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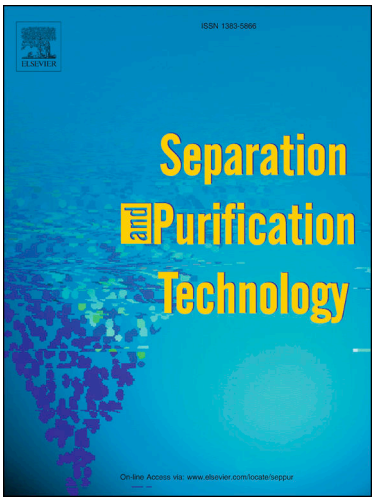

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Sequential recovery of C-phycocyanin and chlorophylls from *Anabaena cylindrica*

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ABSTRACT

Cyanobacteria are attracting the attention worldwide as a reliable and sustainable feedstock for the production of biofuels, food colorants and biochemically active compounds. *Anabaena cylindrica* (*A. cylindrica*) is a cyanobacterium with a significant widespread occurrence in Portuguese freshwaters, with phycocyanin and chlorophylls, compounds with a high market value, being abundantly produced by the species. This work presents the development of a sequential downstream process to obtain C-phycocyanin and chlorophyll from *A. cylindrica*. The C-phycocyanin extraction is carried using Na-phosphate buffer, while aqueous solutions of surface-active compounds (250 mM), as well as ethanol, were screened for the extraction of the chlorophylls. After selecting the best solvents to recover both pigments from *A. cylindrica*, the operational conditions (solid-liquid ratio, time of extraction and temperature) were optimized, allowing the recovery of around 90% and 55% of C-phycocyanin and chlorophylls, respectively. Finally, dextran/copolymer-based ABS were applied to promote the purification of C-phycocyanin from the residual chlorophylls and other contaminant proteins, leading to an increase in the C-phycocyanin purity of 4-fold, without compromising its photostability.

KEYWORDS: Cyanobacteria; *Anabaena cylindrica*; C-phycocyanin; chlorophylls; sequential extraction; purification; aqueous biphasic systems; stability

1. INTRODUCTION

Cyanobacteria are prokaryotic photosynthetic microorganisms harmful for the aquatic environment. Their photosynthetic apparatus is formed by three principal light-harvesting systems, two main photosystems and one phycobilisome. The phycobilisome is composed of phycobiliproteins, which can represent up to 50% of the total cellular protein in cyanobacteria [1]. This class of proteins has been attracting the attention of academia and industry because of their potential application in different fields, such as nutraceutical, pharmaceutical, food, feed and cosmetics [2], being C-phycocyanin the most abundant in cyanobacteria. Besides the protein content, cyanobacteria are also composed of carbohydrates, pigments (e.g. chlorophylls and carotenoids), lipids and fatty acids [3,4].

Anabaena cylindrica is a widespread native cyanobacterium frequently found worldwide and particularly in Portuguese freshwaters [4,5]. This filamentous cyanobacterium belongs to a genus known for its ability to fix atmospheric nitrogen and produce oxygen using the enzyme nitrogenase [6]. It has the ability to adapt its pigment composition to the environmental conditions, with C-phycocyanin and chlorophylls being produced abundantly [7]. C-phycocyanin is a water-soluble fluorescent phycobiliprotein with a variety of bioactivities, which can be used as an effective ingredient of food additives, healthcare, food, cosmetics, and pharmaceuticals [8–10]. Chlorophylls have been extensively studied as natural colorants in the food and cosmetic industries and in energy and medicinal applications [11], being currently approved by EU as food dyes (E140 and E141) [12]. However, the use of *A. cylindrica* as a source of these biocompounds has attracted little attention despite its potential.

Several methods and procedures have been used to isolate C-phycocyanin from cyanobacteria and microalgae combining the disruption of cell walls and the extraction of the water-soluble phycobiliproteins into aqueous media [13,14]. The extraction of C-

phycocyanin from fresh cyanobacteria is commonly performed by freezing and thawing, followed by a solid-liquid extraction using a phosphate buffer [13,15]. More recently, other strategies have been reported combining both ultrasonication and freezing/thawing, resulting in a higher extraction efficiency of C-phycocyanin from dried *Spirulina* sp. biomass [16,17]. Microwave-assisted extraction has also been used to improve the extraction efficiency and purity of C-phycocyanin [14,18].

The commercial applications of C-phycocyanin depend on its purity, which is evaluated based on the ratio between absorbance at 620 (A_{620}) and 280 nm (A_{280}). For purity ratios (A_{620}/A_{280}) of 0.7, C-phycocyanin is considered as of food grade with market prices around \$0.13 USD *per* mg, while purity ratios of 3.9 and greater than 4.0 are considered as of reactive and analytical grade[13], with market prices reaching \$15 USD *per* mg [19]. In this sense and, considering not only the demands of the application but also the relation price - purity, purification strategies need further development. In literature, the purification procedures for C-phycocyanin from cyanobacteria usually involve a high number of steps including precipitation, centrifugation and dialysis, followed by ion-exchange chromatography, gel filtration chromatography and ultrafiltration as final purification unit [1,8]. However, and despite the large number of steps that leads to low yields, the scale-up of these methods has been also reported as an expensive and difficult task.

Aqueous biphasic systems (ABS) have been emerging as an alternative method of purification [20]. Beyond its simple and fast operation, ABS afford an economic, scalable and biocompatible environment for molecules' isolation. Polymer-based ABS have been employed in the separation of distinct proteins [21–23]. One of the most common polymer/polymer ABS is formed by polyethylene glycol (PEG) and dextran, the latter being a biodegradable and biocompatible biopolymer [24]. Nevertheless, conventional

polymer/polymer systems display limited polarity differences between the phases, which limits their performance in separation processes. The use of copolymers constitutes one of the alternative strategies to overcome this obstacle. The PEG-PPG copolymer (random or block forms) has been widely used in the formation of ABS due to its ability to tune the phase polarity by changing the PPG/PEG ratio, as well as its thermoresponsive properties [25–28]. Patil et al.[29] have demonstrated the ability of ABS composed of polymer/salt to purify the phycocyanin in single (purity index of 3.52) and multiple extractions (purity index of 4.05) with an overall yield of 85%. More recently, Chang and co-workers showed that ionic liquid-based ABS may be also efficient for the purification of C-phycocyanin from *Spirulina* sp. [30], even in continuous-operation extractors [31].

