libraries. Statistically significant differences among groups were indicated using different lowercase and uppercase subscript letters.

3. RESULTS

The effects of solvent and subsequent extraction cycles on phycocyanin recovery from dried Spirulina sp. biomass were compared using traditional extraction methods (solvent-based and freeze-thawing) and green extraction techniques (ultrasound-assisted, microwave-assisted, and supercritical carbon dioxide). The results were evaluated based on the colourimetric features of phycocyanin extracts, phycocyanin content (mg/mL), yield (mg/g), and purity (%).

3.1. Summary of Literature Search for Phycocyanin Extraction Methods

Based on a literature review, the methods yielding the highest phycocyanin extraction yields, along with the solvents used for each method, were listed in Table 1. The findings from Table 1 suggested that the solvent plays a significant role in phycocyanin extraction methods. However, an evaluation of this role across four subsequent extractions has not been reported to date.

Table 1. Phycocyanin extraction methods with the highest recovery from *Spirulina sp*. Powder

Extraction Method	Solvent	Phycocyanin Yield % (mg/g) DW or FW	Reference
Freeze-Thawing Extraction	Sodium acetate and Sodium chloride (pH 5.1)	21.71	(Chentir et al., 2018)
	Distilled water	22.56	(Ruiz-Domínguez et al., 2019)
	Sodium phosphate buffer (pH 7.0)	16–19.8	(Sukhinov et al., 2021)
Solvent-based Mixing and Homogenization Method	Calcium chloride (glass bead extraction)	10.92	(Kuhnholz et al., 2024)
	Phosphate buffer (pH 6.8)	2.9–11.9	(Tavanandi et al., 2018)
	Phosphate buffer (pH 7.0)	4.1–14.99	(Chaiklahan et al., 2018)
	Calcium chloride, Sodium phosphate buffer (pH	0.3–9.41	(İlter et al., 2018)
	Distilled water	0.03–8.54	(Larrosa et al., 2018)
	Distilled water	10.4	(Nakagawa et al., 2016)
	Sodium phosphate buffer (pH 7.0)	3.23–31.26	(Pan-utai et al., 2018)
	Hexane	8.4	(Seo et al., 2013)
	Distilled water	15.9	(Q. Chen et al., 2022)
Ultrasound-Assisted Extraction	Calcium chloride, Sodium phosphate buffer (pH	8.83–9.24	(İlter et al., 2018)
	Distilled water	4.37	(Moraes et al., 2011)
	Sodium Phosphate buffer (pH 7.0)	1.0–6.0	(Pan-utai and Iamtham, 2019)
	Phosphate buffer saline (pH 7.0)	9.4–25.12	(Park et al., 2018)
	Distilled water	0.7–3.70	(Salazar-González et al., 2024)
Microwave-Assisted Extraction	Calcium chloride, Sodium phosphate buffer (pH	5.62–9.66	(İlter et al., 2018)
	Distilled water	3.51–21.5	(Ruiz-Domínguez et al., 2019)
SFE Extractions	Liquefied CO_2, co-solvent (ethanol)	2.8–11.46	(Deniz et al., 2016)
	Distilled water	21.7–25.2	(Marzorati et al., 2020)

3.2. Evaluation of Visual Appearance of Phycocyanin Extracts

The visual appearance of the phycocyanin extracts showed a gradual decrease in pigmentation over the subsequent extraction cycles for each method. In all the extraction methods analyzed, the positive control (+) took on a dark blue color characteristic of phycocyanin-rich extracts. In contrast, the negative control (-) exhibited a yellowish color indicating the absence of phycocyanin. Figure 1 shows the visual appearance of phycocyanin extracts in 1 mL cuvettes obtained from *Spirulina sp*. using the freeze-thawing (A1 and A2), the solvent-based (B1-B6), the ultrasound-assisted (C1 and C2), the microwave-assisted (D1 and D2) over four subsequent extraction cycles, and the supercritical carbon dioxide (SC-CO_2) extraction methods (E1) under four distinct trials.

For extracts obtained via freeze-thawing extraction using distilled water (Figure 1A1), color intensity progressively decreased with increasing extraction cycles. The first extraction cycle (Figure 1A1–Cycle 1) yielded a distinctly blue extract, whereas subsequent cycles (Figure 1A1–Cycles 2–4) exhibited a gradual transition toward greenish and yellowish hues, indicating reduced phycocyanin recovery in later extraction stages. A comparable pattern was observed for sodium acetate buffer extracts. In the initial extraction cycle of the freeze-thawing method (Figure 1A2–Cycle 1), sodium acetate buffer produced an intensely blue extract closely resembling the positive control. However, a gradual decline in color intensity was evident across subsequent cycles (Figure 1A2–Cycles 2–4), with the extracts transitioning from deep blue to lighter blue and pale blue–green tones by the fourth cycle. Collectively, these observations demonstrate that phycocyanin extraction was most efficient during the initial freeze-thawing cycles, while pigment recovery progressively declined in subsequent extractions. Moreover, the stronger and more persistent blue coloration observed in sodium acetate buffer extracts indicates enhanced pigment stability and/or extraction efficiency relative to distilled water.

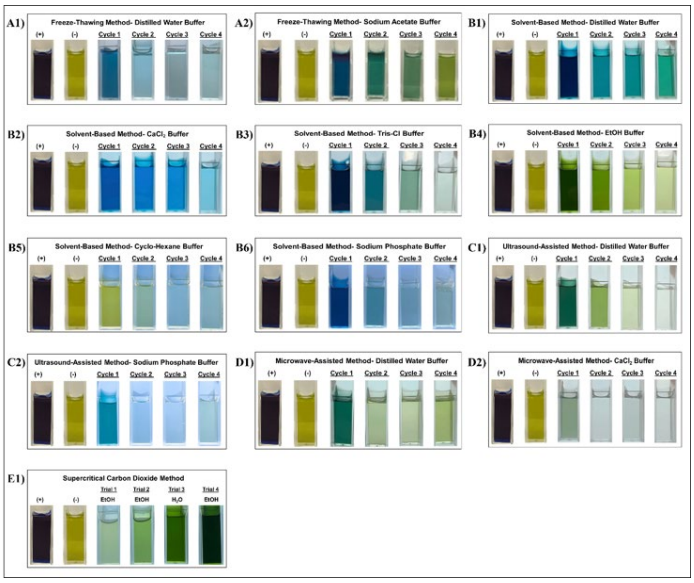

Figure 1. Phycocyanin extracts obtained from *Spirulina sp*. using the freeze-thawing (A1 and A2), the solvent-based (B1-B6), the ultrasound-assisted (C1 and C2), the microwave-assisted (D1 and D2), and the supercritical carbon dioxide (SC-CO_2) extraction methods (E1) over four subsequent extraction cycles and under four distinct trials, respectively. (+) and (-) are positive and negative control extracts, respectively. A1: Distilled Water Buffer; A2: Sodium Acetate Buffer; B1: Distilled Water Buffer; B2: $CaCl_2$ Buffer; B3: Tris-Cl Buffer; B4: EtOH Buffer; B5: Cyclohexane Buffer; B6: Sodium Phosphate Buffer; C1: Distilled Water Buffer; C2: Sodium Phosphate Buffer; D1: Distilled Water Buffer; D2: $CaCl_2$ Buffer; E1-Trial 1, 2,4: EtOH Buffer; E1-Trial 3: Distilled Water Buffer.

