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Spirulina (*Arthrospira platensis*) immobilization in calcium-alginate beads can provide a way to produce food-grade C-phycocyanin following a biorefinery perspective

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ABSTRACT

C-phycocyanin (C-PC), a water-soluble blue pigment, is the primary phycobiliprotein in Spirulina. In this study, Spirulina was immobilized in calcium-alginate (SAC) beads as an innovative method to recover C-PC in the crosslinking bath while retaining the biomass within the beads. This approach simplifies the separation process and reduces costs. SAC beads were prepared via ionic gelation with alginate and $CaCl_2$ at 2 % (PC2) and 4 % (PC4) concentrations. Different Spirulina to $CaCl_2$ (S:CA) ratios (1:33, 1:42, 1:83, 1:125 w:v) were tested. PC4 extracts surpassed the food-grade purity threshold ($\geq$ 0.7), achieving the highest purity of 0.83 at a 1:42 S:CA ratio. For PC2, the highest purity was 0.68, observed at a 1:83 S:CA ratio. Overall, this method effectively releases C-PC into the $CaCl_2$ bath, attaining food-grade purity with significant extraction yields ($>$ 50 mg/g biomass). Additionally, the SAC beads exhibited high protein levels ($>$ 25 g/100 g d.w.) and can be further utilized within a biorefinery framework, either directly as a food supplement or for cascade extractions to recover the remaining lipid and protein fractions.

1. Introduction

Spirulina (*Arthrospira platensis*) is a photosynthetic cyanobacterium well-known for its high nutritional value and richness in valuable biocompounds. It has high protein content (50–70 %) and phycobiliproteins, which are pigment-protein complexes. Light-harvesting pigments are found in Cyanobacteria (blue-green algae), Rhodophyta (red algae), and Cryptophyta (cryptomonads). They can be classified into three main types: phycocyanin, allophycocyanin, and phycoerythrin. Among them, phycocyanin is the most abundant in Spirulina, also known as C-phycocyanin (C-PC), achieving up to 20 % of the microalgae dry weight [1,2].

C-PC is a water-soluble blue pigment widely utilized in various industries, particularly food and beverages. Owing to the growing consumers' demand for natural and sustainable ingredients, C-PC is consolidating its role as a natural alternative to synthetic colorant counterparts (e.g., blue brilliant (E133)), also having the advantage of holding functional properties, namely anti-inflammatory, anticancer, and antioxidant [3–5].

The use of C-PC in food products was reported in studies addressing ice cream [6], beverages [7], confectionary products [8], jelly candy [9], and food emulsions [10,11], showing promising results as a blue colorant alternative. At the industrial level, M&Ms.® from Mars and Smarties® from Nestlé are examples of chocolate confectionery industries that have replaced the blue synthetic colorants with C-PC from Spirulina [1].

C-PC is commercially available in powder or liquid forms, with the food-grade product commonly called "Blue Spirulina". In 2020, the global market was $155.3 million and is projected to grow to $409.8 million by 2030, with an annual growth rate (CAGR) of 9.6 % from 2021 to 2030. Although C-PC is employed in pharmaceutical, cosmetic, and nutraceutical fields, the food & beverages sector held a significant share, 67.9 % in 2020, corroborating the high exploitation potential of C-PC as a natural colorant for the food industry [12].

C-PC extraction from cyanobacteria can be done through several methods, typically using buffer and saline solutions as the extraction

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media. Examples include sodium phosphate buffer, $CaCl_2$, and NaCl. An optimal method should enable the release of the target compound at high selectivity, within short extraction times, and with low energy consumption. The most usual methods are freezing/thawing, bead milling, shaker or magnetic stirrer mixing, and homogenization techniques. Recently, emerging technologies like ultrasound, microwave, high-pressure homogenization, and pulsed electric fields have been applied. Nonetheless, these techniques are mostly time- and energy-consuming, often leading to low-purity extracts [13]. The level of C-PC purity ratio, expressed as the ratio between the absorbance at 615 nm (maximum absorbance of C-PC) and the absorbance at 280 nm (maximum absorbance of other proteins present in the biomass) (Abs_{615}/Abs_{280}), is a critical parameter ruling its application [14]. A value between 0.7 and 3.9 corresponds to a reagent-grade product and when ≥4.0 to analytical grade, with food applications requiring a purity ≥0.7 [13,15,16].

Alginate-based matrices are commonly applied in microalgae research for environmental purposes, i.e., biosorption. These systems act as biosorbents utilizing microalgae immobilized in biopolymeric matrices to remove heavy metals from wastewater. Promising results were shown for Spirulina entrapped in calcium alginate beads to remove Pb(II) from aqueous solutions, achieving an enhanced adsorption efficiency compared to Spirulina alone and blank alginate beads [17].

These alginate-based systems can also be employed to entrap proteins targeting protection from environmental conditions and delivery, e.g., in the gastrointestinal tract. Zhang et al. [18], which encapsulated whey proteins in calcium-alginate beads, analyzed their release in different pHs, showing the ability to design the beads to retain or release proteins depending on the application. Guarienti et al. [19] immobilized Spirulina biomass in calcium-alginate beads to attain thermal stability and preserve antioxidant properties, performing a study of C-PC controlled release in different pH media. It was concluded that 85 % of C-PC could be released under conditions simulating the alkaline gut environment. This is a significant achievement, highlighting that immobilization in alginate beads can be explored to selectively extract C-PC from Spirulina biomass, offering advantages for C-PC production.

In this work, Spirulina immobilization into calcium-alginate beads produced by ionic gelation with $CaCl_2$ was studied as a straightforward process to extract C-PC targeting food-grade purity. C-PC was recovered in the $CaCl_2$ crosslinking bath, double-acting as a stabilizing medium, and the SAC beads were recovered by filtration. The effects of the Spirulina to $CaCl_2$ ratio and $CaCl_2$ concentration on C-PC extraction yield, efficiency, and purity were analyzed. In addition to seeking a biorefinery framework, SAC proximate composition was evaluated to facilitate subsequent protein or lipid extraction or to utilize the whole material as a food supplement. To the authors' best knowledge, this is the first study to explore Spirulina immobilization in alginate beads, focusing on C-PC extraction for production purposes, targeting a food-grade product.