The development of a cost-effective process able to work on a multi-product scenario remains a challenge for biorefinery. As previously mentioned, besides C-phycocyanin, *A. cylindrica* is also able to produce chlorophylls in abundance. Chlorophyll is a chlorin that contains a long hydrophobic tail, consequently with lower affinity for hydrophilic solvents. Among the approaches employed to extract chlorophylls, organic solvents are the most employed due to their higher hydrophobicity, simplicity and efficiency [32]. Commonly, the extraction process involves the organic solvent penetrating through the cell membrane and dissolving the lipids as well as the lipoproteins of the chloroplast membranes. In that sense, the success of this extraction method depends on the degree of cell disruption that can be achieved. Methanol, ethanol and acetone are some of the most studied solvents for chlorophyll extraction, the first two being considered better than acetone. In that case, cell disruption is usually achieved through grinding, homogenisation or sonication [32]. Alternatively, aqueous solutions of surfactants were proposed to extract chlorophylls from spinach leaves [33] and from the cyanobacteria *Synechocystis* sp. [34], since they can disrupt the cell membrane and help extracting hydrophobic

components from the inner cell. Due to their high solvation capability, ionic liquids (ILs) have been used in aqueous solutions as promising solvents to distinct biomolecules [35]. In addition, these solvents present widely tunable properties that can be adjusted to suit the requirements of a particular application. Some ILs (typically containing long alkyl chain substituents) are also tensioactive, being termed as surface-active ILs. In that sense, the use of these compounds could be a useful strategy to improve the biorefinery concept within the context of the present study.

The main goal of this work is to establish a sustainable and sequential process to extract both C-phycocyanin and chlorophylls from *A. cylindrica*, followed by the application of ABS to improve the purification of C-phycocyanin. For its extraction, the use of Na-phosphate buffer is studied. To further valorize the biomass, a second extraction step was suggested to recover the chlorophylls. In addition, the operational conditions (solid-liquid ratio (SLR), time of extraction and temperature) were optimized to develop a more efficient strategy to sequentially extract C-phycocyanin and chlorophylls. Finally, ABS based on copolymers and dextran were applied to improve the purity and stability of C-phycocyanin. In the end, a sequential process to recover two valuable pigments from the same biomass source was proposed.

2. EXPERIMENTAL SECTION

2.1. Materials

The solvents ethanol (analytical reagent grade) and ethyl acetate (99 wt% of purity) were supplied by Fisher Scientific and Carlo Herba, respectively. Sodium-phosphate buffer was prepared using sodium phosphate dibasic, Na_2HPO_4 (98 wt% of purity) and sodium phosphate monobasic, NaH_2PO_4 (99 wt% of purity), purchased from Sigma-Aldrich and Panreac, respectively. Included in the ILs studied to extract chlorophylls are

the 1-alkyl-3-methylimidazolium chloride series, [$C_n$mim]Cl, including the 1-decyl-3-methylimidazolium chloride, [C_{10}mim]Cl (98 wt% of purity), 1-dodecyl-3-methylimidazolium chloride, [C_{12}mim]Cl (98 wt% of purity) and 1-methyl-3-tetradecylimidazolium chloride, [C_{14}mim]Cl (98 wt% of purity), acquired from IoLiTec. Tributyltetradecylphosphonium chloride, [$P_{4,4,4,14}$]Cl (95 wt% of purity) was supplied by IoLiTec. Trimethyltetradecylammonium bromide, [$N_{1,1,1,14}$]Br (98 wt% of purity) and dodecyltrimethylammonium bromide, [$N_{1,1,1,12}$]Br (99 wt% of purity) were purchased from Alfa Aesar. The anionic surfactant, sodium dodecyl sulfate, SDS (99 wt% of purity), and non-ionic surfactants, polyoxyethylene sorbitan monolaurate, Tween 20 (lab grade) and polyethylene glycol tert-octylphenyl ether, Triton X-114 (lab grade), were acquired from Acros Organics. The chemical structure and acronyms of the surface-active compounds studied in this work are depicted in Figure 1. For the preparation of ABS, Dextran T6 (Mw around 6000 Da) and Dextran T40 (Mw around 40000 Da), Pluronic L35, PEG-ran-PPG monobutyl ether (PPB) and PEG-ran-PPG-ran-PEG (UCON) were acquired from Sigma. Pluronic PE 6400 (PO-EO-block polymer with approximately 40% polyethylene glycol in molecule) was purchased from BASF.

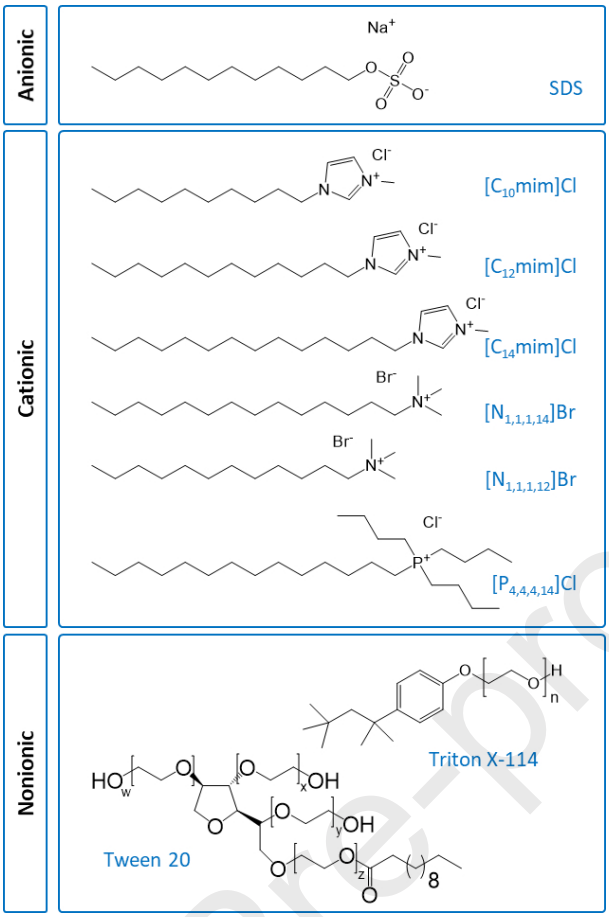

Figure 1. Chemical structure and acronyms of the surface-active compounds studied in this work.

2.2. Methods

Cyanobacteria cultivation

The cyanobacterium *A. cylindrica* was maintained in Erlenmeyer flasks (50 mL) in an incubator chamber at (20 ± 2) °C under 16:8 light/dark cycles with light intensity of 130 $\mu mol_{PAR} \cdot m^{-2} \cdot s^{-1}$, provided by cool white fluorescent tubes. The culture was grown in Woods Hole MBL culture medium [36]. The culture media components were sterilized together with the cultivation apparatus (30 min, 120 °C, 1 atm), except for vitamins, which were added posteriorly. For culture growth purposes, a 13-day inoculum was used to inoculate 4 L of MBL culture medium in 5 L glass vessels (Schott Duran). This culture

was incubated under the same conditions as the inoculum for a batch period of 13 days. Agitation was provided by sterile aeration. At the end of the batch culture period, the cells were harvested by self- sedimentation and soft centrifugation at 4000 rpm for 7 min at 4 °C, and then the supernatant was discarded. The fresh biomass was stored at -20 °C in dark for further processing.