In all solvent-based extraction systems, the first extraction cycle produced the most intense coloration, with extracts ranging from deep blue to blue–green depending on the solvent employed. For aqueous and buffered solvents, including distilled water, sodium phosphate, Tris–Cl, and $CaCl_2$, a progressive reduction in color intensity was observed from the first to the fourth extraction cycle (Figure 1B1–B5). The extracts gradually shifted from deep blue in the initial cycles to lighter blue or pale blue–green hues in later cycles, reflecting decreased pigment recovery during sequential extraction. Among these solvent systems, $CaCl_2$ and Tris–Cl buffers (Figure 1B2 and B3) retained visibly stronger blue coloration across multiple cycles compared with distilled water (Figure 1B1), indicating superior pigment preservation. In contrast, organic solvents displayed markedly different visual characteristics. Ethanol extracts (Figure 1B4) exhibited predominantly greenish coloration with comparatively weak blue intensity throughout all extraction cycles, whereas cyclohexane extracts (Figure 1B5) produced pale green to yellowish solutions with minimal blue coloration even during the first cycle. Overall, these findings demonstrate that solvent composition substantially influenced both the intensity and persistence of

phycocyanin coloration in solvent-based extraction systems, with buffered aqueous solvents providing superior pigment retention compared with distilled water and organic solvents.

For ultrasound-assisted extraction using distilled water (Figure 1C1), the first extraction cycle generated a greenish-blue solution with lower color intensity relative to the buffered extraction system. A pronounced reduction in coloration was observed across subsequent cycles, with extracts from later cycles (Figure 1C1–Cycles 2–4) appearing pale green to nearly colorless. In contrast, ultrasound-assisted extraction using sodium phosphate buffer (Figure 1C2) produced an intensely blue extract during the first extraction cycle, closely resembling the positive control. Although color intensity gradually decreased in subsequent cycles (Figure 1C2–Cycles 2–4), the extracts maintained visibly stronger blue coloration compared with those obtained using distilled water. These results indicate that ultrasound-assisted extraction was most effective during the initial extraction cycle and that sodium phosphate buffer substantially enhanced phycocyanin recovery and pigment intensity relative to distilled water. In both solvent systems, however, phycocyanin recovery visibly declined with increasing extraction cycles.

For microwave-assisted extraction using distilled water (Figure 1D1), the first extraction cycle produced a greenish extract with relatively high color intensity compared with the buffered extraction system. However, a pronounced reduction in color intensity was observed during subsequent extraction cycles (Figure 1D1–Cycles 2–4), with extracts from the third and fourth cycles appearing pale green to weakly colored. Similarly, microwave-assisted extraction using calcium chloride buffer (Figure 1D2) yielded a light blue–green extract during the initial extraction cycle, although the coloration remained visibly less intense than that of the positive control. Across subsequent extraction cycles (Figure 1D2–Cycles 2–4), the extracts progressively lost color intensity and approached a nearly transparent appearance by the fourth cycle. Overall, these observations indicate that microwave-assisted extraction provided comparatively limited phycocyanin recovery relative to the other extraction methods evaluated, with the highest apparent pigment recovery occurring during the initial extraction cycle. In both distilled water and calcium chloride buffer systems, phycocyanin recovery visibly declined with successive extraction cycles, suggesting progressive pigment degradation and reduced extraction efficiency during repeated microwave-assisted treatment.

Distinct differences in extract coloration were observed among the SC-CO_2 extraction trials (Figure 1E). In Trial 1, the extract exhibited a pale blue–green appearance, indicating limited phycocyanin recovery under the applied extraction conditions. Trial 2 displayed a slightly more intense greenish coloration, suggesting a moderate increase in chlorophyll-associated pigment extraction relative to Trial 1. In contrast, Trial 3 produced a visibly stronger green coloration, reflecting enhanced recovery of chlorophyll-related pigments compared with the preceding trials. The highest color intensity was observed in Trial 4, in which the extract exhibited a dark green appearance, indicating the greatest apparent chlorophyll pigment concentration among the SC-CO_2 extraction conditions evaluated. Overall, these visual observations demonstrate that the efficiency of supercritical CO_2 extraction was highly dependent on the applied operating parameters, with progressively stronger chlorophyll pigmentation observed in the later extraction trials. Compared with the positive controls, phycocyanin recovery under SC-CO_2 extraction conditions remained relatively limited, indicating that the tested conditions favored the co-extraction of chlorophyll pigments rather than selective phycocyanin recovery. These findings suggest that further optimization of extraction parameters, particularly pressure conditions and co-solvent composition, may be necessary to enhance phycocyanin extraction efficiency and pigment selectivity in SC-CO_2-based extraction systems.

3.3. Colorimetric Measurement of Phycocyanin Extracts Using the CIE-L*a*b* System

Based on the CIE Lab* color measurements, pronounced differences in color characteristics were observed among the extraction methods, solvent systems, and extraction cycles, as presented in Figure 2 and Supplementary Table 1. Within the CIE-LAB color system, the L* parameter represents lightness–darkness, positive a* values correspond to the red–blue chromatic axis, and positive b* values represent the yellow–green chromatic axis. The positive control (+C), representing purified phycocyanin pigment, exhibited relatively low L* values together with high positive a* values and moderate b* values, corresponding to the characteristic dark blue coloration of phycocyanin. In contrast, the negative control (−C) displayed higher L* values accompanied by near-zero or negative a* values and positive b* values, indicating a yellow–green coloration associated with the absence of phycocyanin.

The comparative evaluation of pigment behavior, degradation tendencies, and solvent performance was derived from the CIE-LAB color measurements. Across all extraction methods, the initial extraction cycles generally exhibited lower L* values, reflecting darker and more intensely colored extracts. In contrast, subsequent extraction cycles showed progressively increasing L* values, indicating gradual lightening of the extracts. This trend suggests a reduction in phycocyanin concentration during sequential extraction. Positive a* values were consistently associated with phycocyanin-rich extracts. In contrast, a progressive shift toward lower or negative a* values were observed in later extraction cycles and in less effective solvent systems, reflecting degradation and loss

of blue pigmentation. Similarly, b* values either decreased toward zero or increased toward positive values depending on the solvent system employed, indicating shifts along the yellow–blue chromatic axis. Buffered extraction systems, including sodium acetate, Tris–Cl, sodium phosphate, and $CaCl_2$, generally maintained color coordinates closer to those of the positive control compared with distilled water and nonpolar solvents. These findings indicate that buffered systems provided improved phycocyanin stability and pigment retention during extraction, whereas nonpolar solvents were comparatively less effective in preserving the characteristic chromatic properties of phycocyanin.