2. Materials and methods

2.1. Samples, standards, and reagents

The dried Spirulina (*Arthrospira platensis*), chosen due to advantages like not requiring refrigeration during transportation, was generously provided by Algikey - Algae-based Solutions S.A., a Portuguese company, stored at −24 °C and protected from light. For Spirulina biomass immobilization, sodium alginate and calcium chloride ($CaCl_2$) were obtained from Sigma-Aldrich (Saint Louis, MO, USA). n-Hexane, used for lipid extraction in the proximate composition determination, was acquired from Carlo Erba Reagents (Barcelona, Spain). Distilled water was used in the experiments.

2.2. C-phycocyanin extraction procedure

The extraction procedure, based on the immobilization of Spirulina biomass within alginate beads formed through ionic gelation, is schematically represented in Fig. 1. It comprises two stages: bead formation and C-PC release. Two series of assays, PC4 and PC2, were prepared, reflecting the $CaCl_2$ (CA) concentration used in the crosslinking bath and the sodium alginate (A) concentration used in the Spirulina (S) suspension, i.e., 4 % and 2 % (*w/v*). The beads formation stage comprises the preparation of 30 mL of the S suspension with a fixed S:A ratio of 2:1 (*w*/w) at the corresponding A concentration. For that, the required S and A amounts were weighted, followed by water addition and homogenization using a magnetic stirrer for about 30 min. This suspension was added dropwise at a flow rate of 1.003 mL min^{-1} into a CA crosslinking bath with the corresponding concentration. For each series, different S:CA ratios were tested (1:33, 1:42, 1:83, and 1:125 (w:v)), achieved by changing the crosslinking bath volume. The CA concentration and S:CA ratios were selected based on the group's prior experience and data from relevant literature [13,19].

To facilitate the C-PC release (second stage), following the addition of the 30 mL of the Spirulina suspension (dropwise), the $CaCl_2$ bath was stirred (200 rpm) for 24 h at room temperature, shielded from light. Subsequently, the Spirulina-alginate-calcium (SAC) beads were retrieved via filtration, rinsed three times with distilled water, and subjected to freeze-drying for 48 h (Labogene, ScanVac CoolSafe, Lillerod, Denmark). The liquid C-PC extracts were collected and safeguarded from light at 4 °C. The pH levels of the extracts were gauged at the onset and conclusion of the extraction process utilizing a pH meter (WTW Inolab® pH 720, Washington, USA).

To estimate the maximum extractable amount of C-PC from Spirulina biomass, a serial extraction of freezing and thawing steps was performed using the procedure reported by Tavanandi et al. [20]. For that, the Spirulina biomass was dissolved in a $CaCl_2$ solution (2 % or 4 %) using the most favorable S:CA ratio (w:v), i.e., the one giving rise to the higher C-PC extraction purity, according to the immobilization technique, namely 1:83 for PC2 and 1:42 for PC4. The solution was frozen (−24 °C) for 1 h and thawing (room temperature) for 30 min. Since after 4 cycles, no significant amount of phycocyanin was released, these conditions were considered optimal. The samples were centrifugated (Eppendorf, 5810R, Hamburg, Germany), and the supernatants were recovered and pooled for C-PC quantification.

2.3. Characterization of the extraction process and extracts

The C-PC concentration and purity in the liquid extracts were determined using UV-VIS (Jasco Inc., V-730, Tokyo, Japan). The concentration, expressed in mg/mL, was calculated using Eq. (1) according to Bennett and Bogorad [21], where Abs_{615} is the maximum absorbance of C-phycocyanin measured at 615 nm, and Abs_{652} is the maximum absorbance of allophycocyanin measured at 652 nm. The C-PC purity was determined by Eq. (2) according to Abalde et al. [14], where Abs_{280} is the maximum absorbance of other proteins measured at 280 nm. Thereafter, C-PC extraction yield ($mg/g_{biomass}$) was determined by Eq. (3), where C-PC concentration is the C-PC concentration (mg/mL) in the volume V (mL) of the $CaCl_2$ crosslinking bath, and S weight the initial weight of the Spirulina biomass (g). For the extraction efficiency, the obtained yield was compared with the one obtained from the serial extractions (maximum yield) for PC4 and PC2 extracts, as applicable (Eq. (4)) [20].

$$\text{C} - \text{PC concentration (mg/mL)} = \frac{Abs_{615} - 0.474 \times Abs_{652}}{5.34} \quad (1)$$