Solid-liquid extraction

The methodology used in this work for the C-phycocyanin extraction was based on the work previously developed by Sarada and coworkers [37]. Briefly, the fresh biomass was thawed and homogenized in Na-phosphate buffer aqueous solutions at 20 mM and pH 7.0 (SLR of 0.1) and placed in an Eppendorf Thermomixer Comfort equipment for 45 min, at 25 °C and 1500 rpm. During the solid–liquid extraction step, all samples were protected from light exposure due to the high photosensitivity of the pigments. Then, the solution was centrifuged in a Thermo Scientific Heraeus Megafuge 16 R centrifuge at 12000 rpm for 10 min. The C-phycocyanin-rich supernatant was collected (C-phycocyanin crude extract) and its amount quantified using a UV-Vis microplate reader (Synergy HT microplate reader – BioTek) at 620 nm (calibration curve in the Table S1 in ESI). Aqueous solutions of distinct surface-active compounds (250 mM), as well as ethanol, were screened for the extraction of chlorophylls from the biomass pellet obtained from the C-phycocyanin extraction. The screening was performed at a SLR of 0.1 and 25 °C for 51 min, and in the absence of light. The quantification of chlorophylls was determined using a UV-Vis microplate reader at 670 nm (calibration curve in the Table S1 in ESI). Included in the set of surface-active compounds, cationic, anionic and non-ionic were tested, these allowing to study the structural impact of different alkyl chain lengths. Different solutions of ethanol, namely ethanol 100 %, ethanol 75 % and ethanol 50 %,

were prepared to study the effect of ethanol/water ratio in the extraction performance. All the conditions were tested in duplicate. The yields of extraction of C-phycocyanin and chlorophylls were expressed in $mg_{phycocyanin} \cdot g_{fresh\ biomass}^{-1}$ and $mg_{chlorophyll} \cdot g_{fresh\ biomass}^{-1}$, respectively. The purity index of C-phycocyanin was evaluated based on the ratio between absorbance at 620 nm (A_{620}) and at 280 nm that represents the total amount of proteins (A_{280}), as described by Equation 1 [8].

$$purity\ index = \frac{A_{620}}{A_{280}} \qquad (1)$$

Optimization of the extraction conditions for C-phycocyanin and chlorophylls: Response Surface Methodology (RSM)

The operational conditions of SLR, time of extraction and temperature were optimized aiming to develop a more efficient strategy to extract sequentially C-phycocyanin and chlorophylls. These assays were performed using Na-phosphate buffer (20 mM, pH 7.0) for the extraction optimization of C-phycocyanin. Moreover, and after the selection of the best solvent to extract chlorophyll, the optimization of the process variables (SLR, extraction time and temperature) was performed. These conditions were simultaneously analysed by a Response Surface Methodology (RSM) [38]. This methodology allows to conduct a single experimental design considering the simultaneous study of the effect of different extraction parameters. The agitation was maintained at 1500 rpm and all the assays were conducted protected from light. These extractions were performed using samples from the same batch produced to eliminate the errors associated. Through an experimental design of 2^3 factorial, a total of twenty experiments were carried out, with six replicates at the central point (Table S2 and S3 in ESI).

The results obtained were statistically analyzed considering a confidence level of 95%. The Statsoft Statistica 8.0© software Statsoft© was applied in the statistical analysis and

preparation of the response surface plots. The surface responses were plotted considering the changes in two variables within the experimental range. After analyzing the RSM results, the best conditions for the pigment' extraction were estimated and experimentally validated.

Phase diagrams and tie-lines

Binodal data were determined by cloud-point titration at (25.0 ± 0.5)°C and atmospheric pressure following methods previously described [39]. Briefly, aqueous solutions of dextran (Dextran T6 at 50 wt% and Dextran T40 at 40 wt%) and aqueous solutions of the different copolymers from 60 to 80 wt% were prepared to determine the binodal curves. Each point of the binodal curve was determined by weight (±10^{-4} g). The experimental solubility curves were fitted by the equation originally described by Merchuk et al. [40]:

$$[copolymer] = A \exp\left[\left(B\,[dextran]^{0.5}\right) - \left(C\,[dextran]^{3}\right)\right] \quad (2)$$

where A, B and C are constants obtained by the regression of the experimental binodal data, and [copolymer] and [dextran] correspond to the copolymer and dextran weight fraction percentages, respectively.

The tie-lines (TLs) associated with each binodal curve were determined by a gravimetric method [40]. Different initial mixture compositions (copolymer + dextran + water) at the biphasic region were gravimetrically prepared (±10^{-4} g), vigorously stirred, and allowed to reach the equilibrium by the separation of the two phases for at least 24 h at (25.0 ± 0.5) °C. After the phase separation, both top and bottom phases were separated and weighted. Each TL was determined by the lever-arm rule [40] according to Equations 3−6:

$$[copolymer]_T = A \exp\left[\left(B\,[dextran]_T^{0.5}\right) - \left(C\,[dextran]_T^{3}\right)\right] \quad (3)$$

$$[copolymer]_B = A \exp\left[\left(B\,[dextran]_B^{0.5}\right) - \left(C\,[dextran]_B^3\right)\right] \quad (4)$$

$$[copolymer]_T = \frac{[copolymer]_M}{\alpha} - \frac{1-\alpha}{\alpha}[copolymer]_B \quad (5)$$

$$[dextran]_B = \frac{[dextran]_M}{\alpha} - \frac{1-\alpha}{\alpha}[dextran]_B \quad (6)$$

where the subscripts M, T, and B represent the initial mixture and the top and bottom phases, respectively. The α corresponds to the ratio between the weight of the top phase and the total mass of the system. In all systems, the top phase is enriched in copolymer (copolymer-rich phase), whereas the dextran is majorly enriched in the bottom phase. Each TL length (TLL) was determined by the following equation:

$$TLL = \sqrt{([dextran]_T - [dextran]_B)^2 + ([copolymer]_T - [copolymer]_B)^2} \quad (7)$$