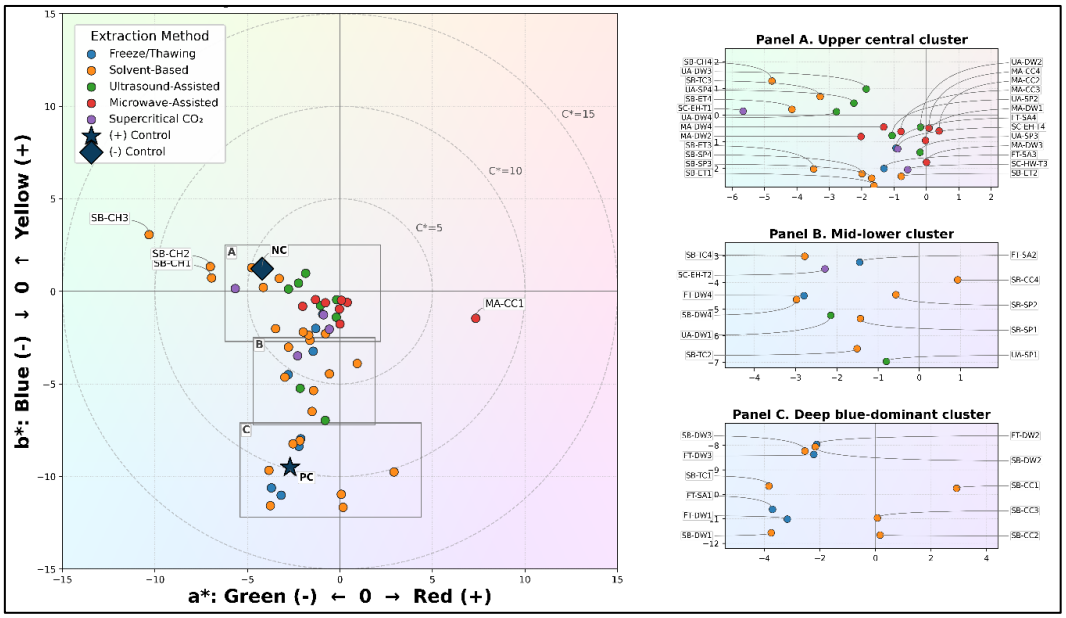

Figure 2. Positions of phycocyanin extracts in the CIE L*a*b* color space. The L* value represents lightness-darkness, the positive a* values indicate red–blue chromatic contribution, whereas positive b* values indicate yellow–green contributions. (+) and (-) are the positive and negative controls, respectively. FT: Freeze-Thawing; SB: Solvent-Based; UA: Ultrasound-Assisted; MA: Microwave-Assisted; SC: Supercritical Carbon Dioxide; DW: Distilled Water; ET: Ethanol; SP: Sodium Phosphate; SA: Sodium Acetate; CH: Cyclohexane; TC: Tris-Cl; CC: Calcium Chloride; PC: Positive Control; NC: Negative Control; T1-T4: Trial 1-4; 1: Cycle 1; 2: Cycle 2; 3: Cycle 3; 4: Cycle 4.

Freeze-thawing extracts were predominantly clustered within the positive a* and low-to-moderate b* region during the initial extraction cycles, indicating strong blue pigmentation associated with high phycocyanin content. With increasing extraction cycles, the data points shifted progressively toward lower a* values and reduced chromatic intensity, reflecting the gradual depletion of phycocyanin during sequential extraction. Extracts obtained using sodium acetate buffer in the freeze-thawing extraction method remained positioned closer to the positive control region than those obtained using distilled water, suggesting improved preservation of pigment characteristics. In sodium acetate buffer extracts, L* values increased markedly with each successive extraction cycle, indicating progressive lightening of the extracts. Although the first extraction cycle exhibited relatively high a* values, the red component gradually diminished in subsequent cycles and became nearly absent in the later extraction stages. Similarly, b* values decreased to comparatively low levels across the extraction cycles. These findings suggest that while sodium acetate buffer was effective at solubilising phycocyanin during the initial extraction stages, it was less effective at maintaining long-term pigment stability during repeated extraction cycles.

Solvent-based extraction exhibited a broader distribution within the a*–b* color space, particularly in extracts obtained using organic solvents. Aqueous solvent systems generated extracts positioned closer to the positive control, whereas ethanol and cyclohexane extracts shifted toward higher b* and lower a* values, indicating diminished blue pigmentation accompanied by increased yellow–green coloration. In the solvent-based extraction system using Tris–Cl buffer, the first extraction cycle showed relatively high a* values; however, a* progressively decreased in subsequent cycles and reached negative values by the third cycle, suggesting substantial structural degradation of the pigment during extraction. Although b* values remained comparatively low and stable throughout the extraction cycles, the overall findings indicate that Tris–Cl did not provide a sufficiently stable environment for effective phycocyanin preservation.

Sodium phosphate buffer used in the solvent-based extraction method produced elevated L* values during the first two extraction cycles, followed by a decline in the third and fourth cycles, resulting in darker-colored extracts. The

continuous decrease in a* values across successive cycles indicated progressive pigment destabilization, whereas consistently low b* values suggested retention of a relatively bluish coloration. In contrast, cyclohexane-based extraction produced markedly high L* values, indicating very light-colored extracts. Persistently negative a* values reflected dominant greenish tones, while substantially elevated b* values indicated pronounced yellowness. These findings demonstrate that cyclohexane was unsuitable for phycocyanin extraction, likely due to its inability to effectively solubilize the pigment, with the observed coloration primarily arising from non-phycocyanin compounds present in the extract.

In ethanol-based extracts, L*, a*, and b* values fluctuated throughout the extraction cycles. The a* values remained generally low and shifted to negative values during the final extraction cycle, indicating a transition toward greener coloration. Concurrently, increasing b* values reflected enhanced yellowness, suggesting reduced phycocyanin purity and stability. Calcium chloride ($CaCl_2$) buffer, applied in both solvent-based and microwave-assisted extraction methods, was among the most effective solvent systems for preserving pigment characteristics. Predominantly negative b* values confirmed that $CaCl_2$ maintained blue coloration more effectively than the other solvent systems evaluated. Furthermore, relatively high a* values, particularly during the initial extraction cycles, indicated preservation of a strong red component associated with pigment integrity. Although some degree of pigment degradation became evident during the final extraction cycle, $CaCl_2$ overall provided a favorable extraction medium for maintaining phycocyanin stability.

Distilled water, which was used across all extraction methods, exhibited a substantial increase in L* values with successive extraction cycles, resulting in progressively lighter-colored extracts. Simultaneously, a* values shifted from positive to negative values, indicating declining pigment stability, whereas increasing b* values reflected enhanced yellowness of the extracts. Collectively, these findings demonstrate that both solvent composition and extraction cycle strongly influenced pigment stability and color characteristics during phycocyanin extraction.

Ultrasound-assisted extraction generated early-cycle extracts that were clustered in regions close to the positive control, particularly when buffered solvent systems were employed. However, subsequent extraction cycles exhibited a systematic shift of the data points toward the origin of the a*–b* color space, indicating progressive reductions in chromatic intensity and diminished phycocyanin recovery. In the ultrasound-assisted extraction method using sodium phosphate buffer, L* values increased consistently across successive extraction cycles, reflecting progressively lighter-colored extracts. Although the first extraction cycle exhibited relatively high a* values, the parameter shifted to negative values in later cycles, indicating a substantial loss of the red chromatic component associated with pigment integrity. Concurrently, b* values increased slightly throughout the extraction process, suggesting a greater yellowness in the extracts. This increase in yellowness is likely attributable to pigment deformation and structural degradation induced by ultrasonication. Collectively, these findings demonstrate that ultrasonic treatment destabilises and degrades phycocyanin pigment, thereby significantly reducing its chromatic properties and visual quality during repeated extraction cycles.

Microwave-assisted extraction displayed the greatest deviation from the positive control. Several samples exhibited low a* values and, in some cases, negative b* values, indicating substantial pigment degradation or reduced stability under microwave conditions. The widespread points reflected strong sensitivity to the extraction cycle and solvent composition, which made the pigment loss more evident. Supercritical CO_2 extracts covered a distinct region characterized by low-to-moderate a* values and variable b* values. Extracts obtained under optimized conditions shifted closer to the positive control, whereas less favorable conditions produced points nearer the origin, indicating limited phycocyanin recovery. In the solvent mixture of EtOH and CO_2 used in the supercritical CO_2, the L*, a*, and b* values displayed irregular patterns. The first cycle exhibited notably high b* values, indicating pronounced yellowness and low pigment purity. Although yellowness decreased in later cycles, the extract did not present a stable color profile. These findings suggest that the combination of supercritical CO_2 with ethanol is not sufficient on its own to provide a suitable environment for phycocyanin extraction.