$$\text{Purity} = \frac{Abs_{615}}{Abs_{280}} \quad (2)$$

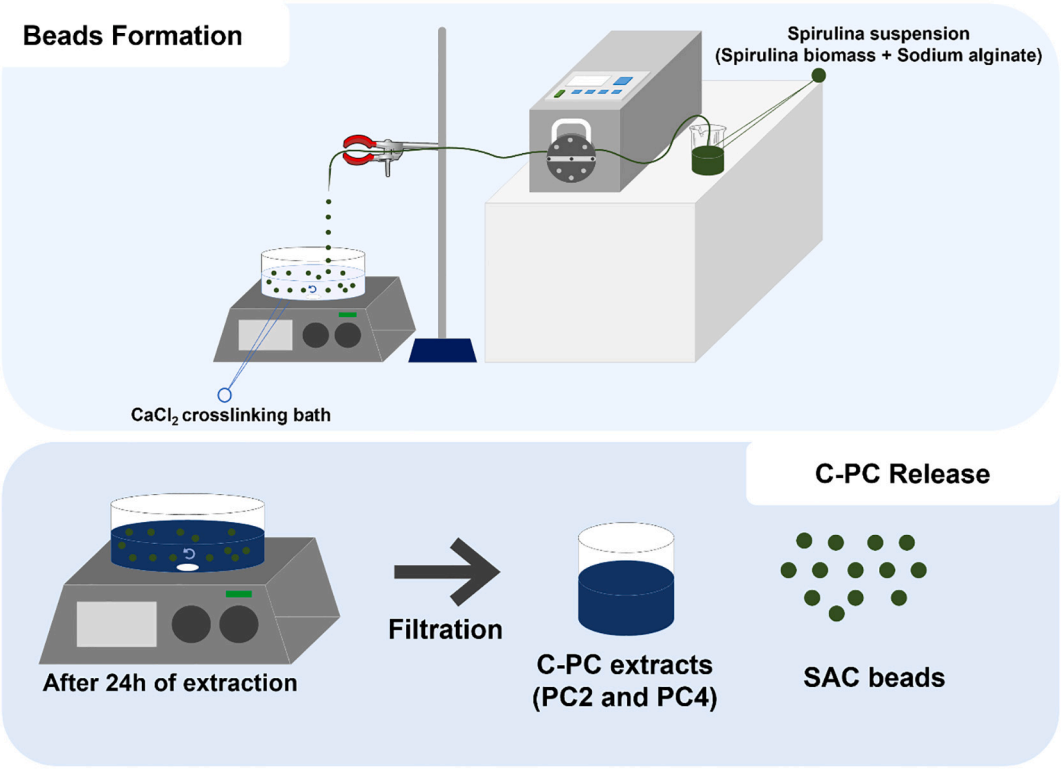

Fig. 1. Schematic representation of the two main stages of C-PC extraction using Spirulina immobilization in calcium-alginate beads.

$$\text{C}-\text{PC yield (mg/g)} = \frac{\text{C}-\text{PC concentration (mg/mL)} \times \text{V (mL)}}{\text{S weight (g)}} \quad (3)$$

$$\text{C}-\text{PC efficiency (\%)} = \frac{\text{C}-\text{PC yield immobilization technique}}{\text{C}-\text{PC yield serial extraction}} \times 100 \quad (4)$$

The C-PC extract colorimetric parameters (L^*, a^*, and b) (CIELAB) were obtained with a Konica Minolta CR-400 equipment (Chiyoda, Tokyo, Japan).

2.4. Proximate composition of Spirulina biomass and Spirulina calcium-alginate beads

The proximate composition of the Spirulina biomass and the SAC beads was determined following AOAC protocols [22]. Briefly, protein content was measured by the macro-Kjeldahl method using an N-factor of 5.95 according to López et al. [23]; fat content was determined using a Soxhlet apparatus with n-hexane extraction; ash content by incineration at 550 ± 15 °C; and moisture by drying the samples in an oven until constant weight. Carbohydrates were calculated by the difference considering moisture, protein, fat, and ash content subtraction. All the results were expressed in g/100 g d.w.. The SAC-beads' average size was determined using a caliper and by averaging the diameter of 30 beads right after the C-PC extract separation step.

2.5. C-phycocyanin extraction kinetics

According to the literature, the solid-liquid extraction processes, such as the case of C-PC extraction from SAC beads, is effectively represented by second-order rate kinetics models, as described by Eq. (5) [20,24].

$$\frac{dC_L}{dt} = k(C_S - C_L)^2 \quad (5)$$

where "$\frac{dC_L}{dt}$" is the extraction rate (mg/mL·h), "C_L" is the concentration of C-PC (mg/mL) in the extraction medium at the specific time "t" (hours), "C_S" is the equilibrium concentration of C-PC (mg/mL) in the extraction medium, and "k" is the second order extraction rate constant (mL/mg·h). The integration under the limits 0 to t for t, and 0 to C_S for C_L, results in the linearized equation represented by Eq. (6). By plotting t/C_L against t, k and C_S can be determined from the slope and intercept of the linear plot, respectively. Moreover, the initial extraction rate (R_i) (mg/mL·h) can be obtained when t and C_S tend to 0 (Eq. (7)).

$$\frac{t}{C_L} = \frac{t}{C_S} + \frac{1}{kC_S^2} \quad (6)$$

$$R_i = kC_S^2 \quad (7)$$

2.6. Statistical analysis

Results were expressed as mean ± standard deviation, and the analyses were performed in triplicate. The data were normalized according to Templeton [25] and a two-way ANOVA (Analysis of Variance) using the general linear model procedure to analyze the effect of CA concentration and S:CA ratio along with their interaction effect on the C-PC extraction yield, C-PC efficiency, and C-PC purity was applied. Moreover, a one-way ANOVA was conducted, followed by post-hoc Tukey's test for data with homoscedastic distributions and Tamhane's T2 test for data with heteroscedastic distributions to compare the differences among the mean values of SpB and SAC beads for proximate composition. A Student's t-test was also performed to identify differences between the mean values of the SAC beads size.

The normalization and statistical analysis were performed using IBM SPSS Statistics 28.0 software (IBM Corp., USA) at a 5 % significant level (p-value $\leq$0.05).

3. Results and discussion

3.1. C-phycocyanin extraction yield and efficiency and extract purity

The extraction yield of C-PC (mg/$g_{biomass}$) and its efficiency (%) were examined for PC4 and PC2 extracts following 24 h of extraction, with the results illustrated in Fig. 2. In the case of PC4, the C-PC extraction yield rose from 56.0 to 67.6 mg/$g_{biomass}$ as the S:CA ratio increased. The same behavior was observed for PC2, with yields ranging from 49.6 to 70.9 mg/$g_{biomass}$. Thus, higher S:CA ratios result in higher C-PC extraction yields, which can be related to higher solvent availability facilitating C-PC diffusion to the extraction medium [13].

The range for the obtained C-PC yield is in accordance with other published results. Tavanandi et al. [20], who tested different extraction methods, found similar values by applying 4 cycles of freezing/thawing (73.7 mg/$g_{biomass}$), maceration (55.9 mg/$g_{biomass}$), high-shear homogenization (52.1 mg/$g_{biomass}$), and ultrasonication (51.5 mg/$g_{biomass}$). These values increased by combining two extraction methods, e.g., a C-PC yield increase was observed when ultrasonication was combined with freezing/thawing (109.6 mg/$g_{biomass}$). İlter et al. [26] found similar C-PC yields (68.1 mg/$g_{biomass}$) using a S:CA ratio of 1.71 % through high-shear homogenization employing frozen Spirulina biomass and a CA concentration of 1.5 %. The authors also noticed a lower C-PC yield (29.1 mg/$g_{biomass}$) when using microwave-assisted extraction. Pan-utai et al. [27] got a C-PC yield of 60 mg/$g_{biomass}$ by applying ultrasound (50 % amplitude; 20 kHz; 5 min) and a phosphate buffer (0.1 M; pH 7.0) using a Spirulina:Solvent (S:SV) ratio of 6.6 %.