C-phycocyanin and chlorophylls partition

In this work, the purification of both classes of pigments was tested by applying ABS. The success of this step was evaluated through the partition coefficient and selectivity. The purity level of C-phycocyanin was determined by using the Equation 1. The partition coefficient of C-phycocyanin ($K_{C\text{-}phycocyanin}$) was calculated as the ratio between the amount of C-phycocyanin present in the top and bottom phases, as described in Equation 8. The partition coefficient of chlorophylls ($K_{chlorophylls}$) and total proteins ($K_{total\ proteins}$) was determined in the same way.

$$K_{phycocyanin} = \frac{[C-phycocyanin]_T}{[C-phycocyanin]_B} \quad (8)$$

The selectivity ($S_{phycocyanin/total\ proteins}$ and $S_{phycocyanin/chlorophylls}$) of the ABS under study was determined according to Equations 9 and 10:

$$S_{phycocyanin/total\ proteins} = \frac{K_{C-phycocyanin}}{K_{total\ proteins}} \quad (9)$$

$$S_{phycocyanin/chlorophylls} = \frac{K_{C-phycocyanin}}{K_{chlorophylls}} \quad (10)$$

C-phycocyanin stability

The photostability of the C-phycocyanin was evaluated considering the system promoting the highest values of purification. For that, the relative concentration of C-phycocyanin (C_R, %) i.e., the remaining concentration of C-phycocyanin as a percentage of its initial concentration in the sample, was determined by Equation 11:

$$C_R = \frac{C}{C_0} \times 100 \quad (11)$$

where C and C_0 are the concentrations of C-phycocyanin at time t and the beginning of assays, respectively.

3. RESULTS AND DISCUSSION

3.1. Sequential extraction of C-phycocyanin and chlorophylls

The aim of this work is to develop a sequential extraction to obtain C-phycocyanin and chlorophylls from *A. cylindrica*. For the extraction of C-phycocyanin, Na-phosphate buffer will be used as a traditional/control solvent extracting proteins [41]. Based on literature, fresh *A. cylindrica* was thawed and homogenized in Na-phosphate buffer aqueous solutions at 20 mM (pH 7.0), with a SLR of 0.1, for 45 min, at 25°C and 1500 rpm. The yield of C-phycocyanin extraction obtained was 67 $mg_{phycocyanin} \cdot g_{fresh\ biomass}^{-1}$. To improve the extraction yield, the operational conditions were optimized, namely temperature [(16.6 - 43.4) ± 0.5 °C], extraction time (11.5 – 48.0 min) and SLR (0.05 - 0.15 $g_{fresh\ biomass} \cdot mL_{solvent}^{-1}$) by applying a Response Surface Methodology. The yield of extraction of C-phycocyanin was used as the dependent variable in the definition of the predictive model represented by Equation 12:

$$(Y_1)_{\ yield_{phyco.\ extraction}} = -89.05 + 5.96(X_1) + 0.81(X_3) - 0.07(X_1)^2 - 508.48(X_2)^2 \quad (12)$$

where X_1, X_2 and X_3 represent the independent variables, temperature, SLR and extraction time, respectively. The response surface plots are represented in Figure 2, with the yields of extraction of C-phycocyanin shown in Table S2 from ESI.

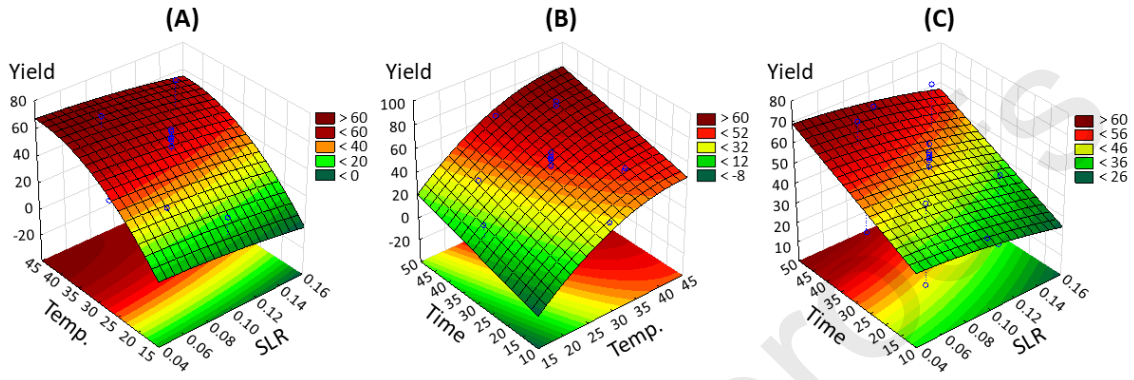

Figure 2. Response surface plots showing the yield of extraction of C-phycocyanin ($mg_{phycocyanin} \cdot g_{fresh\ biomass}^{-1}$) with the combined effects of (A) Temperature and SLR; (B) Extraction time and Temperature; (C) Extraction time and SLR. Temperature, SLR and extraction time are in °C, $g_{fresh\ biomass} \cdot mL_{solvent}^{-1}$ and min, respectively.

The accuracy and precision of the equations were validated by comparison of the experimental and predicted values of the yield of extraction under the conditions previously selected. The coefficient of determination obtained was 0.88, a high value when considering the use of fresh biomass. No significant differences (ANOVA *p*-value = 0.05) were observed between the theoretical and experimental responses as shown in Figure S1 from ESI. Temperature was the independent variable with higher impact on the C-phycocyanin extraction yield (Figure 2). Its yield of extraction increases with the temperature (Figure 2, A and B), reaching its maximum around the highest temperature tested. Nevertheless, the temperature also plays an important role in the stability of C-phycocyanin, being reported a dramatically increase on its degradation for temperatures

above 40°C [42]. The same behavior was observed for extraction time (Figure 2, B and C), with the best extraction yield obtained between 40 and 50 min. Regarding the SLR (Figure 2, A and C), the results showed a negligible impact of this independent variable on the yield of extraction. The responses observed in Figure 2 are also supported by the Pareto chart shown in Figure S2 from ESI. Considering the results obtained, the model was further validated using the selected conditions (SLR of 0.1 at 35 °C for 45 min). The experimental value of the extraction yield under these conditions was 63 ± 1 $mg_{phycocyanin} \cdot g_{fresh\ biomass}^{-1}$, which corresponds to 3.3% of deviation from the theoretical value, evidencing the high confidence and accuracy of the model.

Using the Na-phosphate buffer, it was possible to recover around 90% of the content of C-phycocyanin present with a purity index of 0.521 ± 0.003 (Equation 1): this was achieved using a much shorter time of extraction (45 min) than the ~48 h reported in literature (87% of phycocyanin extracted from cells of *Synechococcus bacillaris* applying a centrifugation, a freeze–thaw cycle and sonication) [43].