Overall, the CIE Lab* color analysis demonstrated that extraction method, solvent system, and extraction cycle significantly influenced the chromatic characteristics of the phycocyanin extracts. Buffered aqueous solvent systems generally produced extracts with color coordinates more closely resembling those of the phycocyanin-positive control, indicating improved pigment preservation and stability. In particular, high-polarity solvent systems, including calcium chloride, sodium phosphate, and distilled water, promoted more effective pigment solubilization and generated comparatively consistent color parameter values throughout the extraction process. In contrast, organic solvent systems such as ethanol and cyclohexane, as well as high-energy extraction approaches involving intensive ultrasonication, exhibited reduced effectiveness in preserving the characteristic chromatic properties of phycocyanin. The comparatively low polarity of these solvent systems, together with the structural stress induced by ultrasonication, likely contributed to insufficient pigment solubilization and enhanced pigment degradation. Consequently, these extraction conditions produced extracts with weaker blue pigmentation, increased yellow–green coloration, and reduced overall chromatic stability.

3.4. Total Protein Content of Phycocyanin Extracts

A BSA standard curve was established using the Bradford assay method to determine the total protein content in all phycocyanin extracts from Spirulina powder. The BSA standard curve was created by measuring the absorbance values of BSA stock solutions prepared at different concentrations. The BSA standard curve showed an acceptable linear relationship (R^2 = 0.94) and was used for estimating total protein concentrations. Absorbance values of each phycocyanin extract were measured at a wavelength of 595 nm and then used to calculate the total protein content of each extract according to the formula (y = 0.0951x + 1.1395) determined by the BSA standard curve (Supplementary Table 2). One-way ANOVA revealed that the extraction cycle significantly influenced total protein concentration in most extraction method–solvent systems ($p < 0.05$). A gradual decrease in total protein concentration was observed across successive extraction cycles, indicating progressive depletion of extractable protein fractions. Solvent-based, ultrasound-assisted, and microwave-assisted extraction methods exhibited statistically significant variations in protein recovery among cycles. The SC-CO_2 extraction method demonstrated variability among trials, reflecting the sensitivity of protein extraction to solvent composition and extraction conditions.

In the freeze-thawing extraction method (Figure 3), total protein content gradually decreased with increasing extraction cycles. This reduction does not necessarily indicate direct protein loss; rather, it suggests that a substantial proportion of soluble proteins was transferred into the extract phase during the initial extraction cycles, thereby reducing the pool of extractable proteins available in subsequent cycles. Such behavior is characteristic of sequential extraction processes and reflects the efficiency of the extraction method in recovering soluble intracellular components during the early extraction stages. Similarly, solvent-based extraction methods exhibited relatively stable total protein concentrations, with only minor decreases observed across successive extraction cycles. This trend indicates that the majority of soluble proteins were effectively recovered during the initial stages of extraction. Among the tested solvent systems, sodium phosphate buffer and ethanol produced comparatively consistent and reproducible total protein values, suggesting their effectiveness in maintaining protein solubility and extraction stability throughout the extraction process.

In ultrasound-assisted and microwave-assisted extraction methods, moderate fluctuations in total protein content were observed, likely attributable to the physical and thermal effects associated with these extraction techniques on cellular integrity. Ultrasonic treatment enhances cell disruption through cavitation phenomena, thereby facilitating the release of intracellular compounds. In contrast, microwave-assisted extraction may generate rapid and localized temperature increases, resulting in substantial thermal stress within the extraction medium. Such thermal stress can induce protein denaturation through disruption of the three-dimensional protein structure, consequently reducing protein solubility. Because denatured proteins may become less detectable in spectrophotometric analyses, the apparent decrease in measured total protein concentration is more plausibly associated with heat-induced structural alterations rather than with actual protein degradation or loss. In supercritical CO_2 extraction, variations in total protein content among extraction cycles indicate that the extraction efficiency of the method was highly sensitive to solvent–matrix interactions and operating parameters. Factors such as extraction pressure, co-solvent composition, and solvent contact time likely played critical roles in determining protein extractability under supercritical conditions.

Overall, the variations in total protein content observed across successive extraction cycles for all extraction methods appear to be primarily associated with progressive depletion of the extractable protein fraction and with method-specific processing effects, rather than with direct protein loss.

3.5. Phycocyanin Concentration, Purity, and Yield of Phycocyanin Extracts

To measure phycocyanin content of all extracts from freeze-thawing, solvent-based, ultrasonic-assisted, and microwave-assisted extraction methods, with four subsequent cycles with different solvent buffers and four different trials of the supercritical-CO2 extraction method, the absorbance values of the extracts were measured at 280, 620, and 652 nm wavelengths (Supplementary Table 3). Phycocyanin (PC) concentration, purity, and yield values were calculated based on the absorbance measurements (OD_{620}, OD_{652}, and OD_{280}) (Table 2).

The freeze-thawing method was conducted using distilled water and sodium acetate buffer. In the extraction with distilled water, the PC concentration was 0.30 mg/mL in the first cycle, decreasing to 0.29 mg/mL in the second cycle, and further dropping to 0.11 mg/mL and 0.04 mg/mL in the third and fourth cycles, respectively. The purity ranged from 4.54% to 5.74%, while the yield peaked at 9 mg/g in the first cycle and decreased to 1.2 mg/g in the fourth cycle. When sodium acetate buffer was used, concentration values ranged from 0.182 to 0.267 mg/mL, purity from 2.85% to 9.78%, and yield from 5.46 to 8.01 mg/g. These results indicated that the freeze-thawing method provides relatively higher concentrations with distilled water, while higher purity is achievable with sodium acetate buffer. However, the yields from both buffers were limited, particularly when compared to the more efficient solvent-based and ultrasonic methods, suggesting that the freeze-thawing method may be more

suitable for applications prioritizing purity over quantity, but is constrained in producing high amounts of PC.

Solvent-based extraction was performed using various solvents, and the extraction performance of each solvent was compared. In solvent-based extraction with distilled water, PC concentration ranged from 0.229 mg/mL to 0.416 mg/mL, with purity of 3.81% to 7.21% and yield of 45.8 mg/g to 83.2 mg/g. The 1.5% $CaCl_2$ solution stood out for its high yield and purity, with concentrations in the first three cycles ranging from 0.404 mg/mL to 0.409 mg/mL, purity from 4.86% to 9.79%, and yield from 80.8 mg/g to 81.8 mg/g; however, a significant drop in yield was observed in the fourth cycle (20.6 mg/g). The Tris-Cl_2 buffer provided high yields (83 mg/g to 84.6 mg/g) and moderate concentrations (0.195 mg/mL to 0.423 mg/mL) in the first two cycles, but both yield and concentration significantly decreased in the third and fourth cycles. Ethanol extraction exhibited low purity (1.15% to 2.81%) and variable concentrations, particularly with a sharp decline in yield in the third and fourth cycles (12.4 mg/g to 19 mg/g). Extraction with cyclohexane showed limited performance with low concentration (0.029 mg/mL to 0.052 mg/mL), low yield (29 mg/g to 52 mg/g), and low purity (1.52% to 3.29%). Solvent-based extraction with sodium phosphate buffer yielded significantly higher PC yields (76 mg/g to 477 mg/g) and moderate purity (2.60% to 3.88%). These findings suggest that the ionic structure of the buffer directly affects the solubility of PC and the extraction efficiency.