Only a few studies have evaluated the effect of different S:SV ratios on the C-PC extraction yield, as this is a relevant parameter for the extraction process. In this framework, according to a review work performed by Jaeschke et al. [13], the most used S:SV ratios are 1:25, 1:20, 1:15, and 2:25, even though other studies reported the values 1:6, 1:8, 1:10, 1:50, and 1:25 [20,28]. In this work, the S:SV ratio ranged from 1:33 to 1:125. Although higher S:SV ratios can result in improved extraction yields, they can generate low-purity C-PC extracts since solvent availability can lead to impurities extraction, such as interfering proteins [13]. The results of this work can corroborate this behavior since the lowest purities were found for the higher-used S:SV ratios, both for the PC2 and PC4 series (Fig. 2A). Tavanandi et al. [20] also reported this trend when evaluating 3 ratios (1:6, 1:8, and 1:10) using sodium phosphate buffer (0.1 M, pH 6.8), achieving the highest extraction yield for the 1:10 ratio. In contrast, the highest purity was obtained for the lowest one (1:6).

Sodium phosphate buffer (pH 6–8), distilled water, and saline solutions (CA and NaCl) have been commonly used for C-PC extraction [13]. İlter et al. [26] compared distilled water, sodium phosphate buffer (pH 7.4), and CA solutions (1.5 %) at a S:SV ratio of 1 % (1:100), using a homogenizer (UltraTurrax®), ultrasound and microwave devices. The authors concluded that the CA solution presented the deepest blue color and the highest amount of C-PC. Herrera et al. [29] also performed C-PC extraction using CA (10 g/L, 25 °C, pH 6.8), obtaining a C-PC yield of 143 mg/$g_{biomass}$ and a purity of 0.74 after 240 min of extraction. On the other hand, Silveira et al. [30] achieved no significative differences in the extraction yields using water, phosphate buffer (pH 7.0), NaCl (0.15 M), and CA (10 g/L) solutions. In this work, CA was chosen since this salt is food-grade and non-toxic and gave excellent performance in encapsulation studies [31]. Moreover, it enables ionic crosslinking with alginate, the base of the herein-developed process.

The optimal pH range for C-PC extraction is between 6 and 7, as C-PC can become unstable at pH levels below 5 and above 8 [13]. Zhang et al.

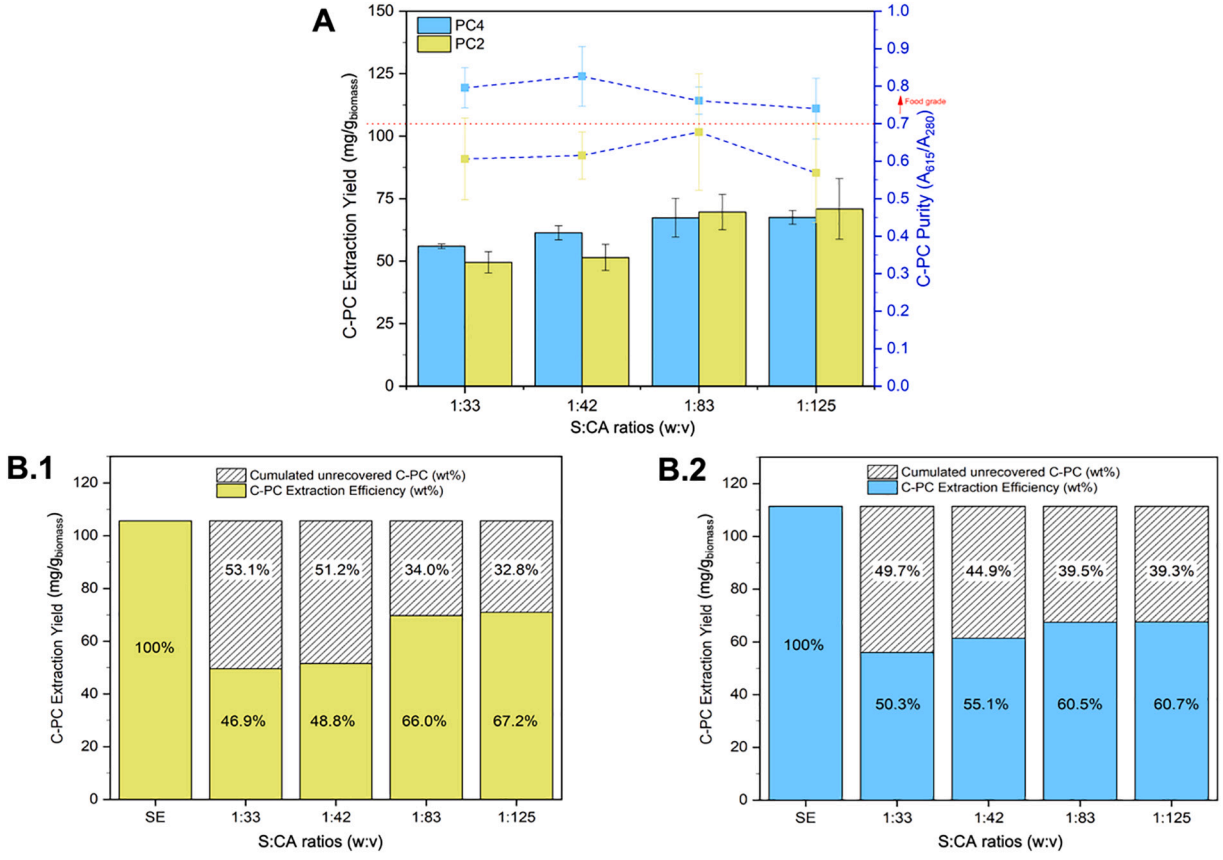

Fig. 2. (A) C-PC extraction yield (mg/$g_{biomass}$) and C-PC purity ratio for different calcium-alginate contents (2 % (PC2) and 4 % (PC4)) and S:CA ratios (w:v) (1:33, 1:42, 1:83 and 1:125). (B.1) C-PC extraction efficiency (wt%) together with the cumulated recovered and unrecovered C-PC (wt%) of the serial extraction (SE) and PC2 and (B.2) PC4 extracts.

[18], by performing a study of whey protein encapsulation in calcium-alginate beads, demonstrated that protein retention increased at low pHs due to electrostatic attraction, and protein release increased at higher pH levels due to enhanced electrostatic repulsion. They also demonstrated that a rapid protein release occurred within 30 min at pH 5–7. In this study, the crosslinking solutions' natural pH was 6.60 ± 0.01 for both PC2 and PC4 series, which is within the optimal range. After 24 h, the pH of the C-PC extract ranged from 4.43 ± 0.01 to 5.56 ± 0.01 for PC2 and from 4.55 ± 0.02 to 5.36 ± 0.06 for PC4, slightly below the lower limit of the stability range.

To determine the C-PC extraction efficiency, C-PC content in the used biomass was estimated using a serial freezing and thawing extraction (Fig. 2B.1 for the PC2 extracts and Fig. 2B.2 for the PC4 extracts). Better efficiencies were attained for higher S:CA ratios aligned with the C-PC yield. For the PC4 series, efficiency ranged from 50.3 % to 60.7 %, and for PC2, from 46.9 % to 67.2 %, signifying that at least 50 % of the C-PC content in the Spirulina biomass was extracted. These findings align with those reported in existing literature. Tavanandi et al. [20] also estimated the maximum extractable C-PC by serial freezing and thawing extraction, obtaining C-PC efficiencies of circa 40 % for maceration, homogenization, and ultrasonication. When ultrasonication was combined with 2 cycles of freezing/thawing, up to 92.08 % was achieved. On the other hand, Ayekpam et al. [32] found a lower C-PC efficiency (28.5 %) when employing a CA-based extraction (3 mg/mL) using a S:CA ratio of 1:6, pH 6.8, 35 °C and an extraction time of 160 min.

C-PC extract purity is a relevant parameter that defines the final application of the extract depending on the obtained value. Most extraction methodologies cannot generate high-purity C-PC extracts, demanding subsequent purification steps, which can increase costs significantly. In this work, higher purities were found for the PC4 series compared with the PC2 ones. For all the studied S:CA ratios, PC4 extracts presented purities higher than those established for food-grade use (≥ 0.7). Also, although higher efficiencies were observed for PC2 extracts, they exhibited lower purities (Fig. 2A). According to the review work of Gombotz and Wee [31], an increase in the alginate concentration decreased the diffusion rate of proteins from the gel beads. In this work, beads produced using the higher alginate concentration (PC4 extracts) seemed to have favored C-PC release over other proteins, i.e., C-PC selectivity towards other Spirulina biomass proteins, giving rise to higher purity levels.

To better understand the effect of CA concentration and S:CA ratio on the C-PC yield, C-PC efficiency, and C-PC purity, a two-way ANOVA was conducted. The results revealed that there was a statistically significant interaction effect of CA concentration and S:CA ratio on C-PC yield ($F(3, 64) = 6.388$ p-value = 0.001), C-PC efficiency ($F(3, 64) = 5.504$ p-value = 0.002) and C-PC purity ($F(3, 64) = 2.714$ p-value = 0.052). These findings indicate that the impact of CA concentration on these variables depends on the S:CA ratio and vice-versa. The Estimated Marginal Mean (EMM) plots are displayed in Fig. S1 – Supplementary material, where intersecting lines indicate the interactions.

Regarding the main effects of each variable, CA concentration is statistically significant on C-PC yield ($F(1, 64) = 8.063$ p-value = 0.006), giving rise to higher yields when 2 % CA was used. The S:CA ratio effect is statistically significant for both C-PC yield ($F(3, 64) = 46.326$ p-value = 0.000) and C-PC efficiency ($F(3, 64) = 50.352$ p-value = 0.000), showing higher values when higher S:CA ratios are employed, corroborating the discussion above.

In summary, for PC4, a superior purity level (0.83) was achieved with the 1:42 S:CA ratio (further referred to as PC4_B), while for PC2, a higher purity (0.68) was observed with the 1:83 S:CA ratio (further referred to as PC2_B) (Fig. 2A). Although the S:CA ratio effect was not statistically significant on C-PC purity, these two conditions were chosen as the best ones to obtain C-PC extracts from Spirulina biomass, displaying attractive C-PC yields and efficiencies.

3.1.1. Effect of extraction time

To analyze the effect of the extraction time on the C-PC extraction yield (mg/$g_{biomass}$) and purity (Abs_{615}/Abs_{280}) and to perceive if food grade purity can be achieved before the used reference time of 24 h, the samples PC2_B (S:CA 1:83) and PC4_B (S:CA 1:42) were analyzed at 1-h intervals along this total time. The obtained results are included in Fig. 3. C-PC yield increased with time for both PC2_B and PC4_B extracts, with PC2_B giving rise to higher yields. Although PC4_B extract showed lower C-PC yields, its purity was higher. Overall, it is important to highlight that for the PC4_B extract, purities higher than 0.7 (food-grade) were found after 14 h, whereas for the PC2_B extract, this target was not achieved within the studied period.

3.1.2. C-phycocyanin extraction kinetics

Release mechanisms and kinetics describe the rate and way encapsulated substances are released over time. Protein release from alginate matrices typically occurs through two primary mechanisms: diffusion through the beads' pores and degradation of the polymeric structure [33]. In the present study, diffusion might be the dominant release mechanism, as the beads remained intact at the end of the extraction period. This process is strongly influenced by the concentrations of alginate and calcium used during bead formation, as well as environmental factors like the pH of the medium, which was consistent across all tested samples.

C-PC concentrations (mg/mL) obtained at various extraction times (hours) were graphed using Eq. (6) and illustrated in Fig. 4. Additionally, the kinetic parameters, including the equilibrium concentration (C_S, representing extraction capacity), the extraction rate constant (k), and the initial extraction rate (Ri) at different S:CA ratios, are presented in Table 1.

C_S for PC4_B surpassed the extraction value obtained after 24 h, suggesting a higher potential for extracting C-PC. In the case of PC2_B, the C_S was marginally higher, with no significant difference noted. Although Ri for PC4_B exceeded that of PC2_B, k was lower, indicating a slower release of C-PC with a 4 % $CaCl_2$ compared to 2 %. In the conditions of PC2_B, the release of C-PC occurs faster than with PC4_B. This might be due to the increased rigidity of the SAC beads resulting from the later concentration. As previously mentioned, this system also yields lower purities, suggesting that protein contaminants might be released into the medium. These observations imply that higher alginate and calcium chloride concentrations may be advantageous for producing C-PC extracts with food-grade purity. However, the phycocyanin is slowly released into the medium.

Su et al. [24], using a second-order kinetic model, evaluated the effects of pH and temperature on the kinetic parameters of C-PC solid-liquid extraction from *Spirulina*. They obtained an extraction rate constant of 0.0198 L/g·min (1.18 mL/mg·h) and an equilibrium concentration of 2.36 g/L (2.36 mg/mL) ($r^2 = 0.99$) using sodium phosphate buffer (10 mM, pH 7.0) at 50 °C under magnetic stirring and a S:SV of 1:20. The higher temperature, reduced biomass size (particle size <25 μm) and lower S:SV ratio may have synergistically contributed to a faster extraction rate and a higher equilibrium concentration compared to this work.

3.2. A comparative analysis of C-phycocyanin purity

Fig. 5 depicts a comparison of the C-PC purities obtained in this work by immobilization (IM) against those obtained using other techniques, namely freezing/thawing (FT), high-shear homogenization using UltraTurrax® (UT), mixing using rotary shaker/magnetic stirrer (MX), contact with salt-based solutions (SB), bead milling (BM), homogenization using ultrasound (probe) (US), high-pressure homogenization (HPH), pulsed-electric fields (PEF), along with different extraction conditions. No purification steps were considered for all the applied techniques. The main objective was to provide an overview of the C-PC extract purity reported in the literature, acknowledging that the

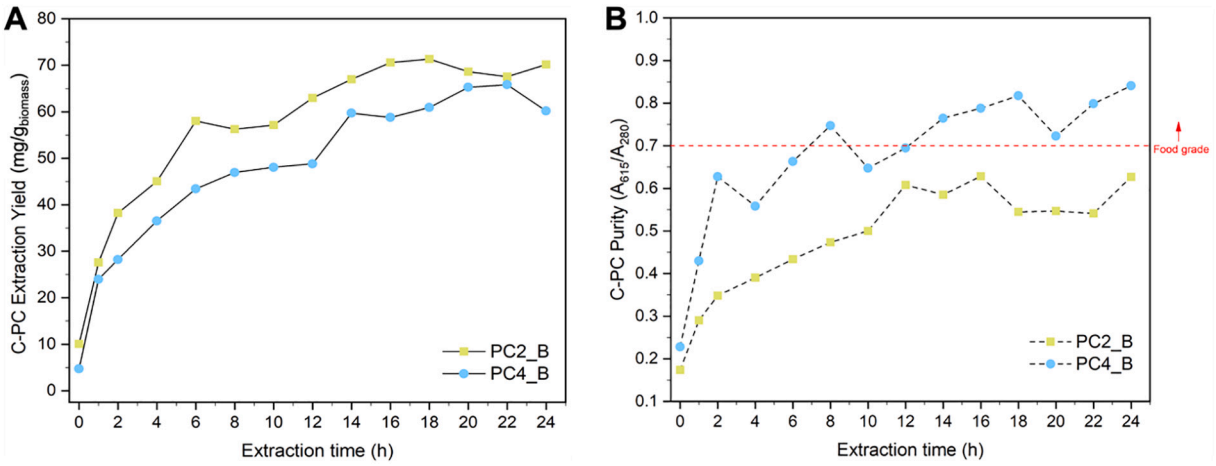

Fig. 3. (A) C-PC extraction yield (mg/$g_{biomass}$) and (B) C-PC purity ratio of the best conditions extracts (PC2_B and PC4_B) over time.

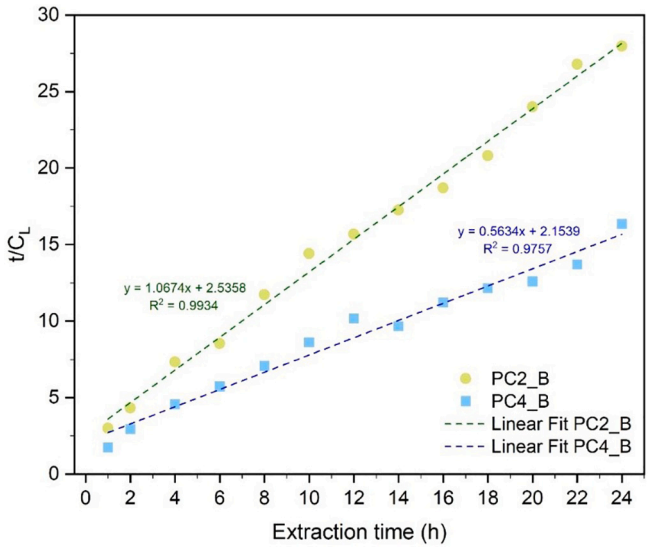

Fig. 4. C-PC extraction kinetics for the best conditions of PC2 and PC4 extracts.

comparison involves different processes under varying conditions, which may introduce limitations. Table S1 (Supplementary material) shows the complete information, including extraction time, Biomass: Solvent (w:v) ratio, type of solvent, and biomass. For most of the used technologies food-grade purities (≥ 0.7) were achieved, with HM and US giving rise to purities lower than 0.7. According to the selected works, HPH provided purities ranging from 0.32 [34] to 1.18 [15] and PEF from 0.46 [35] to 0.89 [36]. SB extract purities ranged from 0.62 [29] to 1 [32], and for MX, from 0.46 [30] to 0.93 [37]. In this work, the IM technique resulted in purities ranging from 0.57 (PC2; 1:125 S:CA ratio) to 0.83 (PC4; 1:42 S:CA ratio).

All the presented methods have their advantages and drawbacks. Although HPH and PEF result in higher purities, they are considered high-energy demanding technologies, increasing C-PC extraction costs. MX and SB methods are known for their time-consuming nature while freezing/thawing poses challenges when scaling up. Although BM is considered a rapid cell disruption method, it produces extracts with low purity (below 0.7). Moreover, all these techniques imply a final centrifugation step to separate the liquid extract from the spent biomass. Centrifugation is an energy-intensive process; e.g., it accounts for 20–30 % of biomass production costs in microalgae harvesting [38,39]. The presented developed technique (IM) offers a more efficient alternative, requiring only gravity filtration to separate the extract from the biomass. Moreover, the immobilized biomass can also be valued as a marketable product. The constraints of this approach can be related to the processing time required to achieve food-grade purities.

3.3. C-phycocyanin extracts and Spirulina calcium-alginate beads characterization

Fig. 6A illustrates the colorimetric parameters of C-PC extracts obtained under the optimal conditions using the IM technique, specifically PC2 (PC2_B) and PC4 (PC4_B). All extracts showed similar L* (lightness) and a* (greenness) values, with minor variations in b* values (blueness). Regarding the visual appearance of the extracts, a lower S:CA ratio resulted in a bluer extract, which correlates with the higher C-PC concentration (mg/mL) achieved when using a smaller solvent volume (Fig. 6B).

The proximate composition of SAC beads is shown in Fig. 7A. For comparison purposes, the one of Spirulina biomass was also added. The SAC beads resulting from PC2 and PC4 conditions, respectively SAC2 and SAC4, presented a similar proximate composition. The SAC beads exhibited higher ash content than the initial Spirulina biomass due to calcium salts, with statistically significant differences. SAC4 had slightly higher ash content (27.5 ± 0.42 g/100 g d.w.) than SAC2 (26.8 ± 0.2 g/100 g d.w.). Additionally, the beads contained higher fat content. Despite the extraction of C-phycocyanin, substantial protein content remained in the SAC beads (> 25 g/100 g d.w.), which was statistically significantly lower (p-value ≤ 0.05) than in the original Spirulina biomass (54.3 ± 0.92 g/100 g d.w.). Given the significant proximate composition of the SAC beads (SAC2 and SAC4), they can be utilized as a food supplement, thereby completing the C-PC production chain. Alternatively, they can be further processed to extract the remaining

Table 1
Kinetic parameters of the C-PC extraction from the best PC2 and PC4 extract conditions.

C-PC extract	S:CA ratio	Slope	C_S = (1 / Slope)	C_L (24 h)	Intercept	R_i = (1 / Intercept)	$k = (R_i / C_S^2)$	R^2
PC2_B	1:83	1.07	0.94	0.86	2.54	0.39	0.45	0.99
PC4_B	1:42	0.56	1.77	1.47	2.15	0.46	0.15	0.98

C_s: mg/mL; Ri = mg/mL·h; k = (mL/mg·h).

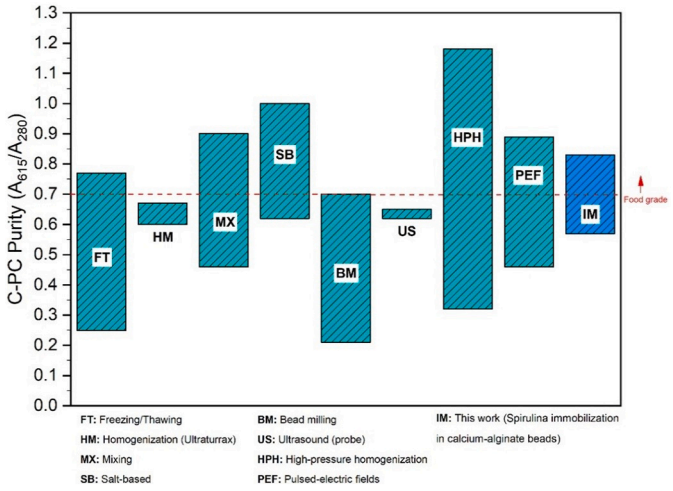

Fig. 5. General comparison of C-PC purity obtained from commonly applied extraction methods. The purities values were retrieved from the cited references: FT: [14,40]; HM: [20,41]; MX: [30,37]; SB: [29,32]; BM: [35,42]; US: [20,37]; HPH: [15,34]; PEF: [35,36]. For complete information, the authors encourage verifying Table S1 (Supplementary material).

lipid and protein fractions, conforming to the cascade principle [43]. From a biorefinery perspective, this approach maximizes resource utilization and minimizes waste, contributing to a more sustainable production process.

The average size of the SAC beads was measured immediately after extract separation (Fig. 7B.1 and B.2). Both beads exhibited a dark green color and similar sizes, with SAC4 (3.0 ± 0.20 mm) being statistically larger than SAC2 (2.4 ± 0.32 mm). Moreover, SAC4 beads were more rigid and spheric than SAC2 owing to the higher alginate concentration used, conforming to the results reported in other studies [31].

4. Conclusions

This study demonstrated the effectiveness of using Spirulina biomass (S) immobilized in calcium-alginate (SAC) beads for C-phycocyanin (C-PC) extraction, targeting a food-grade product. The results highlight the effect of calcium (CA) concentration and S:CA ratio on C-PC yield, efficiency, and purity. The S:CA ratio significantly influenced extraction yield and efficiency, while CA concentration affected purity levels. Extracts with 4 % CA (PC4) consistently achieved food-grade purity (≥ 0.7), surpassing the purity levels of 2 % CA extracts (PC2). However, PC2 extracts yielded higher C-PC yields and efficiencies. The highest purity (0.83) was achieved with PC4 at a 1:42 S:CA ratio, yielding 61.4 $mg/g_{biomass}$ at 55.1 % efficiency. The maximum yield (70.9 $mg/g_{biomass}$) and efficiency (67.2 %) were obtained with PC2 at a 1:125 S:CA ratio, though at lower purity (0.57). Kinetic analysis indicated that C-PC extraction was faster with PC2, although PC4 maintained higher purities over time. Significant C-PC yields (>30 $mg/g_{biomass}$) were achieved within 4 h for both PC2 and PC4. However, food-grade purities (≥0.7) were attained only with PC4 after 14 h, while PC2 was always below the food-grade threshold.

Immobilizing Spirulina in calcium-alginate beads resulted in C-PC purities comparable to or higher than those achieved with conventional and emerging technologies. Although further optimization might be needed to reduce extraction time, this method simplifies the separation of the liquid extract from the spent biomass, eliminating energy-intensive centrifugation and reducing extraction costs. From a biorefinery perspective, this approach maximizes resource efficiency and minimizes waste by leveraging bead valorization (e.g., as a nutritional supplement) and considering the potential for cascade extraction of the remaining proteins and lipids in the beads, thereby supporting a sustainable and economically viable C-PC production process. A more detailed bead characterization could enhance their potential and further consolidate the developed concept, including functional properties (e.g., antioxidant, anti-inflammatory, and anticancer activities), digestibility, and lipid and protein profiles. In addition, a techno-economic analysis

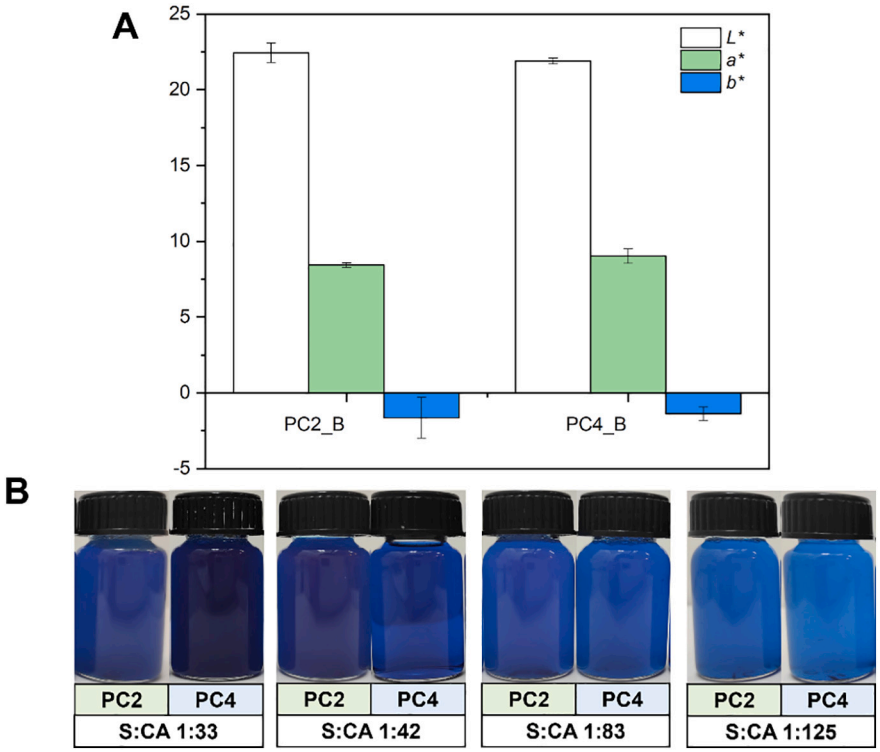

Fig. 6. (A) Colorimetric parameters (L^*, a^* and b^*) of the PC2 and PC4 extracts obtained by Spirulina immobilization in calcium-alginate beads and (B) visual appearance of the C-PC extracts for both PC4 (4 % of CA) and PC2 (2 % of CA) over different S:CA ratio (w:v) after 24 h of extraction.

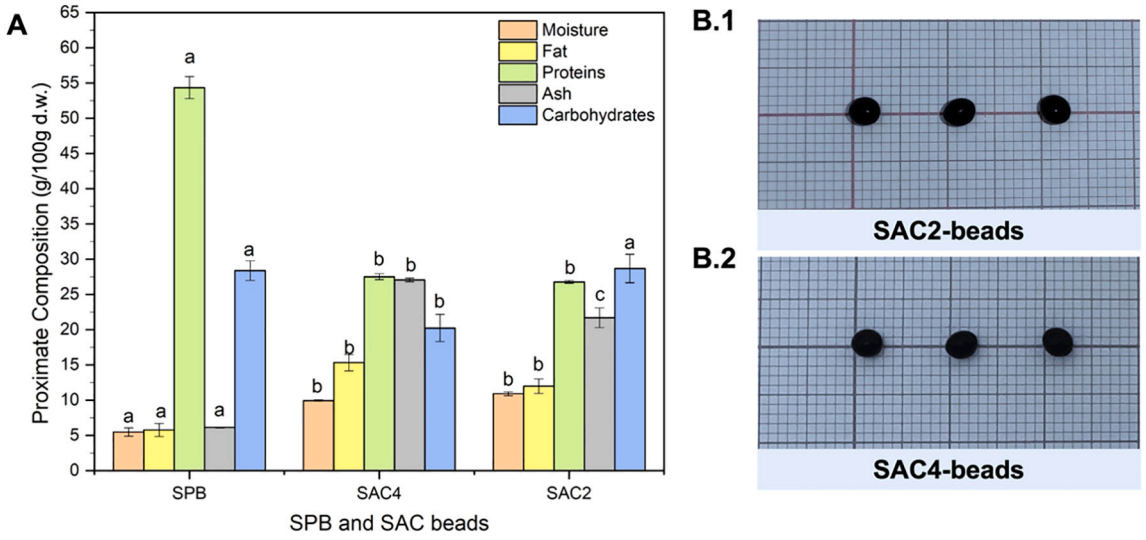

Fig. 7. Proximate composition (g/100 g d.w.) of the Spirulina biomass and SAC beads (A) and the average size of the SAC2 (B.1) and SAC4 (B.2) beads. Different letters indicate significant differences ($p \leq 0.05$).

could play a crucial role in strengthening commercial viability by evaluating cost-effectiveness and scalability.

CRediT authorship contribution statement

Samara C. Silva-Pituco: Writing – original draft, Methodology, Investigation, Formal analysis, Conceptualization. **Leandro L. Aquino:** Investigation, Formal Analysis, Writing – review & editing. **Madalena M. Dias:** Writing – review & editing, Supervision, Conceptualization. **M. Filomena Barreiro:** Writing – review & editing, Supervision, Resources, Conceptualization.

Author statement

The authors declare that this manuscript or a very similar manuscript has not been published, nor is under consideration by any other journal.

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Declaration of competing interest

The authors declare no competing interests.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.algal.2025.103916.

Data availability

Data will be made available on request.

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