After the extraction of C-phycocyanin, the same biomass samples were subjected to another step of solid-liquid extraction to recover the chlorophylls. For that purpose, aqueous solutions of ten ILs and surfactants were investigated, where different surface-active compounds (cationic and anionic, and non-ionic) and ethanol (used as control at three different concentrations) were used. This screening was performed under the following conditions: SLR of 0.1, extraction time of 45 min, at (25.0 ± 0.5) °C, and in the absence of light, also due to the photostability of chlorophylls. The concentration of aqueous solutions of tensioactive compounds was fixed at 250 mM.

In this work, distinct structures of surface-active ILs were evaluated on the extraction of chlorophylls, namely imidazolium ($[C_{10}mim]Cl$, $[C_{12}mim]Cl$ and $[C_{14}mim]Cl$), phosphonium ($[P_{4,4,4,14}]Cl$) and ammonium ($[N_{1,1,1,12}]Br$ and $N_{1,1,1,14}]Br$). Among the ILs

studied, the most hydrophobic IL, the $[P_{4,4,4,14}]Cl$ [44] was the most successful extracting chlorophylls, with a yield of extraction of 0.5 ± 0.1 $mg_{chlorophyll} \cdot g_{fresh\ biomass}^{-1}$. From $[C_{10}mim]Cl$ to $[C_{14}mim]Cl$ and $[N_{1,1,1,12}]Br$ to $[N_{1,1,1,14}]Br$ there is an increase in the yield of extraction of chlorophylls, which is explainable by the increase of hydrophobicity when the alkyl chain is elongated (Figure 3).

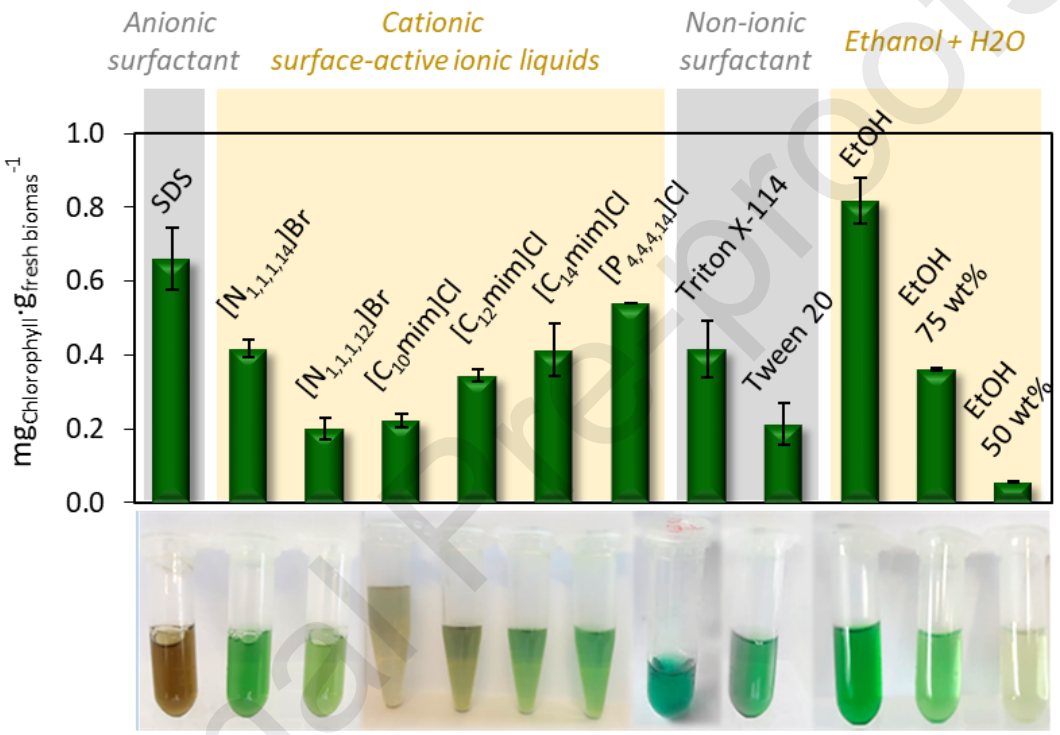

Figure 3. Yield of extraction of chlorophylls from the biomass pellet obtained after the C-phycocyanin recovery using distinct surface-active compounds (250 mM) and ethanol at different concentrations (pure, 75 wt% and 50 wt%).

The effects of Tween 20, Triton X-114 and SDS were also tested. Some of these surfactants have been reported to efficiently extract hydrophobic compounds like chlorophylls and carotenoids from various sources [45]. Both non-ionic surfactants studied (Tween 20 and Triton X-114) presented low yields of extraction (around 0.21 ±

0.06 $mg_{chlorophyll}.g_{fresh\ biomass}^{-1}$ and 0.42 ± 0.08 $mg_{chlorophyll}.g_{fresh\ biomass}^{-1}$, respectively), while the anionic surfactant (SDS) showed a higher yield of extraction of 0.66 ± 0.08 $mg_{chlorophyll}.g_{fresh\ biomass}^{-1}$. Nevertheless, according to Figure 3, the crude extract presents a brown color that may be indicative of a high percentage of carotenoids present in the extract, suggesting that SDS can be an important extractive solvent to recover carotenoids, or at least to recover the most hydrophobic pigments present in this cyanobacteria. In addition, the brown colour may also be related with some extent of chlorophyll degradation. In this sense, the conventional extraction of chlorophylls using absolute ethanol appears as the best system among those investigated (0.82 ± 0.06 $mg_{chlorophyll}.g_{fresh\ biomass}^{-1}$), allowing the extraction of 40% and 20% more than $[P_{4,4,4,14}]Cl$ and SDS, respectively. Thus, absolute ethanol was selected for further optimization tests towards more effective extraction of chlorophylls. For that, a RSM was applied considering the extraction time (11.4 - 68.6 min), SLR (0.05 - 0.15 $g_{fresh\ biomass}{\cdot}mL_{solvent}^{-1}$) and temperature (14.9 - 35.1°C). The yield of extraction of chlorophyll was used as the dependent variable in the definition of the predictive model represented by Equation 13:

$$(Y_1)_{\ yield_{chloro.\ extraction}} = -3.80 + 0.23(X_1) + 31.60(X_2) - 0.004(X_1)^2 - 121.59(X_2)^2 \quad (13)$$

The analysis of variance (ANOVA) and the Pareto chart are shown in ESI, namely in Figures S3 and S4. Again, the accuracy and precision of the model equations were validated by comparison of the experimental and the predicted values of the extraction yield under the conditions previously selected. No significant differences (ANOVA *p*-value = 0.05) were observed between the theoretical and experimental responses as shown in Figure S4 from ESI.