Ultrasonic-assisted extraction was conducted using distilled water and sodium phosphate buffer. In the extraction with distilled water, the concentration in the first cycle was measured at 0.432 mg/mL, with a purity of 4.50% and a yield of 432 mg/g; however, a rapid decrease in both concentration and yield was observed in subsequent cycles. Ultrasonic extraction with sodium phosphate buffer yielded a higher concentration and 480 mg/g in the first cycle. Purity values varied between 1.62% and 4.73%. These results demonstrated that ultrasonic extraction effectively disrupts cell walls, enabling rapid, high-efficiency extraction, although purity varies depending on the buffer selected.

Microwave-assisted extraction was performed using distilled water and 1.5% $CaCl_2$. In the extraction with distilled water, the concentration in the first cycle was 0.328 mg/mL, with a purity of 2.56% and a yield of 82 mg/g. For the 1.5% Ca-Cl_2 extraction, the concentration in the first cycle was 0.280 mg/mL, purity was 3.97%, and yield was 70 mg/g. While microwave extraction facilitated rapid extraction, fluctuations in purity and yield were observed in some cycles, which may be attributed to the effects of high temperature on PC stability.

Supercritical CO_2 extraction was performed solely with a CO_2/ethanol mixture, yielding a concentration of 0.416 mg/mL, a purity of 3.81%, and a yield of 41.6 mg/g in the first cycle. Both concentration and yield slightly decreased in the second and third cycles, while purity remained within the range of 3.81% to 7.21%. The advantage of supercritical extraction lies in its minimal use of organic solvents while providing moderate levels of purity and yield.

Statistical analyses showed that extraction cycle, extraction method, and solvent system significantly affected phycocyanin concentration, purity, yield, and total protein concentration ($p < 0.05$). Significant interactions among extraction method, solvent type, and extraction cycle were also observed, indicating that each solvent's extraction performance varied with the applied technique and stage. The strongest yield responses were generally associated with ultrasound-assisted and solvent-based extractions, whereas freeze-thawing produced lower yield values but retained relatively favorable purity in some cycles. For instance, the maximum yield achieved with freeze-thawing was 9 mg/g, while the ultrasonic extraction with sodium phosphate buffer yielded 480 mg/g and solvent-based extraction yielded 477 mg/g. Ultrasound-assisted extraction with sodium phosphate produced significantly higher PC yield than freeze-thawing and microwave-assisted extraction. In terms of purity, the freeze-thaw method yielded values comparable to those of the microwave and ultrasonic methods, particularly achieving 9.78% purity with sodium acetate buffer. Regarding concentration, the freeze-thawing method lagged behind most other methods, producing 0.04 mg/mL to 0.30 mg/mL, while ultrasonic and solvent-based extractions produced concentrations ranging from 0.076 mg/mL to 0.477 mg/mL. Microwave and supercritical CO_2 extractions yielded moderate levels of recovery and concentration while achieving comparable purity in some cycles compared to the freeze-thawing method. Across nearly all method-solvent combinations, extraction cycle had a statistically significant effect on PC content, PC purity, and/or PC yield ($p < 0.05$), indicating that repeated extraction cycles substantially altered phycocyanin recovery and extract quality. Within extraction methods, solvent type significantly affected PC recovery parameters ($p < 0.05$). Sodium phosphate and aqueous/buffered solvent systems generally produced higher PC content and yield than organic/non-polar systems, while cyclohexane and ethanol showed comparatively weaker performance for phycocyanin recovery.

Overall, extraction method, solvent type, and extraction cycle significantly influenced phycocyanin content. Interaction effects indicate that solvent performance varied depending on the extraction method and the extraction cycle. Significant main and interaction effects suggest that both extraction conditions and solvent systems strongly affected phycocyanin purity and pigment stability. Phycocyanin yield was significantly affected by the extraction method, solvent system, and extraction cycle, with notable interaction effects among factors.

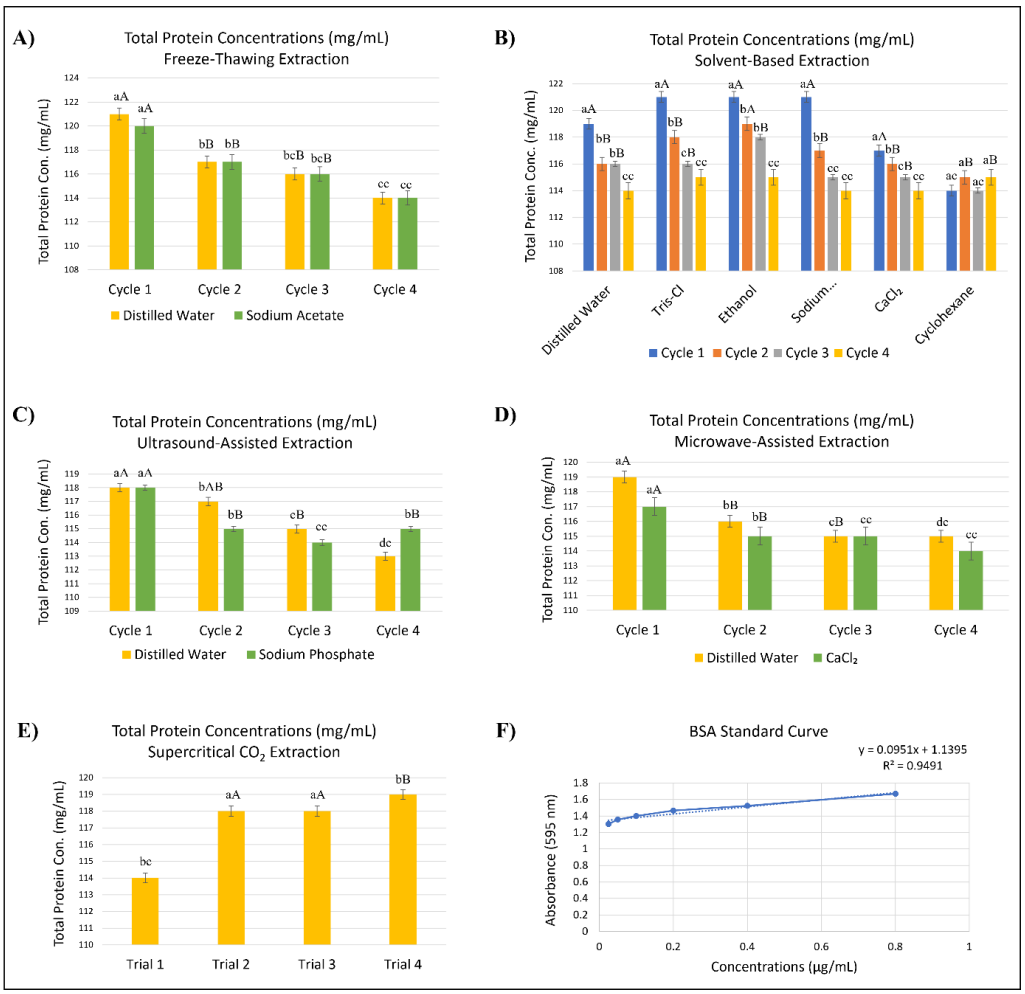

Figure 3. Total protein concentrations (mg/mL extract) for phycocyanin extracts obtained through the freeze-thawing (A), the solvent-based (B), the ultrasound-assisted (C), and the microwave-assisted extraction (D) methods with four subsequent cycles, and the supercritical carbon dioxide (SC-CO_2) extraction (E) method with four trials. F: BSA standard curve used for determining total protein content. Groups sharing the same letter are not significantly different. Different lowercase letters indicate statistically significant differences among extraction cycles/trials within the same extraction method and solvent system. Different uppercase letters indicate statistically significant differences among extraction methods and solvent systems according to one-way ANOVA followed by Tukey's HSD post hoc test ($p < 0.05$).

Table 2. Phycocyanin concentration, purity, and yield of phycocyanin extracts obtained through freeze-thawing, solvent-based, ultrasound-assisted, microwave-assisted extraction over four subsequent extraction cycles, and supercritical carbon dioxide (SC-CO_2) extraction method under four distinct trials.

Extraction Method	Solvent	Cycle	PC Content (mg/mL)	PC Purity (%)	PC Yield (mg/g)
Freeze and Thawing Extraction	Distilled Water	1	0.300 ± 0.004^{aA}	4.54 ± 0.41^{bB}	9.00 ± 0.11^{aB}
		2	0.290 ± 0.000^{bA}	5.74 ± 0.00^{aB}	8.70 ± 0.00^{bB}
		3	0.110 ± 0.000^{cA}	3.04 ± 0.01^{dB}	3.30 ± 0.01^{cB}
		4	0.040 ± 0.000^{dA}	3.84 ± 0.04^{cB}	1.20 ± 0.01^{dB}
	Sodium Acetate	1	0.222 ± 0.004^{bA}	2.85 ± 0.25^{cA}	6.66 ± 0.12^{bB}
		2	0.267 ± 0.002^{aA}	5.61 ± 0.17^{bA}	8.01 ± 0.06^{aB}
		3	0.182 ± 0.001^{dA}	3.11 ± 0.07^{cA}	5.46 ± 0.03^{dB}
		4	0.197 ± 0.001^{cA}	9.78 ± 0.43^{aA}	5.91 ± 0.02^{cB}
Solvent-Based Extraction	Distilled Water	1	0.416 ± 0.001^{aA}	3.81 ± 0.00^{cA}	83.20 ± 0.11^{aB}
		2	0.392 ± 0.001^{bA}	7.21 ± 0.07^{aA}	78.40 ± 0.12^{bB}
		3	0.355 ± 0.001^{cA}	7.14 ± 0.10^{aA}	71.00 ± 0.12^{cB}
		4	0.229 ± 0.001^{dA}	5.04 ± 0.06^{bA}	45.80 ± 0.12^{dB}
	$CaCl_2$	1	0.409 ± 0.001^{aA}	4.86 ± 0.04^{dA}	81.80 ± 0.11^{aB}
		2	0.405 ± 0.001^{aA}	7.29 ± 0.10^{bA}	81.00 ± 0.12^{aB}
		3	0.404 ± 0.006^{aA}	9.79 ± 0.50^{aA}	80.80 ± 1.13^{aB}
		4	0.103 ± 0.001^{bA}	5.79 ± 0.12^{cA}	20.60 ± 0.12^{bB}
	Tris-Cl	1	0.423 ± 0.001^{aA}	2.81 ± 0.01^{bB}	84.60 ± 0.12^{aB}
		2	0.415 ± 0.001^{bA}	4.32 ± 0.03^{aB}	83.00 ± 0.12^{bB}
		3	0.195 ± 0.001^{cA}	2.72 ± 0.02^{cB}	39.00 ± 0.12^{cB}
		4	0.029 ± 0.001^{dA}	1.04 ± 0.03^{dB}	5.80 ± 0.12^{dB}
	Ethanol	1	0.425 ± 0.001^{aA}	2.81 ± 0.01^{aC}	85.00 ± 0.13^{aB}
		2	0.315 ± 0.001^{bA}	2.47 ± 0.01^{bC}	63.00 ± 0.12^{bB}
		3	0.095 ± 0.001^{cA}	1.15 ± 0.01^{dC}	19.00 ± 0.12^{cB}
		4	0.062 ± 0.001^{dA}	2.25 ± 0.05^{cC}	12.40 ± 0.12^{dB}
	Cyclohexane	1	0.029 ± 0.000^{cB}	3.29 ± 0.13^{aC}	29.00 ± 0.26^{cB}
		2	0.052 ± 0.000^{aB}	1.81 ± 0.04^{bC}	52.00 ± 0.41^{aB}
		3	0.030 ± 0.001^{cB}	1.52 ± 0.05^{cC}	30.00 ± 0.59^{cB}
		4	0.040 ± 0.000^{bB}	1.57 ± 0.03^{cC}	40.00 ± 0.41^{bB}
	Sodium Phosphate	1	0.477 ± 0.000^{aA}	2.60 ± 0.01^{cB}	477.00 ± 0.41^{aA}
		2	0.212 ± 0.000^{bA}	3.88 ± 0.02^{aB}	212.00 ± 0.41^{bA}
		3	0.086 ± 0.000^{cA}	3.64 ± 0.05^{bB}	86.00 ± 0.41^{cA}
		4	0.076 ± 0.000^{dA}	3.79 ± 0.06^{aB}	76.00 ± 0.26^{dA}
Ultrasound-Assisted Extraction	Distilled Water	1	0.432 ± 0.001^{aA}	4.50 ± 0.03^{aB}	432.00 ± 0.62^{aA}
		2	0.209 ± 0.000^{bA}	2.52 ± 0.01^{bB}	209.00 ± 0.41^{bA}
		3	0.053 ± 0.001^{cA}	2.34 ± 0.06^{bB}	53.00 ± 0.62^{cA}
		4	0.018 ± 0.000^{dA}	4.13 ± 0.52^{aB}	18.00 ± 0.46^{dA}
	Sodium Phosphate	1	0.480 ± 0.000^{aB}	4.73 ± 0.02^{aB}	480.00 ± 0.41^{aA}
		2	0.059 ± 0.000^{bB}	2.27 ± 0.03^{cB}	59.00 ± 0.41^{bA}
		3	0.050 ± 0.000^{dB}	2.57 ± 0.05^{bB}	50.00 ± 0.41^{dA}
		4	0.054 ± 0.000^{cB}	1.62 ± 0.02^{dB}	54.00 ± 0.41^{cA}
Microwave-Assisted Extraction	Distilled Water	1	0.328 ± 0.000^{aB}	2.56 ± 0.01^{bB}	82.00 ± 0.10^{aB}
		2	0.072 ± 0.000^{cB}	1.59 ± 0.02^{dB}	18.00 ± 0.10^{cB}
		3	0.052 ± 0.001^{dB}	1.85 ± 0.03^{cB}	13.00 ± 0.15^{dB}
		4	0.142 ± 0.000^{bB}	4.13 ± 0.04^{aB}	35.50 ± 0.10^{bB}
	$CaCl_2$	1	0.280 ± 0.000^{aB}	3.97 ± 0.02^{aB}	70.00 ± 0.10^{aB}
		2	0.119 ± 0.000^{bB}	3.83 ± 0.05^{aB}	29.75 ± 0.10^{bB}
		3	0.081 ± 0.000^{cB}	2.84 ± 0.04^{cB}	20.25 ± 0.10^{cB}
		4	0.045 ± 0.000^{dB}	3.40 ± 0.10^{bB}	11.25 ± 0.10^{dB}
Supercritical CO_2 Extraction	EtOH	Trial 1	0.416 ± 0.000^{aA}	3.81 ± 0.43^{cA}	41.60 ± 0.02^{aB}
	EtOH	Trial 2	0.392 ± 0.000^{bA}	7.21 ± 0.00^{aA}	39.20 ± 0.00^{bB}
	H_2O	Trial 3	0.355 ± 0.000^{aA}	7.14 ± 0.01^{aA}	17.75 ± 0.01^{aB}
	EtOH	Trial 4	0.229 ± 0.000^{cA}	5.04 ± 0.01^{bA}	11.45 ± 0.01^{cB}

PC: Phycocyanin. Values are presented as mean ± SD. Different lowercase superscript letters following each value indicate significant differences among cycles/trials within the same extraction method and solvent system for the same parameter. Different uppercase superscript letters indicate significant differences among method–solvent combinations for the same parameter. Statistical comparisons were performed using one-way ANOVA followed by Tukey's HSD post hoc test ($p < 0.05$).