Temperature and SLR were the independent variables with the highest influence on the extraction of chlorophylls (Figure 4), which is also demonstrated in the Pareto chart shown in Figure S3 from ESI. A parabolic response achieving a plateau is observed for

the combined effects of temperature and SLR (Figure 4 A). A maximum extraction yield was observed for temperatures between 24.0 and 30.0 (± 0.5 °C), as shown in Figure 4, A and B. Regarding the SLR, the best performance was found between 0.12 and 0.15 $g_{fresh\ biomass} \cdot mL_{solvent}^{-1}$ (Figure 4, A and C). The results showed a negligible impact of time of extraction on the yield of extraction, from 11.4 to 68.6 min (Figure 4, B and C).

Considering the obtained results, the model was further validated using selected conditions (SLR of 0.13 at 25°C for 45 min). The experimental value of the yield of extraction under these conditions was 1.46 ± 0.06 $mg_{chlorophyll} \cdot g_{fresh\ biomass}^{-1}$, corresponding to a relative deviation of 2.9% to the theoretical value, thus evidencing the high confidence and accuracy of the model. In the end, around 54.7% of the content of chlorophylls present in the biomass was efficiently recovered in just one step.

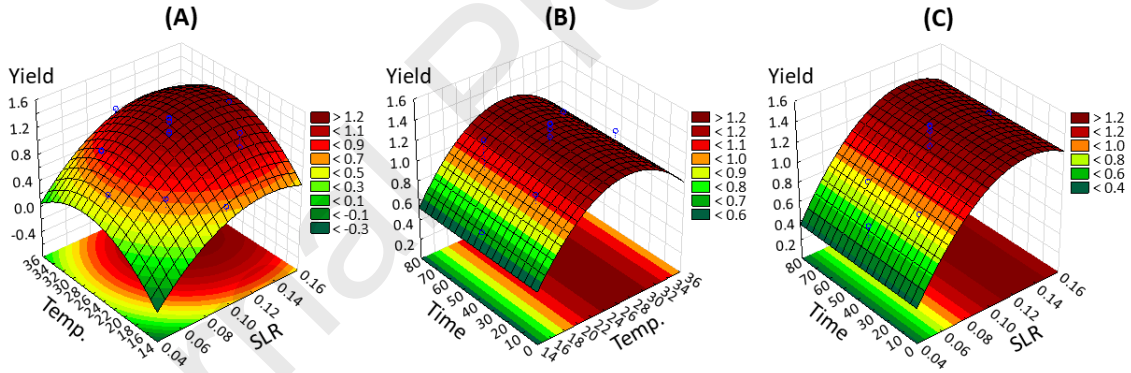

Figure 4. Response surface plots showing the Yield of extraction of chlorophyll ($mg_{chlorophyll} \cdot g_{fresh\ biomass}^{-1}$) with the combined effects of (A) Temperature and SLR; (B) Extraction time and Temperature; (C) Extraction time and SLR. Temperature, SLR and extraction time are in °C, $g_{fresh\ biomass} \cdot mL_{solvent}^{-1}$ and min, respectively.

3.2. Purification of C-phycocyanin using ABS

In the previous section, the extraction of chlorophylls and C-phycocyanin from the cyanobacteria cells was optimized. However, further purification optimization is required to improve the purity index of C-phycocyanin (up from the value of 0.521). The presence of contaminant proteins and some content of chlorophylls was demonstrated. The purification platforms selected ally the biocompatibility of dextran and the appropriate hydrophobic/hydrophilic balance. Thus, ABS based on copolymers and dextran were applied. In this work, the systems composed of copolymers (Pluronic PE 6400, Pluronic L35, PEG-ran-PPG monobutyl ether and PEG-ran-PPG) and dextran (T6 and T40, with Mw around 6000 and 40000 Da, respectively), were investigated to purify C-phycocyanin (see more details in Table S4 from ESI and the parameters of the Merchuk equation fitting in literature [46]). The partition coefficients of C-phycocyanin, chlorophylls and total protein, as well as the respective selectivity and purity of C-phycocyanin are depicted in Table 1. To evaluate the influence of dextran molecular weight, as well as the impact of the copolymer composition on the partition and purification of C-phycocyanin, distinct mixture points were prepared and studied (Table 1). Considering the Dextran T40-based ABS, the preferential partition of C-phycocyanin for the copolymer-rich phase was observed, with the system composed of copolymer PPb being the exception. Considering the hydrophilic nature of this fluorescent protein, these results can be explained by the higher hydrophobic character of the copolymer PPb, which leads to a similar partition of C-phycocyanin between the two phases. The opposite behavior was observed for dextran T6-based ABS. This preferential partition of C-phycocyanin for the dextran-rich phase is in good agreement with the reduction of the hydrophobicity of that phase due to lower dextran molecular weight. The results clearly demonstrate the ability of the dextran/copolymer-based ABS to enhance the purity of C-phycocyanin.

Table 1. Mass fraction composition of each mixture point tested, partition coefficient of C-phycocyanin ($K_{phyc.}$), chlorophylls ($K_{chlor.}$) and total protein (K_{TP}), selectivity data ($S_{phyc/chlor}$ and $S_{phyc/TP}$) and purity index of C-phycocyanin in top and bottom phases. The maximum deviation associated to purity data is ± 5%.

		wt% $_{dextran}$	wt% $_{copolymer}$	K_{phyc}	$K_{chlor.}$	K_{TP}	$S_{phyc/chlor}$	$S_{phyc/TP}$	$Purity_{TOP}$	$Purity_{Bot}$
Dextran T40	**PE 6400**	10.10	7.26	2.43	15.88	3.05	6.53	1.25	0.52	0.59
	L35	10.00	9.98	3.36	1.52	0.85	2.21	3.93	0.67	0.17
	PPb	6.86	6.56	0.65	0.84	0.83	1.30	1.28	0.47	0.60
	UCON	9.96	8.03	2.23	7.71	6.53	3.46	2.93	0.61	1.80
Dextran T6	**PE 6400**	19.52	15.16	0.04	2.35	0.07	61.92	0.07	0.11	1.61
		9.36	15.14	0.05	2.48	0.03	49.52	0.81	0.14	1.46
		9.50	17.50	0.03	0.34	0.39	10.71	12.39	0.13	1.60
		15.00	15.00	0.05	0.05	0.03	0.87	0.62	0.13	1.01
		9.50	13.00	0.08	0.02	0.05	0.29	0.63	0.25	2.16
	L35	11.72	16.34	0.08	0.73	0.03	9.77	0.42	0.21	1.09
	PPb	16.03	5.02	0.07	0.24	0.06	3.33	0.79	0.05	0.69
	UCON	19.56	9.15	0.09	0.29	0.02	0.31	4.05	0.25	0.97

Among the ABS studied, the one based in 9.50 wt% of dextran T6 + 13.00 wt% of PE 6400 seems to be the best system for the selective partition of C-phycocyanin, leading to a 4-fold increase in its purity, a result that may be explained by the hydrophobic differences between the various proteins present and the phase forming components.

3.3. Stability of C-phycocyanin

Since the use of ethanol was proved to be innocuous for the chlorophylls chemical structure [47], the photostability of these pigments was not evaluated herein. Meanwhile, and besides the purity level, one of the main challenges for the commercialization of C-phycocyanin is its photostability [42]. As previously mentioned, in the best ABS (dextran T6 + copolymer PE 6400 + water), C-phycocyanin preferentially partitioned for the dextran 6000-rich phase. The photostability of this fluorescent protein was thus evaluated in aqueous solutions of dextran T6 at 3 different concentrations. Thus, a solid-liquid extraction using Na-phosphate buffer aqueous solution was carried using the selected conditions from RSM applied on the C-phycocyanin extraction (SLR of 0.1 at 35 °C for 45 min). Then, the solution was centrifuged, and the C-phycocyanin-rich supernatant was collected and placed in contact with 3 different concentrations of dextran T6 (5, 15 and 25 wt%) at (25 ± 1) °C and atmospheric pressure. The stability was determined over 13 days, as shown in Figure 5. Besides the mould formation observed after 13th day, the stability of the phycobiliprotein fell to 40% for the assays with 15 and 25 wt% of dextran T6. Meanwhile, one of the simplest strategies to improve the stability of proteins is the use of preservatives, which also applies to phycocyanin [48]. Sodium azide has been recognized as a good preservative for this fluorescent protein, considering its application for analytical proposes [42]. To determine its efficacy, sodium azide (0.01%) was added to the Na-phosphate buffer aqueous solution used in the solid-liquid extraction. The

results obtained clearly show the great capability of sodium azide to enhance the protein stability, maintaining it around 86, 77 and 70 % after 30 days for 5, 15 and 25% of dextran T6, respectively (Figure 5). Furthermore, neither the solid-liquid extraction step nor ABS performance and C-phycocyanin purification are affected by the addition of sodium azide.

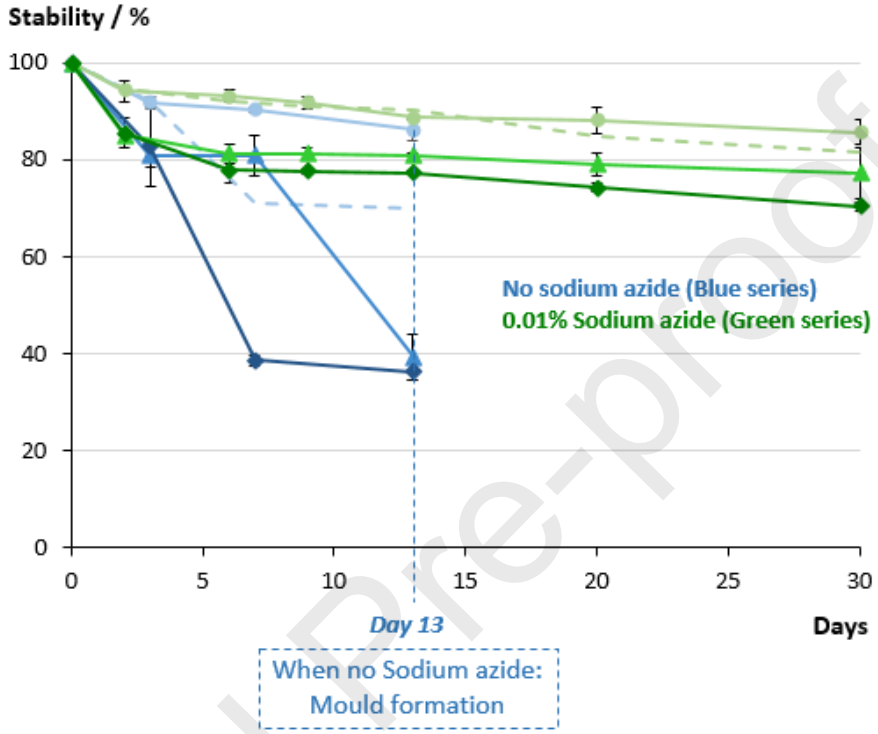

Figure 5. Stability of C-phycocyanin in aqueous solutions of dextran T6 with no sodium azide (blue series) and with presence of 0.01% of sodium azide (green series): (○) 5%, (Δ) 15% and (◊) 25% of dextran 6000. Dashed lines indicate the absence of dextran T6.

3.4. Final process: sequential extraction of C-phycocyanin and chlorophylls followed by C-phycocyanin purification and isolation

After selecting the best solvents to recover chlorophylls and C-phycocyanin from *A. cylindrica* and the best system to promote the C-phycocyanin purification without compromising its photostability, an integrated process was designed. In this case, and considering the different applications envisioned for chlorophylls and C-phycocyanin,

different approaches were considered mainly for the last step of the process, which comprises the preparation of the final product of C-phycocyanin, considering the food and analytical sectors as two examples. The final process is schematically represented in Figure 6 with all the parameters obtained for each product properly described.

As the schematically representation shows, the sequential process comprises two sequential solid-liquid extraction units designed, respectively, for the extraction of chlorophylls using ethanol absolute (recovery = 54.7% representing 1.46 ± 0.06 $mg_{chlorophylls} \cdot g_{fresh\ biomass}^{-1}$) and then C-phycocyanin by using a Na-phosphate buffer (recovery = 90% representing 63 ± 1 $mg_{phycocyanin} \cdot g_{fresh\ biomass}^{-1}$) from the fresh cells of *A. cylindrica*. In addition to the highest values of extraction yields, these solvents are biocompatible and industrially approved, which facilitates the scale-up of the process. Since the purity index of C-phycocyanin was low (purity = 0.521 ± 0.003) after the extraction unit and considering the demands of the different fields of application, a second step comprising the purification of this phycobiliprotein was envisioned by applying a biocompatible ABS based in Dextran T6 + PE 6400. After its application, the purity index of C-phycocyanin was significantly improved (purity index = 2.16, maximum deviation of ± 5%). Meanwhile, an ultrafiltration step may be applied to increase even more the purity of C-phycocyanin which will depend on the demands of the application (step not experimentally tested in the end of the process but recurrently used to refine the purity of proteins) [49]. After the purification step two different approaches were developed, these are represented in Figure 6 as *Via 1* & *Via 2*, regarding the specific demands of analytical and food sectors selected in this work as drivers for the process design. Both fields have different demands regarding the purity level but also the restrictions towards the use of some compounds highlighted as toxic after ingestion. The analytical field requires the stability of C-phycocyanin against the appearance of microorganisms like molds which

is facilitated by the presence of the carbohydrate in the final product and for that, the addition of sodium azide at 0.01% was envisioned [42]. However, for food related applications this is not a viable strategy since this preservative is toxic [42]. In this case, a second approach may be followed, comprising Dextran T6 precipitation by using ethanol [50] for the isolation of C-phycocyanin. In the end, the ethanol may be evaporated for recycling and the phycobiliprotein resuspended in the appropriate solvent required by the application.