4. DISCUSSION

In this study, solvent type and number of subsequent extraction cycles for different extraction methods used for phycocyanin from *Spirulina sp*. dry biomass were comparatively characterized by experimental analysis, including phycocyanin yield, concentration, purity, and colorimetric analysis. UV-spectrophotometric analyses confirmed the presence of phycocyanin in all extracted samples through characteristic absorbance peaks at approximately 615 nm. The results obtained under different extraction conditions and solvent systems revealed significant differences among the applied extraction methods. Statistical analyses demonstrated that the extraction cycle, the extraction method, and the solvent system significantly influenced phycocyanin concentration, purity, yield, and total protein content. Significant interaction effects among extraction method, solvent type, and extraction cycle were also observed, indicating that the effectiveness of each solvent system varied depending on the extraction technique and extraction cycle applied. In general, the initial extraction cycles yielded significantly higher phycocyanin recovery and protein concentrations than subsequent cycles, reflecting progressive depletion of extractable compounds during sequential extraction. Among the tested techniques, ultrasound-assisted extraction produced the highest phycocyanin yield, whereas freeze-thawing extraction provided higher pigment purity and stability. Solvent composition also significantly influenced pigment solubility, protein extraction, purity, and stability. Extraction efficiency with sodium phosphate, CaCl2, and aqueous buffer systems was significantly higher than with organic solvent systems such as cyclohexane and ethanol.

The results summarized in Table 1 revealed pronounced differences in phycocyanin extraction yield among the various extraction strategies applied to Spirulina biomass. Conventional solvent-based techniques, including freeze-thawing cycling and mechanical mixing in distilled water or phosphate-based buffers, generally resulted in moderate to high phycocyanin recovery, with yields typically remaining above 15%. In contrast, assisted extraction methods such as ultrasound, microwave, and high-pressure processing markedly improved phycocyanin recovery. Ultrasound-assisted extraction, in particular, consistently enhanced phycocyanin content relative to conventional approaches, likely due to cavitation-induced cell wall disruption that facilitates pigment diffusion while limiting thermal degradation. Furthermore, aqueous solvents, particularly distilled water and phosphate buffers at near-neutral pH, were associated with superior extraction performance across all techniques, underscoring their compatibility with the physicochemical stability of phycocyanin.

The freeze-thawing extraction method, using distilled water and sodium phosphate buffer systems, resulted in the lowest PC yield among all evaluated methods. However, samples obtained by this method exhibited relatively higher purity values, more stable blue color characteristics in color analyses, and cleaner protein band profiles in SDS-PAGE analyses. These findings indicated that although freeze-thawing extraction was limited in terms of yield, it provides advantages in preserving pigment integrity and protein purity. The freeze-thawing method has been widely reported as an effective strategy for the extraction of phycocyanin from cyanobacterial biomass. For example, Ruiz-Domínguez et al. demonstrated that resuspending cyanobacterial dry biomass (20 mg per sample) in 5 mL of solvent followed by repeated freeze-thawing cycles between −22°C and 20°C enabled efficient pigment release. After centrifugation to remove cellular debris, analysis of the supernatant revealed a phycocyanin yield of approximately 225 mg g^{-1}, indicating that freeze-thawing cycles can serve as a simple and effective cell-disruption approach for pigment recovery (Ruiz-Domínguez et al., 2019). Under experimental conditions applied in this study, the measured phycocyanin yield obtained through the freeze-thawing extraction was 9.00 mg g^{-1}. The considerable difference in phycocyanin yield between the two studies highlighted the strong influence of extraction parameters on the efficiency of freeze-thawing processes. Variations in biomass concentration, solvent volume, freeze-thawing temperatures, and centrifugation conditions may significantly affect cell disruption and pigment release. The higher yield reported by Ruiz-Domínguez et al. (2019) suggested that specific temperature shock conditions and optimized processing parameters can substantially enhance pigment recovery. Conversely, the lower yield observed in this study indicated that further optimization of freeze-thawing parameters, potentially in combination with techniques such as ultrasound-assisted extraction, may improve phycocyanin extraction efficiency. In solvent-based extraction methods, distilled water, sodium phosphate buffer, Tris-Cl buffer, 1.5% calcium chloride buffer, cyclohexane, and ethanol buffers were evaluated. Higher PC solubility and yield were observed in extractions performed with sodium phosphate buffer, whereas Tris-Cl buffer maintained protein stability but resulted in comparatively lower yields. Extractions using non-polar solvents such as hexane were found to be ineffective due to the water-soluble nature of phycocyanin.

The efficiency of solvent-based extraction methods for phycocyanin recovery has been widely investigated. For example, Pan-utai et al. evaluated the extraction efficiency of phycocyanin from oven-dried and freeze-dried Arthrospira biomass using sodium phosphate buffer solutions (0.01 and 0.1 M, pH 7.0). In their study, biomass-to-solvent ratios of 0.06, 0.04, and 0.02 g mL^{-1} were incubated at temperatures of 25, 4, and −20°C before centrifugation, resulting in a reported phycocyanin yield of 312.65 mg g^{-1}. In this study, a solvent-based extraction approach was applied using six different solvents, namely distilled water, 1.5% calcium chloride, 100% ethanol, Tris-Cl buffer, hexane, and sodium phosphate buffer. Among

the tested solvents, sodium phosphate buffer produced the highest phycocyanin yield, reaching 477 mg g^{-1}. The higher yield obtained in the present study compared with that reported by Pan-utai et al. may be attributed to differences in extraction parameters, including solvent composition, biomass-to-solvent ratio, and processing conditions (Pan-utai et al., 2018). These findings highlight the importance of optimizing solvent selection and extraction conditions to maximize phycocyanin recovery. Furthermore, these results suggest that solvent-based extraction methods, particularly those employing sodium phosphate buffer, can provide highly efficient recovery of phycocyanin from Spirulina biomass. Future studies are required for further investigation of the influence of solvent concentration, pH, and extraction conditions to enhance the efficiency and scalability of phycocyanin extraction processes.

In this study, ultrasound-assisted extraction with distilled water and sodium phosphate buffer yielded the highest phycocyanin concentration and extraction yield among the analysed methods. Nevertheless, despite its high efficiency, decreases in purity values were observed under certain experimental conditions. Color analysis provided further evidence that ultrasound treatment could partially affect pigment integrity depending on the applied parameters, highlighting the sensitivity of this method to process optimization. The phycocyanin yields obtained by ultrasound-assisted extraction in this study were compared with previously reported values. In particular, the study by W. S. Park et al. provided an important reference regarding the efficiency of ultrasonic extraction. In their work, 100 mg of Spirulina powder was subjected to sonication for 30 min using 30 mL of phosphate-buffered saline (PBS, pH 7.0) as the extraction solvent. Following extraction, the mixture was centrifuged at 20,000 × g for 6 h, and the supernatant was filtered before phycocyanin quantification, resulting in a phycocyanin yield of 251 mg g^{-1} (Park et al., 2018). In contrast, the ultrasound-assisted extraction protocol applied in this study employed a Spirulina powder to sodium phosphate buffer ratio of 1:100 (w/v), followed by sonication for 30 min. The mixture was subsequently centrifuged at 4000 rpm for 15 min, and the filtered supernatant was analyzed for phycocyanin content. Under these conditions, a yield of 432 mg g^{-1} was obtained, which represented a substantial increase compared with the yield reported by Park et al. Several factors may account for the higher extraction efficiency observed in this study. The centrifugation conditions, including the shorter duration and lower speed applied, may have contributed to improved preservation of the phycocyanin pigment. In addition, the use of sodium phosphate buffer likely provided a favorable pH environment that enhanced pigment stability during extraction. These conditions may have collectively facilitated more efficient pigment recovery. Overall, the results highlight the effectiveness of ultrasound-assisted extraction for phycocyanin recovery from Spirulina biomass. Further investigations examining the effects of ultrasonic frequency, sonication duration, and biomass-to-solvent ratios may help refine the extraction process and further improve pigment yield. Our findings suggest that careful optimization of ultrasound-assisted extraction parameters can significantly enhance the recovery of valuable bioactive compounds such as phycocyanin.

Microwave-assisted extraction enabled rapid extraction; however, reductions in purity values and decreases in blue color intensity were detected, particularly in samples extracted using sodium phosphate buffer and distilled water. These observations were attributed to the thermal sensitivity of phycocyanin and potential localized temperature increases during microwave application. A notable discrepancy was observed between the phycocyanin yields reported by Ruiz-Domínguez et al. (2019) and those obtained in this study using microwave-assisted extraction. Ruiz-Domínguez et al. (2019) applied microwave irradiation at 2500 MHz with an input power of 1000 W to a mixture containing 20 mg of Spirulina powder and 5 mL of water. After 30 seconds of microwave exposure, the samples were centrifuged at 3140 × g for 5 min, yielding a phycocyanin yield of 215 mg (Ruiz-Domínguez et al., 2019). This outcome indicates a highly efficient extraction under the applied conditions. In contrast, the microwave-assisted extraction procedure employed in our study used a frequency of 2450 MHz at full power with a Spirulina–distilled water ratio of 1:250. Microwave irradiation was applied for 15 min, followed by centrifugation at 4000 rpm for 10 min. Under these conditions, the measured phycocyanin yield was 82 mg g^{-1}, which is considerably lower than that reported by Ruiz-Domínguez et al. (2019). The lower extraction efficiency observed in the present study may be attributed to several methodological factors. In particular, prolonged microwave exposure likely caused excessive heating of the extraction medium, negatively affecting phycocyanin stability. As phycocyanin is known to be thermolabile, elevated temperatures may disrupt its structural integrity and consequently reduce extraction efficiency. Furthermore, microwave power intensity and exposure duration were critical parameters influencing pigment recovery. Overall, these findings highlight the importance of optimizing microwave-assisted extraction conditions for phycocyanin recovery. Future studies are necessary to systematically evaluate the effects of microwave frequency, power input, and exposure time to determine optimal extraction parameters.

In supercritical carbon dioxide (SC-CO_2) extraction, ethanol was used as a co-solvent, and the extraction yield was found to be highly dependent on the co-solvent ratio. Despite slight yield improvements under certain conditions, SC-CO_2 extraction generally provided limited efficiency and variable phycocyanin purity. Color analysis indicated partial degradation of pigment integrity, suggesting that this method was not optimal for the direct extraction of hydrophilic pigments such as phycocyanin. Supercritical fluid extraction (SFE) has also been explored

as a technique for recovering phycocyanin from Spirulina biomass. For instance, Deniz et al. applied SFE to 30 g of ground Spirulina platensis using a mixture of liquefied CO_2 and ethanol at a flow rate of 10 g min^{-1} for 60 min. The experiments were conducted under different temperature (50, 60, and 80°C) and pressure (150, 250, and 350 bar) conditions, with the highest phycocyanin yield reported as 114.68 mg g^{-1} (Deniz et al., 2016). In the present study, SC-CO_2 extraction was also evaluated through four experimental trials, yielding a maximum phycocyanin concentration of 41.60 mg g^{-1}. The substantially lower yield observed in this study may primarily be attributed to the operational limitations of the extraction system, particularly the restricted maximum pressure that could be applied during the process. Since pressure plays a critical role in enhancing the solvating power of supercritical CO_2, insufficient pressure conditions may significantly reduce extraction efficiency. The comparison between the two studies highlighted the importance of optimized operational parameters, especially pressure and temperature, in supercritical fluid extraction processes. The higher yield reported by Deniz et al. (2016) can likely be attributed to the broader range of pressure conditions and optimized extraction parameters used in their experimental design. Therefore, more research is required to focus on employing extraction systems operating at higher pressures and exploring optimized process conditions to improve the efficiency of phycocyanin recovery using supercritical fluid extraction.

CONCLUSION

Experimental results demonstrated that pigment stability, purity, and protein integrity could not be fully explained by modelling approaches alone. Therefore, the extraction method selection for phycocyanin should be based on a combined evaluation of yield, purity, and stability according to the intended application. Subsequent extraction cycles significantly changed extraction efficiency and pigment characteristics, and the first extraction cycles generally produced significantly higher values than later cycles. For applications prioritizing high extraction yield, ultrasound-assisted extraction using sodium phosphate buffer is recommended, provided that ultrasound power and duration are carefully optimized. For applications requiring high purity and pigment stability, the freeze-thawing extraction method, despite its lower yield, may be preferred, particularly when performed with sodium phosphate buffer or distilled water. Microwave-assisted extraction should be applied with caution due to the thermal sensitivity of phycocyanin, and excessive power or prolonged exposure should be avoided. SC-CO_2 extraction is not recommended as a stand-alone method for phycocyanin extraction due to the pigment's hydrophilic nature; however, it may be more suitable for other bioactive compounds. Future studies are necessary to investigate hybrid extraction approaches, such as combining freeze-thawing and ultrasound techniques, to achieve a balance between yield and purity. When all results are evaluated collectively, the highest phycocyanin yield was achieved through ultrasound-assisted extraction using sodium phosphate buffer, while the freeze-thawing method provided superior purity, stable color characteristics, and cleaner protein profiles despite its lower yield. Therefore, the selection of the extraction method and solvent system for phycocyanin recovery should be simultaneously determined based on the targeted application, considering yield, purity, and pigment stability.

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Author Contributions

Serkan CIFTCI: (c) Literature Review, (f) Data Collection, (h) Drafting Article.

Seyit YUZUAK: (a) Original Idea, (b) Study Design, Methodology, (d) Supervision, (e) Material, Resource Supply, (g) Data Analysis, Interpretation, (h) Drafting Article, (i) Critical Review.

Declaration of Ethical Code

In this study, we affirm that all the necessary rules under the "Regulation on Scientific Research and Publication Ethics in Higher Education Institutions" have been adhered to, and none of the actions mentioned under the title "Actions Contrary to Scientific Research and Publication Ethics" in the mentioned regulation have been conducted.

Conflict of Interest

The authors report there are no competing interests to declare.

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