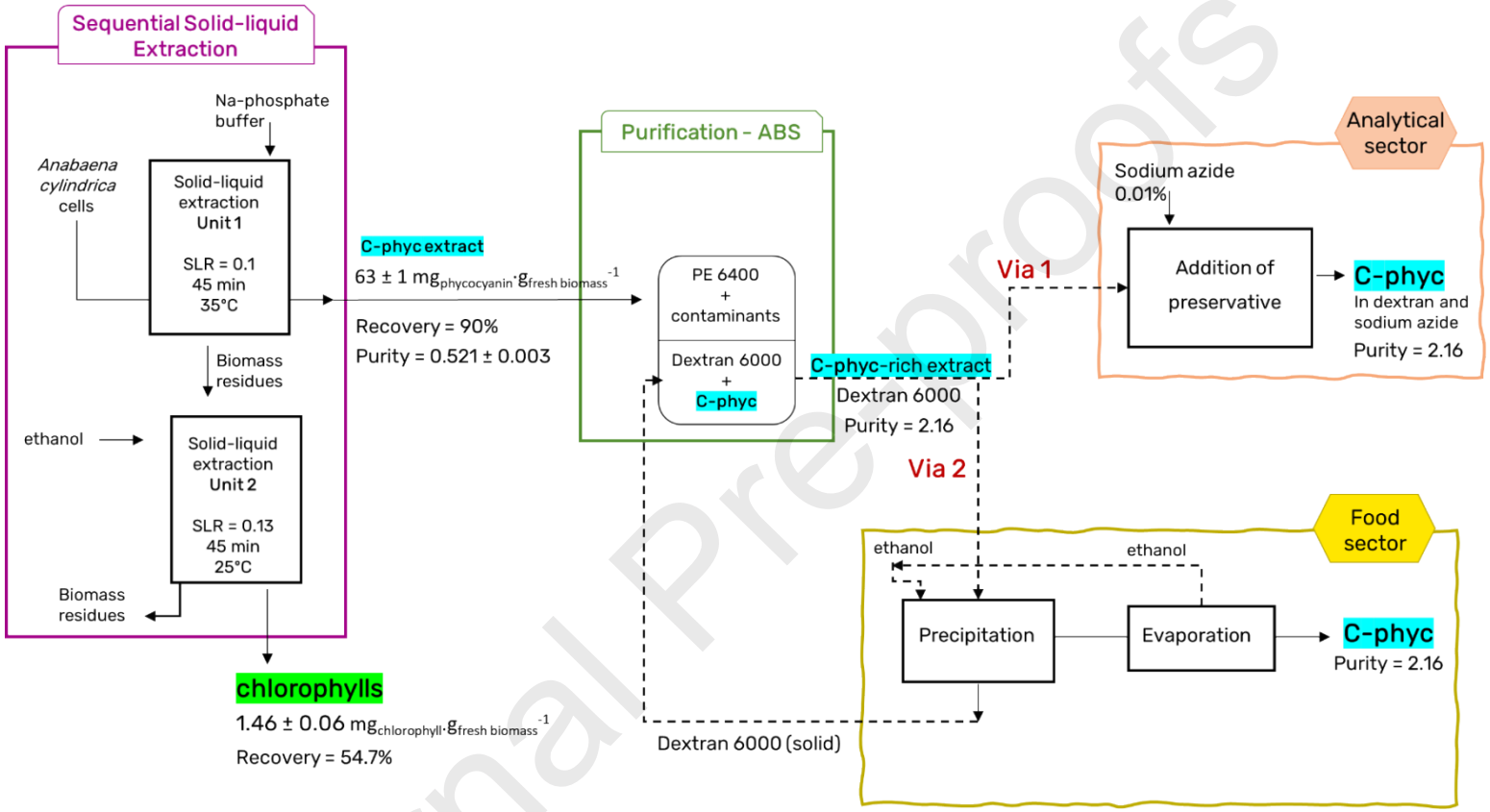

Figure 6. Diagram of the integrated process representing the sequential extraction of pigments (chlorophylls and C-phycocyanin) from *A. cylindrica*, conjugated with the purification of C-phycocyanin using biocompatible ABS and its isolation depending on the application sector. Two strategies, *Via 1* & *Via 2*, were defined for the isolation of C-phycocyanin considering the demands of both the analytical and food sectors, respectively.

4. CONCLUSIONS

In this work, a sequential downstream process to obtain C-phycocyanin and chlorophylls from fresh samples of *A. cylindrica* was proposed and optimized. For that purpose a process comprising two sequential solid-liquid extraction units was designed for the extraction of chlorophylls (maximum recovery of 54.7% achieved in a single step this representing 1.46 ± 0.06 $mg_{chlorophylls} \cdot g_{fresh\ biomass}^{-1}$) by using ethanol and then C-phycocyanin (maximum recovery of 90% representing 63 ± 1 $mg_{phycocyanin} \cdot g_{fresh\ biomass}^{-1}$ achieved in a single step) by using Na-phosphate buffer, both biocompatible and industrially approved solvents. Then, the purification of C-phycocyanin (with a low purity index of 0.521 ± 0.003) was improved by applying a biocompatible ABS based in Dextran T6 + PE 6400, allowing to obtain a chemically stable product with a much higher purity (4-fold). In the end, besides the chlorophylls, two other potential products were defined, these following the demands of analytical and food related applications as two of the most interested in the use of C-phycocyanin.

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Highlights

A sequential process to obtain different pigments from *A. cylindrica* was developed.

C-phycocyanin and chlorophylls were target in this study.

Several conditions were optimized regarding the solid-liquid extraction.

Dextran/copolymer-based ABS were applied to purify C-phycocyanin.

The maximum recoveries of 54.7% and 90% of, respectively, chlorophyll and C-phycocyanin was obtained.

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Declaration of interests

☒ The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

☐The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: