Sustainable strategy based on induced precipitation for the purification of phycobiliproteins

Margarida Martins,[1] Bruna P. Soares,[1] João H. P. M. Santos,[1,2] Pankaj Bharmoria,[1] Mario A. Torres-Acosta,[3] Ana C. R. V. Dias,[4] João A. P. Coutinho, [1] Sónia P. M. Ventura[1*]

[1]CICECO – Aveiro Institute of Materials, Department of Chemistry, Campus Universitário de Santiago, University of Aveiro, 3810-193 Aveiro, Portugal

[2]Department of Biochemical and Pharmaceutical Technology, São Paulo University, Av. Prof. Lineu Prestes n 580 Bloco 16, 05508-000 São Paulo, Brazil

[3]The Advanced Centre for Biochemical Engineering, Department of Biochemical Engineering, University College London, Torrington Place, London, WC1E 6BT, UK

[4]CESAM – Centre for Environmental and Marine Studies, Department of Environment and Planning, Campus Universitário de Santiago, University of Aveiro, 3810-193 Aveiro, Portugal

[*]**Corresponding author:**

Sónia P. M. Ventura, Department of Chemistry, Campus Universitário de Santiago, University of Aveiro, Aveiro, Portugal

Tel: +351-234-370200; Fax: +351-234-370084; E-mail address: spventura@ua.pt

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Abstract

Phycobiliproteins are fluorescent proteins mainly produced by red macroalgae and cyanobacteria. These proteins, essential to the survival of these organisms, find application in many fields of interest, from medical, pharmaceutical, and cosmetic to food and textile industries. The biggest obstacle to its use is the lack of simple environmental and economical sustainable methodologies to obtain these proteins with a high purity. In this work, a new purification process is proposed based on the induced precipitation of the target proteins followed by an ultrafiltration. Purities of 89.5 % of both phycobiliproteins and 87.3 % of R-phycoerythrin were achieved using ammonium sulfate and poly(acrylic acid) sodium salts as precipitation agents (followed by an ultrafiltration step), while maintaining high recovery yields and the protein structure stability. Environmental analysis performed to evaluate the proposed process show that the carbon footprint for the proposed process is much lower than those reported for alternative methodology, and the economic analysis reveals the cost-effective character associated to its high performance. This work is a step towards more sustainable and effective methodologies/processes with high industrial potential.

Introduction

The production of chemicals, materials and fuels from biomass is a growing trend in which academia and industry have invested significant efforts during the last decade.[1] The goal is to reduce the world dependence on a petroleum-based economy, gradually replacing it by a bioeconomy where the so-called biorefinery plays a major role.[2] The development of biorefinery processes is still much focused on the biofuels, power and heat production.[3] However, to achieve a full exploitation of the biomass, a complete cascade of different products should be obtained,[4] following an order that should be dependent on the market value of what is obtained and the sensitivity of the compounds to the conditions of extraction. By guaranteeing the stability of the bioactive compounds, the process value-chain should start by the recovery of low-volume high-value products.[1,5]

Macroalgae are an example of a biomass that could allow the development of a biorefinery focusing on a blue economy. Many high-value products, such as pigments,[6] phenols,[7] lipids,[8] and proteins,[9] are already being explored in what should be the beginning of the biorefinery cascade.[10]

Phycobiliproteins are a family of fluorescent and hydrophilic proteins involved in the light-harvesting processes in red macroalgae. This family of proteins, in red macroalgae, is mainly composed of R-phycoerythrin (R-PE) and R-phycocyanin (R-PC).[11] R-PE has a soft pink color and orange fluorescence, composed of $(\alpha\beta)_6\gamma$ complexes and with 240 kDa, while R-PC has a blue color and red fluorescence, composed of $(\alpha\beta)_3$ complexes.[11,12] Due to their spectroscopic and fluorescent properties, those proteins can be applied in different fields from biotechnology, biomedicine, pharmaceuticals, cosmetics, and food products.[13] More recently, extracts rich in phycobiliproteins were

also studied as natural dyes to use as optical active centers for sustainable luminescent solar concentrators and proving their potential towards cheap and sustainable photovoltaic energy conversion.[14]

Despite the efforts from several researchers on the development of new processes to obtain pure phycobiliproteins, these are still far from industrialization. The purity level required is defined by the application/product demands, and the process to be implemented should take these requirements into account. Conventionally, the purification of phycobiliproteins can be achieved by a set of unit operations that may include (i) a pre-purification step commonly applying ammonium sulfate precipitation, (ii) one or more purification steps applying membrane separation processes (*i.e.* ultrafiltration (UF) and cross-flow ultrafiltration) and/or chromatographic processes which are usually column chromatography (*i.e.* size exclusion-, ion exchange-, hydrophobic interaction-, and affinity-chromatography), and iii) a last step of dialysis to completely remove, replace, or decrease the concentration of salts or solvents from the purified extracts.[15–21] Recently, alternative methodologies of protein purification have been proposed, such as membrane chromatography,[22] centrifugal precipitation chromatography,[23] electrophoretic elution,[24] vortex flow reactor in an adsorption experiment,[25] and aqueous micellar two-phase systems.[26] However, most of them have disadvantages related to complexity, difficulty to scale-up and high associated costs, limiting the applicability of these processes at an industrial scale. This is also true for the process we have previously proposed based on the use of aqueous micellar two-phase systems.[26] Despite the good results achieved for the purification of phycobiliproteins, and R-PE in particular, the process included five main steps, comprising a first solid-liquid extraction, two units of purification applying aqueous micellar two-phase systems

followed by two units of operation to separate the target proteins from the main solvents used. In this context, the present work will attempt the development of a simpler process to purify phycobiliproteins, and also R-PE. The first approach to be used was the elimination of the fourth and fifth steps of our previous process involving the separation of the target proteins after purification from the extraction solvents. For that, the use of induced precipitation seems to be a good strategy. The recovery and purification of proteins by precipitation is one of the most important operations in protein purification, recurrently used in laboratories and also industry.[27] This is achieved by the destabilization of a protein solution that is then separated from the liquid/supernatant by gravity settling, centrifugation, or filtration. The precipitation can be driven by the ionic strength of the medium, but also by size exclusion, pH and temperature variations.[28,29] Much work has been done regarding the use of ammonium sulfate, which is a classic salting-out agent and usually the first choice in protein precipitation.[29] However, and despite its high efficiency promoting precipitation, it is not selective, which means that it will precipitate all the proteins in the solution. It is also known that many other compounds can act as precipitation agents, such as polymers, copolymers, and polyelectrolytes by different phenomena such as crowding or by direct interaction between the protein and the precipitation agent that can tune the solubility decrease of the target protein from a crude extract, thus leading to a selective precipitation.[28–32]

Precipitation is normally used as a pre-treatment,[20,33] meaning that it is complemented by a set of other purification steps, including chromatographic[17] or non-chromatography[34] downstream processing steps. However, in this work, the main objective was to decrease the number of steps required to obtain pure phycobiliproteins

from *Gracilaria gracilis*, in particular, R-PE, thus avoiding the application of other purification steps. The screening of various potential precipitating agents was studied from a large set of polymers, copolymers, and polyelectrolytes. After the design of a simple and efficient process to obtain the phycobiliproteins (and particularly, R-PE), a life cycle analysis was done to compare this process with the one previously proposed by us using aqueous micellar two-phase systems, [26] followed by an economic analysis, based on which the viability and sustainability of this process is discussed.

Experimental

Biomass

The biomass used in this work, fresh *Gracilaria gracilis*, was kindly provided by ALGAplus (Ílhavo, Portugal). ALGAplus farms the macroalgae at Ria de Aveiro lagoon (40°36'44.7" N, 8°40'27.0" W) in coastal Portugal under the EU organic aquaculture standards (EC710/2009). This aquaculture is performed in a land-based integrated multi-trophic aquaculture system (meaning that the nitrogen input is higher than in the outside natural lagoon due to the use of effluent water from fish production). Macroalgae samples were collected between April and December of 2019, washed with tap and distilled water, being frozen until needed, but never for longer than one month.

Chemicals

Ammonium sulfate ($(NH_4)_2SO_4$, 99.5 %) was acquired from Merck. Poly(acrylic acid) sodium salts with average molecular weight of 1200 g.mol^{-1} (NaPA 1200, 45 wt % in water solution) and 8000 g.mol^{-1} (NaPA 8000, 45 wt% in water solution), poly(ethylene glycol) (PEG) with average molecular weight of 8000 g.mol^{-1} (PEG 8000, pure) and

poly(propylene glycol) (PPG) polymer with average molecular weight of 400 g.mol^{-1} (PPG 400, pure) were purchased from Sigma-Aldrich. PEG with average molecular weight of 10000 g.mol^{-1} (PEG 10000, pure) was supplied from Fluka.

Nonionic copolymers composed of PEG and PPG blocks were also used (Figure 1). Their commercial names were adopted throughout this work. Pluronic PE 6800 (PPG-PEG-*blocks* with approx. 8000 g.mol^{-1}, composed of 80 wt % PEG), Pluronic PE 6400 (PPG-PEG-*blocks* with approx. 2900 g.mol^{-1}, composed of 40 wt % PEG) and Pluronic PE 6200 (PPG-PEG-*blocks* with approx. 2450 g.mol^{-1}, composed of 20 wt % PEG) were purchased from BASF. Pluronic P 17R4 (PPG-PEG-PPG-*blocks* with approx. 2700 g.mol^{-1}, composed of 40 wt % PEG), Pluronic L81 (PEG-PPG-PEG-*blocks* with approx. 2800 g.mol^{-1}, composed of 10 wt % PEG), and Pluronic P123 (PEG-PPG-PEG-*blocks* with approx. 5800 g.mol^{-1}, composed of 30 wt % PEG) were acquired from Sigma-Aldrich.

As standard, commercial R-PE (CAS 11016-17-4) supplied by Sigma-Aldrich was used.
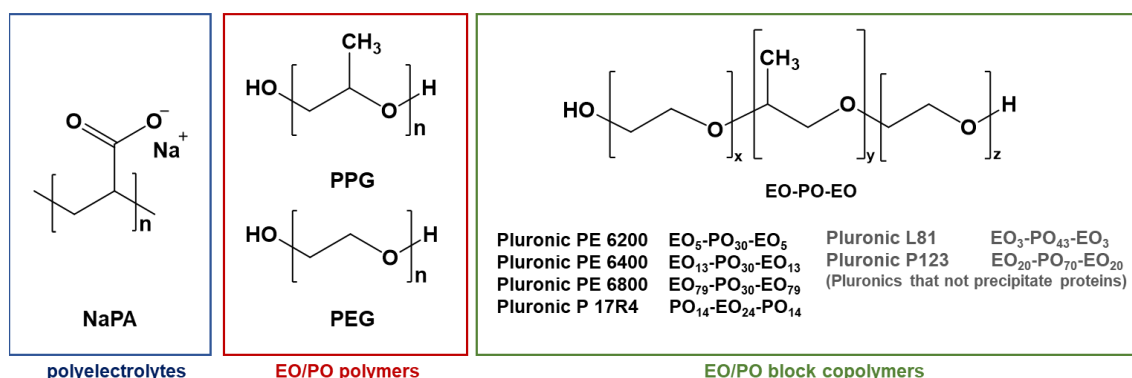
Figure 1. Structures of the precipitation agents studied in this work.

Solid-liquid extraction

The solid-liquid extraction procedure was adopted from Martins et al.[35] but with some modifications. Briefly, the stored fresh *Gracilaria gracilis* at -20 °C was ground in a coffee mill after being deeply frozen with liquid nitrogen for a more efficient extraction. The

extraction was performed using distilled water as solvent in a solid-liquid ratio of 0.5 $g_{\text{fresh biomass}} \cdot mL_{\text{solvent}}^{-1}$ during 20 min at room temperature (20–25 °C) in an orbital shaker (IKA KS 4000 ic control) at 250 rpm and protected from light. The crude extract was obtained after centrifugation at 14000 g, 20 min, at room temperature (20–25 °C) in a VWR microstar 17 centrifuge.

Induced precipitation

Several precipitation agents were tested at three different concentrations (100, 200 and 300 g.L^{-1}). Each precipitation agent was dissolved in the crude extract and left overnight at 4 °C. Pellet and supernatant phases were induced by centrifugation at 900 g, for 15 min at room temperature (20–25 °C) in the VWR microstar 17 centrifuge using the same conditions described in the section Solid-liquid extraction. After centrifugation, the pellet was resuspended in the same initial volume using distilled water. When particles not soluble in water are observed in the resuspended pellets (that happened in PEG 10000 and Pluronic PE 6200), a vigorous centrifugation at 9600 g for 5 min was applied to remove these solids before further analysis.

Ultrafiltration (UF)

500 µL of sample was added in each Amicon Ultra-0.5 mL Centrifugal Filter Unit 100 K. The sample was centrifuged at 14000 g during 15 min. The permeate was discarded and 400 µL of ultrapure water was added to the concentrate and centrifuged in the same conditions, being this last step repeated twice. Lastly, 500 µL of ultrapure water was added to recover the concentrated sample after a centrifugation of 2 min, at 1000 g.

Spectroscopic methods

The absorption spectra of different fractions were measured between 200 and 700 nm using a UV-Vis microplate reader (Synergy HT microplate reader – BioTek). This technique was used in the initial screening of precipitation agents, in which the phycobiliproteins were quantified directly at 565 nm, and the total amount of proteins was quantified by the Bicinchoninic Acid (BCA) method at 562 nm, considering two calibration curves previously prepared (R^2 = 0.999 and R^2 = 0.998, for phycobiliproteins by direct analysis and total proteins by BCA method, respectively). The total protein concentration was determined with the Pierce[TM] BCA Protein Assay and Micro BCA Protein Assay (Thermo Scientific, Schwerte, Germany) according to the supplier recommendations. Bovine serum albumin (from Fisher Scientific) was used as the standard protein. The purity parameter was obtained as the ratio between the phycobiliproteins concentration and the total proteins concentration in the resuspended pellet, with these values presented in percentage. The yield was calculated as the ratio between the phycobiliproteins concentration in the resuspended pellet and the phycobiliproteins concentration in the initial extract.

Parameters as selectivity and *R-PC index* were calculated according to Vicente et al.[26] In order to determine the selectivity, the partition coefficient of R-PE (K_{R-PE}) and total proteins ($K_{Total proteins}$) were firstly calculated (Eqs. 1 and 2, respectively). This parameter is the ratio between the concentration of R-PE (or total proteins) in the purified fraction and the discarded phases along the purification steps. Knowing the partition coefficient of both R-PE and total proteins, the selectivity of the proposed method was determined according to Eq. 3.

$$K_{R\text{-}PE} = \frac{[\text{R-PE}]_{\text{purified fraction}}}{[\text{R-PE}]_{\text{discarded fraction}}} \tag{1}$$

$$K_{Total\ proteins} = \frac{[\text{Total proteins}]_{\text{purified fraction}}}{[\text{Total proteins}]_{\text{discarded fraction}}} \tag{2}$$

$$Selectivity = \frac{K_{\text{R-PE}}}{K_{\text{Total proteins}}} \tag{3}$$

The *R-PC index* and *purity index* relate the amount of R-PC and R-PE, and R-PE and total proteins, in a sample, respectively. Both are calculated by the ratio between the maximum absorbance of R-PC and R-PE, and R-PE and total proteins, respectively (Eq. 4 and 5).

$$R\text{-}PC\ index = \frac{\text{Abs}_{617\ nm}}{\text{Abs}_{565\ nm}} \tag{4}$$

$$Purity\ index = \frac{\text{Abs}_{565\ nm}}{\text{Abs}_{280\ nm}} \tag{5}$$

High-performance liquid chromatography (HPLC) using the equipment Chromaster HPLC system (VWR Hitachi) equipped with a binary pump, column oven, temperature-controlled auto-sampler, DAD detector (HPLC-DAD) and an analytical column Shodex Protein KW-802.5 (8 mm×300 mm) was applied. A 100 mmol.L^{-1} phosphate buffer pH 7.0 was run isocratically with a flow rate of 0.5 mL.min^{-1} and the injection volume was 10 µL. All samples were previously filtered with the 25 mm GHP Acrodisc syringe filters with a pore size of 0.45 µm. The wavelength was set at 280, 565 and 617 nm. All spectra were treated using OriginPro 2018 program. The peaks were deconvoluted and the obtained areas were used, namely the total area and the area of the R-PE and R-PC specific peaks. The purity was obtained by the ratio of the areas of R-PE or R-PC specific peaks and the total area of the spectrum, in percentage. The yield was calculated by the

ratio of the areas of R-PE or R-PC specific peaks in the purified extract and the areas of R-PE or R-PC specific peaks in the initial extract, in percentage.

Circular dichroism spectra were recorded using a Jasco J-815 circular dichroism spectrometer at 298.15 K in the far UV region (λ = 180–260 nm). Spectra were collected in a 0.1 cm path length quartz cuvette at a scan rate of 100 nm.min^{-1} and sensitivity of 100 mdeg. The response time and the bandwidth were 2 s and 0.5 nm, respectively. The samples were solubilized in distilled water up to a dilution where the influence of the sample interferences was negligible, being in those conditions the circular dichroism spectra obtained with high tension voltage below 600 (Figure S1 in Supporting Information).

SDS-PAGE

The phycobiliprotein crude extract was analyzed through electrophoresis that was prepared on polyacrylamide gel (stacking: 4 % and resolving: 20 %) with a running buffer consisting of 250 mmol.L^{-1} of Tris-HCl, 1.92 mol.L^{-1} of glycine, and 1 % of SDS. The proteins were stained with the usual staining procedure [Coomassie Brilliant Blue G-250 0.1 % (w/v), methanol 50 % (v/v), acetic 7 % (v/v), and water 42.9 % (v/v)] in an orbital shaker, at moderate speed, for 2–3 hours at 20–25 °C. The gels were detained in a solution containing acetic acid 7 % (v/v), methanol 20 % (v/v), and water 73% (v/v) in an orbital shaker at ± 60 rpm for 3–4 hours at 20–25 °C. The molecular weight marker used was the NZYColour Protein Marker II from NZYTech.

Environmental evaluation: life cycle assessment

The environmental profile of two scenarios to purify phycobiliproteins was evaluated by life cycle assessment, according to ISO 14040 standard,[36] and covering the impacts from the production of the chemicals used in the processes, water and also the electricity consumption. Table S1 of Supporting Information shows the amounts of chemicals and water consumed during the experimental procedure, as well as the amounts of electricity spent. The latter parameter was calculated for each equipment based on the time of operation, nominal power and fraction of occupancy over total capacity. These amounts are expressed *per* mg of R-PE obtained to allow comparison between the two scenarios proposed in this work. The impact factors associated with the production of chemicals and electricity (Portuguese mix) were taken from the Ecoinvent 3.6 database.[37] The impact factors for distilled and ultrapure water result from tap water production[38] and electricity consumed during the distillation and ultrafiltration.[37] The impact assessment method was the ReCiPe 2008 Midpoint at the Hierarchist perspective,[39] considering the following impact categories: climate change (equivalent to the carbon footprint), photochemical oxidant formation, terrestrial acidification and fossil depletion. The results were compared with the ones obtained by Vicente and collaborators when applying aqueous micellar two-phase systems to purify phycobiliproteins.[26]

Economic evaluation

To further expand this study and understand some of the potential economic constrains of implementing the process optimized in this work into an industrial scenario, an economic analysis was performed considering the traditional approach (using $(NH_4)_2SO_4$) and the alternative precipitation method proposed in this work. In this

analysis, the production cost was calculated *per* mg of R-PE (CoG.mg^{-1}).[6,40] Briefly, three

areas need to be fulfilled to have a complete process: to set up a target output or

production scenarios, then to determine the sequence of unit operations and their

process parameters, and finally to collect the economic datasets to populate the model.

For the process developed in this work, the production scale to be used at the industrial

stage has not been decided and for this reason, five different scales were analyzed,

namely 0.01 kg, 0.1 kg, 1 kg, 10 kg, and 100 kg. This will give a wide range of operations

from the laboratory, to pilot and, finally, industrial scales. The sequence of unit

operations is something that will be discussed in later sections as a result of all the

analyses performed in this work, but briefly, it consists of a water extraction of R-PE

from the biomass, then a centrifugation to remove the spent biomass. For the

precipitation stage, it starts with the mixing of the extract with the precipitant in a tank,

followed by the induced precipitation using a centrifugal step, and a re-suspension of

the pellet. The process ended with an ultrafiltration/diafiltration step to remove the

non-suspended proteins, allowing also the final polishing.

The economic datasets are composed of different areas. For the capital investment

(mainly equipment acquisition costs), cost of equipment was obtained from the

database on the software Biosolve Process (Biopharm Services Ltd., Buckinghamshire,

UK), then different regressions were determined to interpolate the results considering

the different scales needed. The same strategy was employed for consumables (vessel

filters and ultrafiltration/diafiltration membranes). For materials costs (chemicals), as

this analysis comprised small and large scales, their costs were obtained from Sigma-

Aldrich and Alibaba, respectively. Labor has been reported to be approximately 15 % of

the total production costs,[41] so this approach was taken here. Lastly, an additional

economic aspect was denoted as "others", in which utilities and maintenance costs were included. This was calculated following Biosolve Process approach, which estimates these costs as 4 % of the capital investment. Full data for process and economic parameters employed here are included in Table S2 in Supporting Information.

After the completion of the model construction, different analyses were performed to understand how the CoG.mg^{-1} of the R-PE behaves. First, different production scales were evaluated, for the whole range mentioned before (0.01 kg to 100 kg), following incremental steps of 0.1 kg. Then, using only the discrete range of production scales (0.01 kg, 0.1 kg, 1 kg, 10 kg, and 100 kg), a sensitivity analysis was performed by systematically varying the values of the amount of R-PE content in the biomass (mg of R-PE *per* kg of fresh biomass), the materials cost variation and the duration of the process, all of them in a range from 10-fold above and below (± 10X). Additionally, the impact of the overall recovery yield was included, but due to the results obtained, the range was constrained, the worst-case scenario was 30 % less of what is reported in the following sections and the best scenario can only increase up to 100 %. This analysis can provide an insight on how each individual parameter affects the production costs and help potentially to devise strategies to control their variations. As a complement to the sensitivity analysis, a series of Monte Carlo simulations was performed varying the same parameters, with the same ranges, but under a triangular distribution and calculating their respective production costs (CoG.mg^{-1}) for each scenario. Afterwards, a multiple linear regression was calculated to obtain the coefficients and *p*-value for each parameter.

An additional approach was determined in this work, which results in the calculation of the potential income, or *Return*, that the product could provide and to understand how

the different process parameters could affect it. Based on other reports,[42] Eq. 6 was

defined to calculate the *Return* based on the results obtained from this work:

$Return =$

$$\left[C_{prod} \times \$_{prod} - \$_{biom} \right] - \left[(\alpha) \times (\text{Production cost per kg of biomass}) \right] \qquad \textbf{(6)}$$

In Eq. 6, *Return* stands for the *Return per* kg of processed fresh biomass, C_{prod} is the

amount of product *per* kg of biomass, $\$_{prod}$ is the commercial price of R-PE on the market

and $\$_{biom}$ is the cost associated with the acquisition of the biomass. While, in the second

term, the production cost *per* kg of biomass is a conversion of the CoG.mg^{-1} of R-PE into

a CoG.kg^{-1} of processed biomass. To obtain this, it is needed to obtain the production

cost *per* batch (CoG/batch) and to divide it by the amount of biomass processed in that

particular batch. The α is an additional term employed as a multiplier of the CoG.kg^{-1} in

order to increase or decrease its impact consequently allowing us to analyze their effect

in case the real production costs are higher or lower. As part of the *Return* analysis, a

sensitivity analysis was performed by varying the C_{prod} by 0.5X, 1X, or 2X (half or double

of the base concentration) and the α term was varied between 1X, 2X, or 5X.

Additionally, R-PE has a wide range of prices depending on the application, purity and

amount being acquired, and for this reason, the range of € 5 to € 5,000 *per* kg was

analyzed.

Results

Induced precipitation of proteins

Various phenomena can promote protein precipitation however, substances (generally

in high concentration) changing the environment of the protein (e.g. some organic

solvents, salts, and neutral polymers); or substances (generally at low concentration) interacting directly with the protein (e.g. acids, bases, polyelectrolytes and some metal ions), have been reported as the most relevant.[27] In this work, a screening of polymers, copolymers, and polyelectrolytes at different concentrations was performed, being their ability to induce protein precipitation reported in Table 1 and their performance compared with the results obtained for $(NH_4)_2SO_4$ (the conventional precipitation agent here used as control).

Table 1. List of precipitation agents screened according to their ability to precipitate phycobiliproteins from the raw extract at different concentrations. The symbols ✓ and **X** represent, respectively, the systems with and without protein precipitation occurring.

Precipitation agent	Concentration (g.L^{-1})		
	100	200	300
NaPA 1200	✓	✓	✓
NaPA 8000	✓	✓	✓
PEG 8000	**X**	**X**	**X**
PEG 10000	✓	✓	✓
PPG 400	✓	✓	✓
Pluronic PE 6800	✓	✓	✓
Pluronic PE 6400	✓	✓	✓
Pluronic PE 6200	✓	✓	✓
Pluronic P 17R4	✓	✓	✓
Pluronic L81	**X**	**X**	**X**
Pluronic P123	**X**	**X**	**X**
$(NH_4)_2SO_4$	✓	✓	✓

NaPA 1200 and NaPA 8000 are included in the group of precipitation agents interacting directly with the proteins, while the rest of the substances screened, *i.e.* polymers and copolymers, act by promoting changes in the environment of the initial solvent.

Although according to literature,[27] low concentrations of precipitation agents are required when their mechanism of action involves the direct interaction with proteins, both NaPA 1200 and NaPA 8000 were found to be able to induce the precipitation of phycobiliproteins at all concentrations tested. The worst results, without any precipitation of phycobiliproteins, were obtained for PEG 8000, Pluronic L81 and Pluronic P123, independently of the concentration applied.

As previously discussed in the literature,[43] the main phenomena behind the protein precipitation with polymers and copolymers is, in general, a result of the crowding effect, which happens when high concentrations of these molecules are introduced in the system, drastically reducing the volume of water molecules available for protein solvation. In this context, it is well established that PEGs with high molecular weights more easily precipitate proteins, which can explain the difference in the behaviours of PEG 8000 and PEG 10000.

As the polymers, the copolymers can also decrease the solubility of proteins in solution due to their interaction with the water molecules and the volume they occupy in solution. According to the hydrophilic-lipophilic balance which is a parameter that helps to describe the higher or lower capacity of substances to interact with water molecules (data provided by their suppliers and displayed in Table S3 of Supporting Information), the screened Pluronic substances can be ordered as follows: PE 6800 > PE 6400 > P 17R4 ~ PE 6200 ~ P123 > L81. Considering the results of the hydrophilic-lipophilic balance, it is clear that the decrease in the hydrophilicity of the Pluronics screened makes them unable to precipitate the phycobiliproteins, as a result of their reduced capacity to interact with the water molecules present in the crude extract.

After selecting from Table 1 all the compounds able to precipitate the phycobiliproteins, and considering the viscosity of the solutions, and the color intensity in the supernatants (which is a proxy for the residual amounts of phycobiliproteins in solution) only Pluronics, PPG 400 and NaPA 1200 and NaPA 8000 were retained to further evaluate the purity and yield parameters (Figure 2).
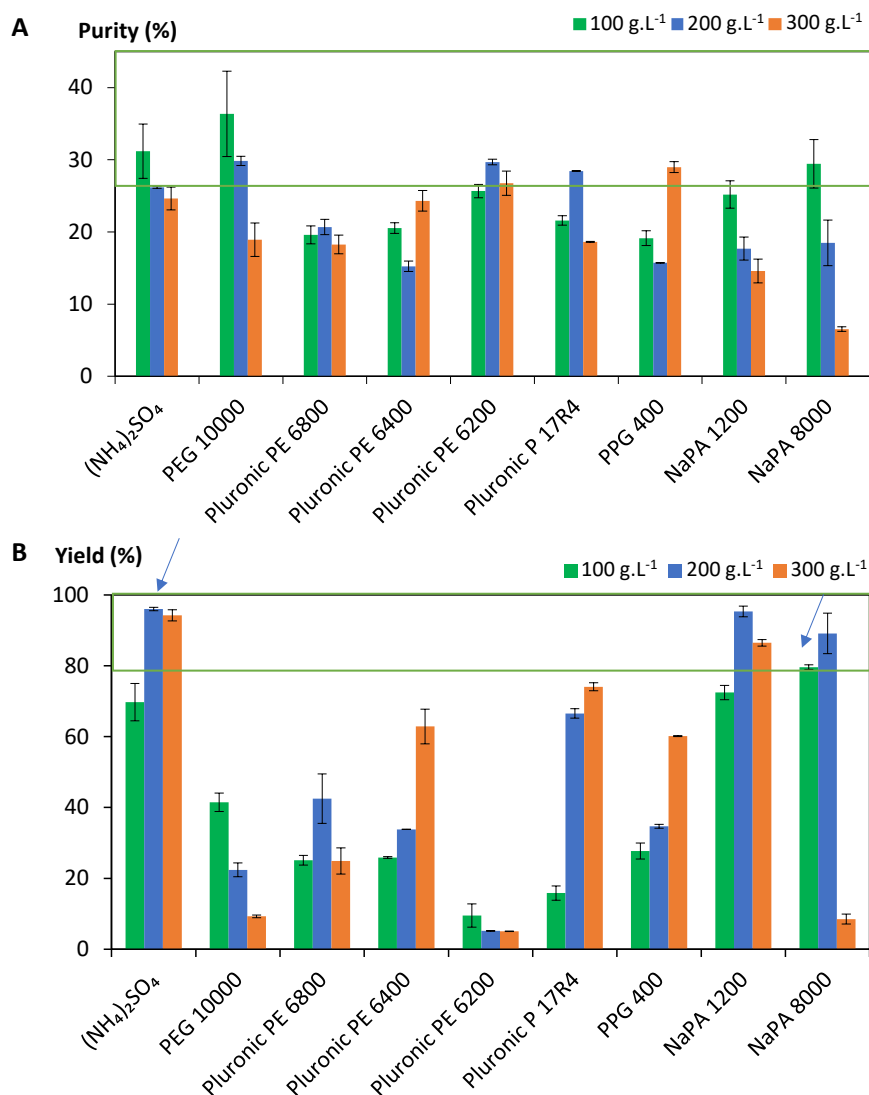
Figure 2. Results obtained for the (A) purity and (B) yield (%) obtained in the resuspended pellets after the precipitation step using different precipitation agents at three distinct concentrations (100, 200 and 300 g.L^{-1}). These analyses were assessed by UV-Vis absorption spectroscopy.

In the view to find the best precipitation agent, a compromise between purity and yield of precipitation was required. The objective was to select the system providing the highest purity levels of phycobiliproteins without reduce the yields of precipitation. After the interpretation of the data presented in Figure 2 and aiming to proceed with the analysis, the criteria selected was the following: to identify the precipitation agents able to simultaneously provide purities and yields higher than 25 % and 80 %, respectively. The systems fulfilling this criteria were the traditional $(NH_4)_2SO_4$ at 200 g.L^{-1} (purity = 26.2 ± 0.1 % and yield = 96.0 ± 0.5 %) and the polyelectrolyte NaPA 8000 (purity = 29 ± 3 % and yield = 79.6 ± 0.7 %). After choosing the best systems and respective concentrations to induce the precipitation of phycobiliproteins, the extracts obtained were further analyzed by HPLC-DAD (Table 2). This analysis identifies which phycobiliprotein (R-PE or R-PC, the two most relevant phycobiliproteins present in the initial extract[26,35]) and in what extent, was precipitated. Moreover, it also enabled us to infer on the selectivity (capacity to separate R-PE from R-PC) of each system (*i.e.* precipitation agent and its concentration).

Table 2. Purity and yield (%) obtained in different fractions separately for R-PE and R-PC based on HPLC-DAD analysis.
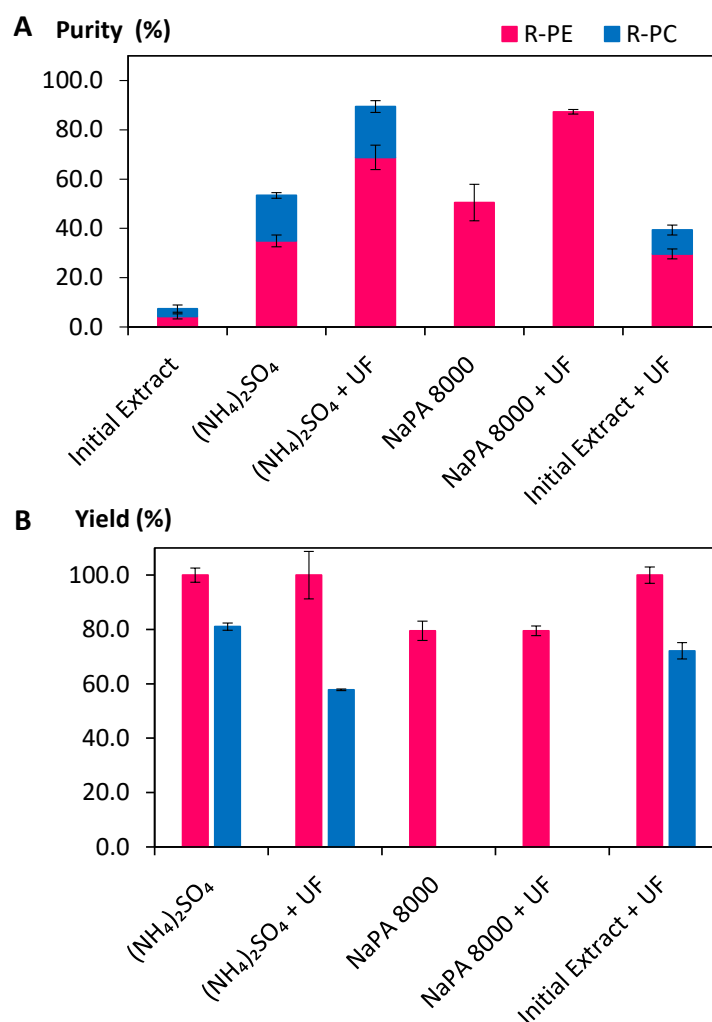
	Purity (%)			Yield (%)	
	R-PE	**R-PC**	**Phycobiliproteins (R-PE + R-PC)**	**R-PE**	**R-PC**
Initial extract	4.4 ± 1.0	3.2 ± 1.5	7.4	-	-
$(NH_4)_2SO_4$ at 200 g.L^{-1}	35.0 ± 2.4	18.5 ± 1.2	53.4	100.0 ± 2.6	81.1 ± 1.3
NaPA 8000 at 100 g.L^{-1}	50.5 ± 7.4	-	50.5	79.5 ± 3.6	-

The results reported in Table 2 show that the $(NH_4)_2SO_4$ at 200 g.L^{-1} can precipitate both R-PE and R-PC, while NaPA 8000 at 100 g.L^{-1} is selective for R-PE, *i.e.* it only causes the precipitation of R-PE, while the other phycobiliproteins remain solubilized in the crude extract. The results of $(NH_4)_2SO_4$ are not surprising, since it is well known that, despite its high capacity to induce the precipitation of proteins, it is not selective. It is efficient in precipitating the R-PE because of its very high molecular weight (240 kDa[20]). Although R-PC (~ 112 kDa[44]) has a lower molecular weight than R-PE, due to the difference between their complexes (($\alpha\beta$)$_3$ for R-PC and ($\alpha\beta$)$_6\gamma$ for R-PE), the R-PC precipitation might be induced due to the proximity between the pH of the aqueous solution of $(NH_4)_2SO_4$ (5.5) and the R-PC isoelectric point, 5.7[44]. In the other hand, NaPA 8000 at 100 g.L^{-1} interacts directly with R-PE, establishing soluble complexes, but not with R-PC, promoting a selective precipitation. Since NaPA 8000 is a polyanion, and at the conditions of the solution, R-PE is negatively charged [pH (8.1) > R-PE isoelectric point (4.2)[45]], site-specific local interactions might be happening, thus justifying the establishment of soluble complexes.[30] At this point, it is also important to mention that, as described in experimental section, all procedures were done protected from light. Besides, the range of temperature (4–25 °C) and pH (5.5–8.1) registered during the precipitation procedures using both NaPA 8000 and $(NH_4)_2SO_4$ do not compromised the stability of phycobiliproteins.[46]

Although the purity has increased after the precipitation step, the extracts are still not very pure (maximum purity up to this point around 50 %). For that reason, the resuspended pellets obtained after the precipitation with NaPA 8000 and $(NH_4)_2SO_4$ were subjected to an additional step of purification using ultrafiltration. As previously detailed in the Experimental section, filters with a cutoff of 100 kDa were applied to

remove the small and medium-size contaminant proteins present in the macroalgae.[26]

Yields and purity obtained before and after ultrafiltration are plotted in Figure 3 (with

more details in Table S4 of Supporting Information).

Figure 3. Summary of the results obtained by HPLC-DAD for the (A) purity and (B) yield

obtained in different fractions, namely the initial extract, the resuspended pellets after

precipitation using $(NH_4)_2SO_4$ at 200 g.L^{-1}, $(NH_4)_2SO_4$ at 200 g.L^{-1} followed by an

ultrafiltration step, and NaPA 8000 at 100 g.L^{-1}, NaPA 8000 at 100 g.L^{-1} followed by an

ultrafiltration step, and lastly initial extract purified by an ultrafiltration step) separately

for R-PE (pink bars) and R-PC (blue bars).

Summing up the results, the initial extract has a purity in phycobiliproteins around 7.4 % (this representing 100 % of both R-PE and R-PC extracted from the biomass). By submitting the extract to a precipitation step using $(NH_4)_2SO_4$ at 200 g.L^{-1}, the purity of both phycobiliproteins increased to 53.4 % without compromising the yield of precipitation. By adding an ultrafiltration step, the purity increased to 89.5 % in phycobiliproteins, without affecting the yield of precipitation of R-PE. On the other hand, and as previously analyzed, after precipitation with NaPA 8000 at 100 g.L^{-1} only R-PE precipitated with a purity of 50.4 % (R-PC remained in solution). Meanwhile, and after applying the ultrafiltration step, the purity of the extract increased from 50.5 % to 87.3 % in R-PE with a yield of 79.5 %.

The selectivity and *R-PC index* of the purified extract obtained from both purification methodologies proposed in this work were also calculated and compared with the results obtained for the process using aqueous micellar two-phase systems[26] for the purification of R-PE (Table 3). In terms of selectivity, it was found that both processes proposed in this work are superior to the systems previously reported by Vicente et al.[26] The *R-PC index* in the extracts purified by $(NH_4)_2SO_4$ (200 g.L^{-1}) precipitation with an additional ultrafiltration step is higher than the NaPA 8000 (100 g.L^{-1}) precipitation with an additional ultrafiltration, supporting the selectivity of the induced precipitation process based in NaPA 8000. Moreover, the induced precipitation with $(NH_4)_2SO_4$ has a higher *R-PC index* than those presented by Vicente et al., showing its ability in preserve the R-PC content. In the other hand, systems of purification with NaPA 8000 have the lowest *R-PC index* in comparison with all systems presented by Vicente and co-authors being in the purity range of the standard R-PE sold by Sigma-Aldrich[47] (which is < 0.03) showing its extremely low contamination with R-PC, as intended.

Table 3. Selectivity and *R-PC index* of both purification methodologies proposed.

	$(NH_4)_2SO_4$ (200 g.L^{-1}) + ultrafiltration (this work)	NaPA 8000 (100 g.L^{-1}) + ultrafiltration (this work)	*AMTPS[26]	Sigma-Aldrich[47]
Selectivity	19.6 ± 0.1	15.3 ± 0.4	13.6 ± 0.0	---
R-PC index	0.23 ± 0.01	0.011 ± 0.001	0.047 ± 0.004	< 0.03
Purity index	3.1 ± 0.3	3.4 ± 0.1	---	> 4.6

*best system proposed by Vicente and co-authors.[26]

Lastly, an ultrafiltration step was applied to the initial extract without any previous precipitation step in order to understand if the same results could be obtained by skipping the precipitation procedure. At this point, the purity obtained was only of 39.4 % in phycobiliproteins, which represents much lower values than those discussed previously with induced precipitation as a first step, thus showing the need of both steps in the proposed process.

To confirm the results represented in Figure 3 on the increase of purity of the extracts in the different scenarios tested, a SDS-PAGE electrophoresis was carried being the results depicted in Figure 4.
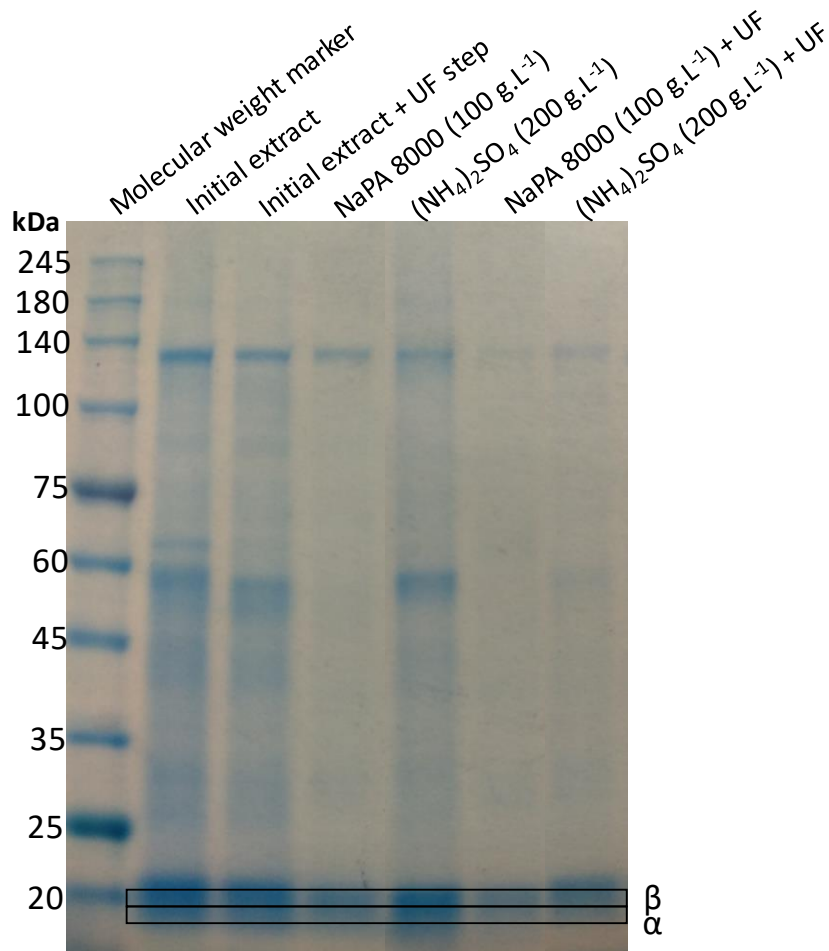
Figure 4. SDS-PAGE analysis of different fractions obtained after testing the different scenarios under study. UF stands for ultrafiltration.

As previously mentioned, R-PE and R-PC are composed of $(\alpha\beta)_6\gamma$ and $(\alpha\beta)_3$ complexes,[12] respectively. Although there are slight differences among the α and β subunits present in the phycobiliproteins, their weight is quite similar, being 18–20 kDa (for α) and 19.4–21 kDa (for β), and for R-PE an additional γ subunit of ~ 30 kDa is also present.[19,44,48] The SDS-PAGE of the standard R-PE can be checked elsewhere.[35] This said, the presence of α and β subunits is a constant in all samples represented in Figure 4. It is also evident the high contamination of the initial extract with other proteins. Despite the removal of some impurities when applied an ultrafiltration step to treat the initial extract, it is not

enough to achieve a significant increment in purity. The step of precipitation of phycobiliproteins by itself (53.4 %) is more effective in the purification than the ultrafiltration alone (39.4 %), as proved by HPLC-DAD (data depicted in Table S4 in Supporting Information). With the application of ultrafiltration after precipitation with polyelectrolyte, an extract with high purity in phycobiliproteins was obtained, with just a tenuous band of contaminating protein (~ 120 kDa) present, which is in agreement with the results depicted in Figure 3. It is then evident that the combination of both steps is able to remove most proteins and peptides apart from α and β subunits, characteristic of phycobiliproteins.

After assessing the purity of the samples by SDS-PAGE electrophoresis, the structural integrity of the phycobiliproteins was checked using circular dichroism. With this technique, the secondary structure of the proteins along the different stages of purification using NaPA 8000 were evaluated and compared with pure commercial R-PE. The results are depicted in Figure 5, with the high-tension voltage graph displayed in Figure S1 in Supporting Information.
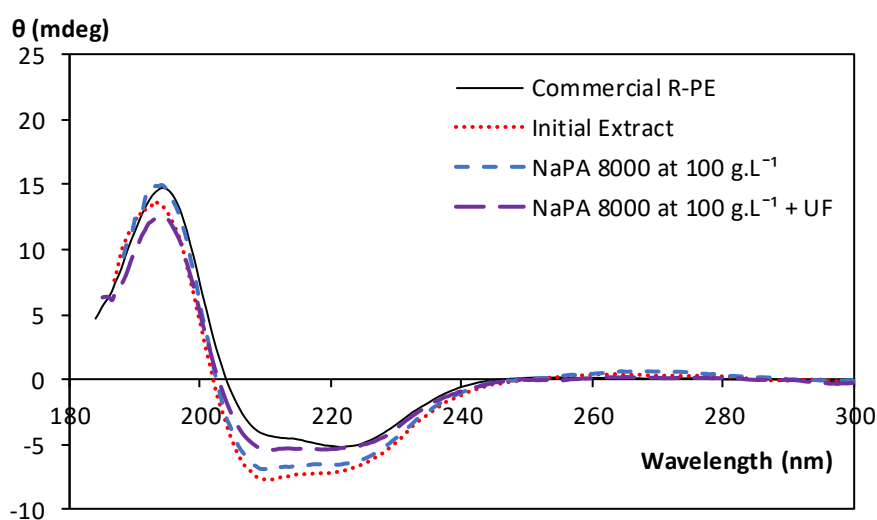
Figure 5. Circular dichroism spectra of the initial extract (dotted line), resuspended pellet after precipitation using NaPA 8000 at 100 g.L^{-1} (smaller dashed line), and resuspended pellet after precipitation using NaPA 8000 at 100 g.L^{-1} followed by an ultrafiltration step (larger dashed line), and commercial R-PE from Sigma-Aldrich (solid line).

The results show that, as the purity of the extracts increases, the better the spectrum fits the commercial R-PE spectra, being indicative of the preservation of the secondary structure of R-PE after purification. The removal of contaminant proteins with different conformations allows the extract to show a spectrum more similar to the commercial R-PE. Besides, and according to literature for R-PE from *Gracilaria chilensis*, the R-PE is mainly composed of α-helixes (71 %) and a minor content in β-sheets and random coils (12 and 17 %, respectively).[49] This also suggests the preservation of the structural integrity of R-PE after precipitation, since the circular dichroism spectra shows the maxima of negative signals at ca. 222 and 210 nm, typical of proteins with a high α-helical content.

In conclusion, the proposed processes (A) for purification of phycobiliproteins and for the (B) selective recovery of R-PE from *Gracilaria gracilis* are represented in Figure 6.
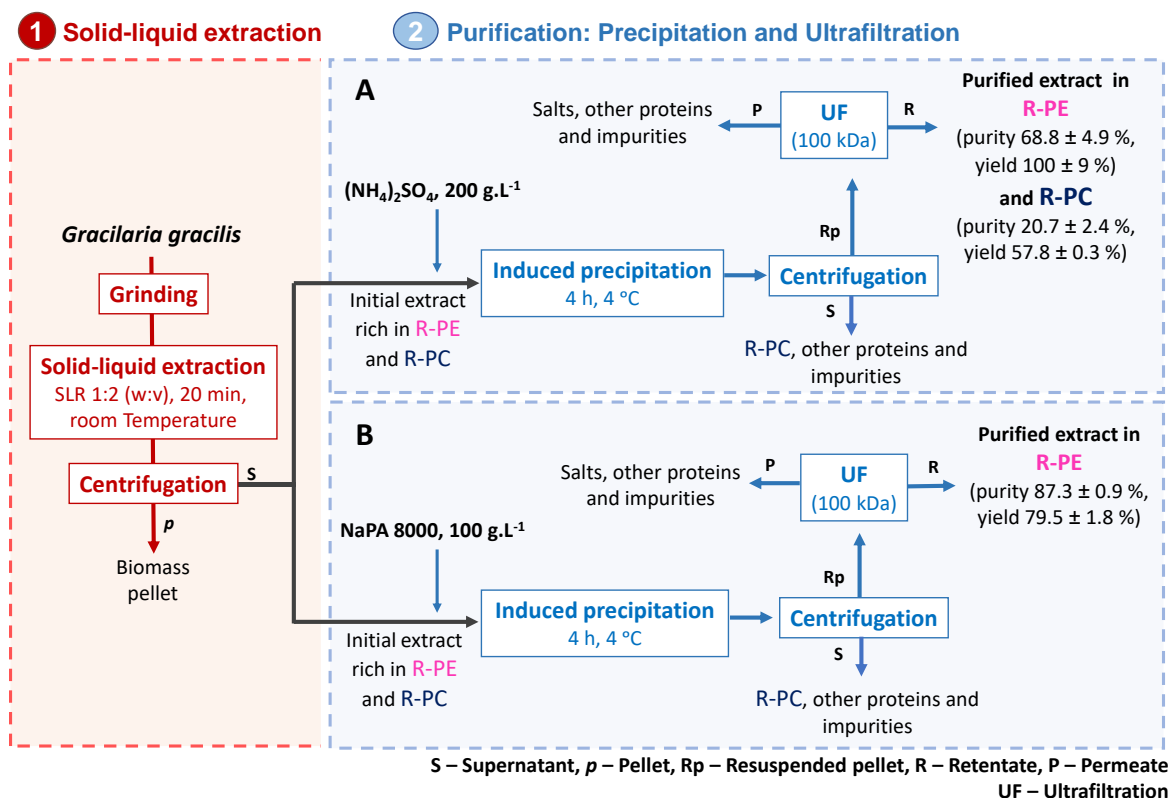
Figure 6. Process flow diagram proposed to obtain a purified extract in phycobiliproteins (A) and an extract with only R-PE (B).

*Environmental evaluation by life cycle assessment*

Aiming to understand the potential environmental impact of the processes developed in this work, and how they do compare with the process already reported using aqueous micellar two-phase systems, the assessment of their environmental impacts was performed. The results of the life cycle assessment, expressed *per* 1 mg of R-PE, show that the impacts of the scenario where $(NH_4)_2SO_4$ is used are 23–25 % smaller than the impacts of the scenario with NaPA 8000 (Table 4 and Figure S2 in Supporting Information). The main reason for this result is the higher yield when $(NH_4)_2SO_4$ is used, which leads to lower values of electricity consumption for obtaining the same amount of R-PE. Another reason is the smaller impacts associated with $(NH_4)_2SO_4$ in comparison

with NaPA 8000. The purification step has the largest impact in both scenarios, mainly
due to electricity consumption during the cycles of ultrafiltration, which contributes to
70–73% of the total impacts (Figure 7).

Table 4. Life cycle assessment for 1 mg of R-PE obtained in both scenarios under study.
Scenario 1 represents NaPA 8000 and scenario 2 represents the $(NH_4)_2SO_4$.

| Life cycle assessment parameters	Scenario 1	Scenario 2
Climate change (g_{CO2eq})	11.6	8.84
Photochemical oxidant formation ($g_{NMVOCeq}$)	0.0453	0.0349
Terrestrial acidification (g_{SO2eq})	0.0803	0.0622
Fossil depletion (g_{oileq})	3.66	2.73
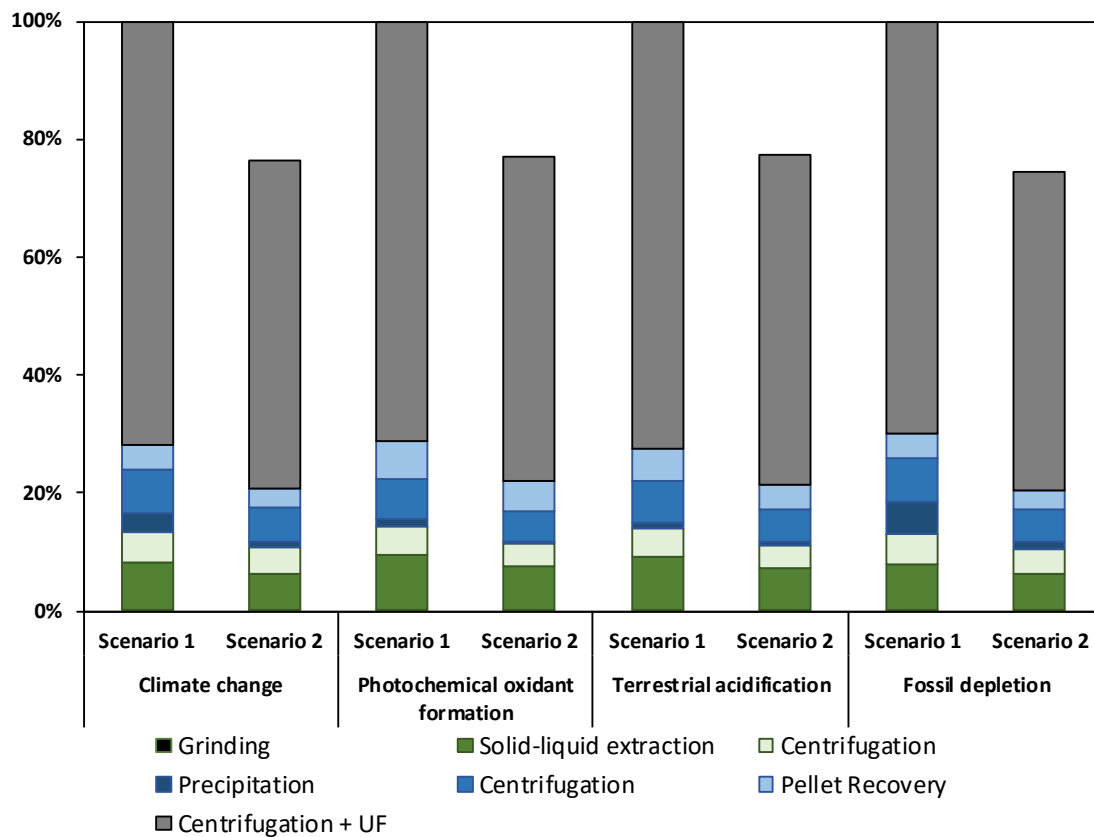
Figure 7. Relative contribution of the operations for the results of life cycle assessment, considering scenario 1 representing NaPA 8000 and scenario 2 representing $(NH_4)_2SO_4$. Greenish bars are related to the recovery of phycobiliproteins from the biomass, blueish bars are related to the precipitation step in the purification approach, and grey bar is related to ultrafiltration.

Despite the small difference between the two scenarios, 1 (NaPA 8000) and 2 $((NH_4)_2SO_4)$, the carbon footprint (corresponding to the climate change results) obtained are much smaller than those reported by Vicente et al.[26] (68.14 and 81.30 kg $CO_{2eq}.mg_{R-PE}^{-1}$) as a result of a much lower electricity consumption in the current process. The process developed in this work proved, not only to be efficient regarding the purification of phycobiliproteins and R-PE in particular, but also to have a low environmental impact.

Economic evaluation

Envisioning the potential industrialization of the process here developed, a detailed economic analysis was performed for both systems, scenario 1 using NaPA 8000 and scenario 2 using $(NH_4)_2SO_4$) as precipitating agents. The production cost *per* mg of R-PE is highly variable and deeply influenced by the process scale (Figure 8). It is important to mention that, as there is not a guide of when to incorporate materials prices for bulk acquisitions, this analysis was performed using the laboratory-scale prices (Table S2 in Supporting Information). Depending on the precipitating agent used, the $CoG.mg^{-1}$ tends to stabilize on 0.93 € *per* mg and 0.32 € *per* mg for NaPA 8000 and $(NH_4)_2SO_4$, respectively.
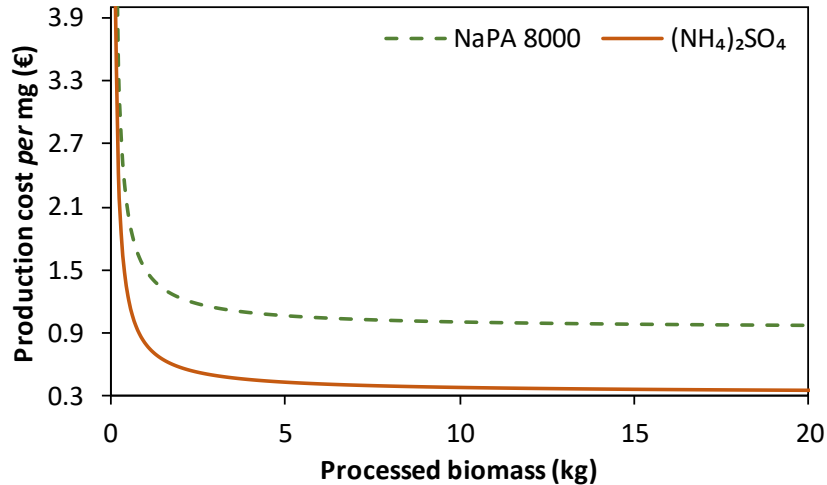
Figure 8. Results obtained from the analysis of production scale (amount of biomass processed).

In practice, different aspects of the bioprocess tend to vary, and thus, a model is very helpful as it is possible to create a wide range of values for different variables to understand how production costs can be affected. For this reason, a sensitivity analysis was performed on the amount of R-PE content in the biomass (mg of R-PE *per* kg of biomass), on the materials cost variation and on the duration of the process. For these three variables, the range of variation was 10-fold (either above or below the amount used for the model construction). Also, the recovery yield of the process was analyzed by a decrease of up to 30 % (worst case scenario), while the best scenario could not be done up to 30 % because of their current level (it will result in recoveries above 100 %), for this reason, the optimal results were fixed at 100 %. The data collected indicated the content of product in the processed biomass as the most important parameter, followed by the materials costs (Figure 9). In general, the impact of all parameters decreases as the production scale increases, which is related to the amount of product being generated, as it dilutes the cost variations. Furthermore, the impact of the amount of

product being generated has been reported continuously to be one of the most important parameters governing the production costs.[50–52] Finally, it is critical to note that for NaPA 8000, the variation on materials costs is more noticeable than for $(NH_4)_2SO_4$. This is because NaPA is a much more expensive material at both laboratory and large scales (Table S2 in Supporting Information).
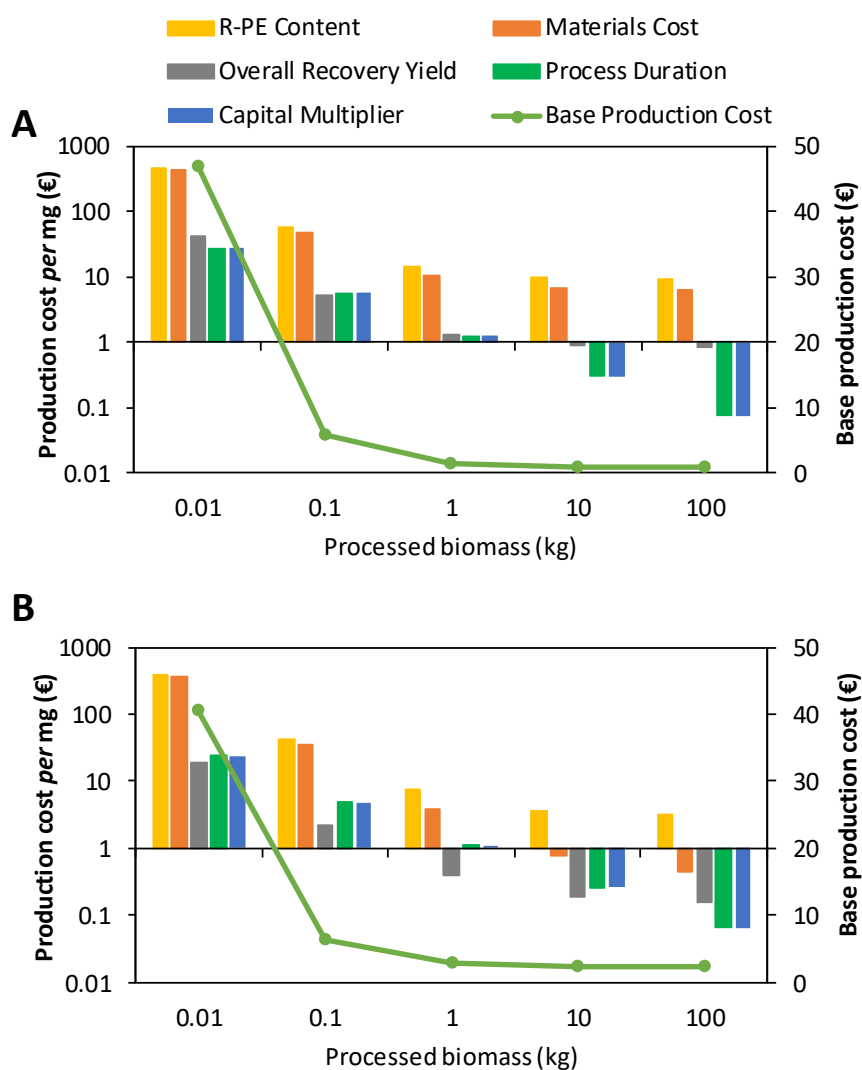
Figure 9. Result for the sensitivity analysis of the complete bioprocess of NaPA 8000 (A) and $(NH_4)_2SO_4$ (B). Results are expressed as the difference of the highest and lowest production costs calculated after varying between the worst and best scenarios.

Additionally, for reference, the base production cost is shown as the green line (right Y-axis). The left Y-axis is presented in a logarithmic scale.

Using the same variables and ranges, a series of Monte Carlo simulations were run to understand how the simultaneous variation of the main parameters affects the production costs. This was done for scales of 0.01 kg and 100 kg (full data is presented in Table S5 in Supporting Information). This results in a collection of statistical data that can show the significance or not of a variable. The main results confirm the importance of the product content in the biomass and of the materials cost variation, but the effect of the second is almost ten times bigger for NaPA 8000 than for $(NH_4)_2SO_4$ at large-scale (Table 5). Interestingly, for all the analyzed scales, the duration of the process is not statistically significant, which means that, if the process is shorter or longer, it will have a negligible effect on the production cost.

Table 5. Results for the Monte Carlo simulations and multiple linear regression. Input variables were in the corresponding multiplier or modifier from the sensitivity analysis. To calculate the CoG.mg^{-1} for R-PE content, materials costs and process duration can be any value that represents a multiplier (used for the modelling were from 0.1X to 10X), while for the recovery yield it is a modifier ($\pm$ 30 %).

	NaPA 8000				$(NH_4)_2SO_4$			
	0.01 kg	*p*-value	100 kg	*p*-value	0.01 kg	*p*-value	100 kg	*p*-value
Intercept (βo)	110.06	1.17×10^{-9}	1.864961	4.19×10^{-11}	78.82741	4.67×10^{-8}	0.344542	2.88×10^{-22}
R-PE content (β1)	-28.5299	1.43×10^{-24}	-0.44852	5.06×10^{-25}	-23.5171	4.22×10^{-30}	-0.06802	1.42×10^{-41}
Overall recovery yield (β2)	-0.94794	0.042111	-0.01485	0.04056	-0.71106	0.202417	-0.00254	0.0505
Materials cost (β3)	22.79631	3.47×10^{-18}	0.340471	4.68×10^{-17}	20.58119	1.39×10^{-25}	0.030776	1.39×10^{-12}
Process duration (β4)	-1.3313	0.586571	-0.0387	0.309369	0.020603	0.991292	0.000104	0.981153

Equations have the form (Eq. 7):

$$Production\ Cost\ [€.\,\mathrm{mg}^{-1}] = β0 + β1 \times W + β2 \times X + β3 \times Y + β4 \times Z \qquad \textbf{(7)}$$

From Eq. 7, *W* represents the R-PE content, *X* the overall recovery yield, *Y* the material cost, and *Z* the process duration. Lastly, the *Return per* kg of processed biomass was performed at laboratorial-scale (0.01 kg) and large-scale (100 kg) using prices from Sigma-Aldrich (for 0.01 kg) and Alibaba (for 100 kg), the latest being considered as an example of a real-life value. Additionally, the amount of product in the biomass (C_{prod}) was varied by 0.5X, 1X or 2X and the $CoG.kg^{-1}$ of processed biomass was varied by a factor of 1X, 2X and 5X. Moreover, the $prod was varied from € 5 *per* kg to € 5,000 *per* kg. Results provide an in-depth look into different scenarios and how they can influence the potential economic return for this process (Figure 10 and Figure S3 in Supporting Information).
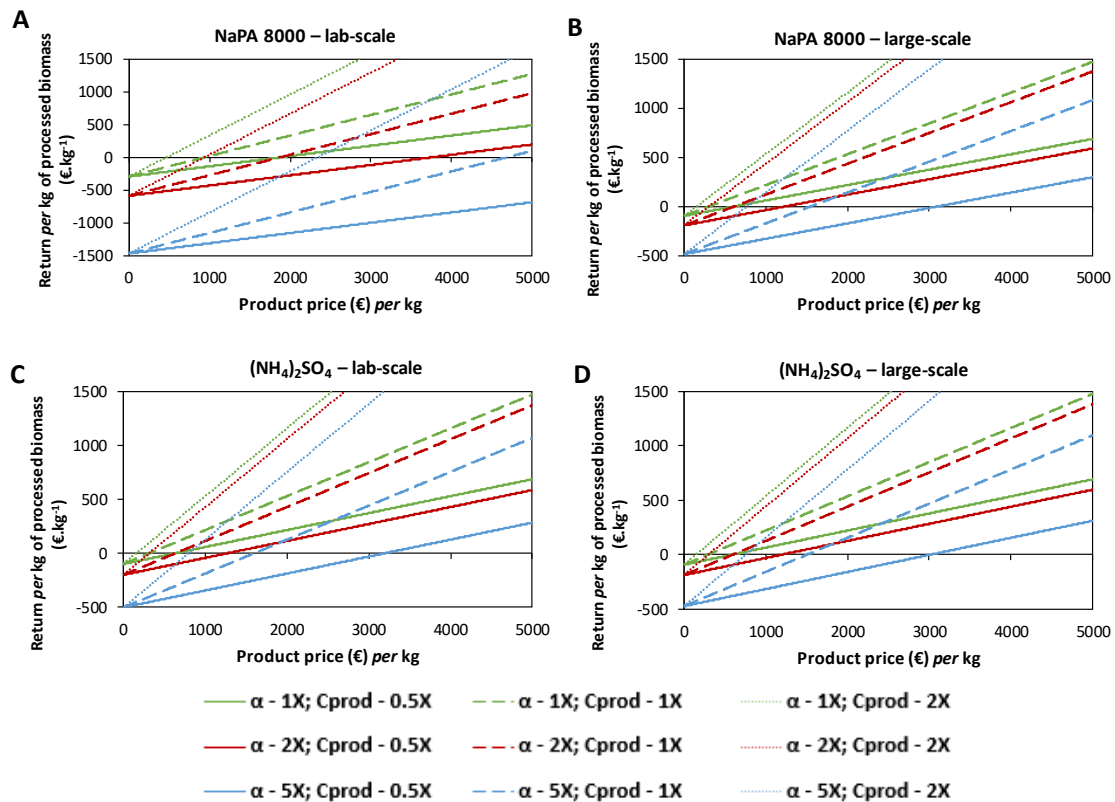
Figure 10. Results for the *Return* (R) analysis for NaPA 8000 and $(NH_4)_2SO_4$. NaPA 8000 results are presented in A) for laboratory-scale (0.01 kg) and B) for large-scale (100 kg), while for $(NH_4)_2SO_4$ are C) laboratory-scale (0.01 kg) and D) for large-scale (100 kg). Green lines are for an alpha of 1X, red for alpha of 2X and blue for alpha of 5X; solid lines for a C_{prod} of 0.5X, dash lines for C_{prod} of 1X and dot lines for C_{prod} of 2X.

Results from this analysis can help to appreciate different issues considered relevant for the efficiency and sustainability of the process. The slope of each line is the influence of the C_{prod} on the *Return*: the higher the C_{prod}, the more vertical the line will be. Additionally, the position where the lines intercept with the y-axis (the point where \$prod is 0), is dictated by the $CoG.kg^{-1}$ of biomass. The most evident result is the abrupt difference on the y-axis intercept for Figure 10A and 10B, indicating the impact that the change in the price of the materials has on the $CoG.kg^{-1}$ of biomass. From the data on

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Table S2 in Supporting Information, the price reduction of NaPA 8000 from laboratory to large-scale is much larger compared to the decrease of $(NH_4)_2SO_4$ price, which can be related to the extensive use of $(NH_4)_2SO_4$. Moreover, this dramatic change becomes the critical aspect for determining, for specific conditions, if there is any *Return* at all.

Given the results obtained here, even after increasing the potential CoG.kg^{-1} of biomass by 5-fold, reducing the C_{prod} by half, it is possible to have a positive *Return* and possible above the € 1,000 *per* kg of product. This can be ensured and enhanced if the bioprocess developed here can increase the purity of the product, then its market price can be increased. As a reference, commercial price of R-PE from Sigma-Aldrich (Product 52412) sells at € 155 *per* mg (€ 155,000,000 *per* kg).

Conclusions

In this work, a new approach, easy to implement, using induced precipitation, is proposed for the purification of phycobiliproteins, in particular R-PE. A set of polymers, copolymers, and polyelectrolytes was screened correlating their ability to selective precipitate proteins from a raw extract of phycobiliproteins regarding the purification of fluorescent proteins. It was found that the most common used precipitation agent in proteins – $(NH_4)_2SO_4$ – at 200 g.L^{-1} is able to precipitate both R-PE and R-PC but it is not selective, while the polyelectrolyte NaPA 8000, even at low concentrations (100 g.L^{-1}), can selectively induce the precipitation of R-PE among the set of phycobiliproteins present in the extract. By further using an ultrafiltration step, purities of 89.5 % and 87.3 % were achieved, respectively for the two phycobiliproteins using the strategy of $(NH_4)_2SO_4$ followed by ultrafiltration and for only R-PE using NaPA 8000 followed by ultrafiltration, having this last one its structural integrity preserved. Summing up, and

despite the regular use of $(NH_4)_2SO_4$, its use did not allow the development of a selective induced precipitation, which is surpassed by the use of NaPA 8000.

Taking into account the results of selectivity for the system using NaPA 8000, the environmental impact was determined and compared with one of the most recent reports of processes optimized for the purification of R-PE using aqueous micellar two-phase systems. The low carbon footprint of the process optimized by using induced precipitation with NaPA 8000, shows that the process here proposed has a lower environmental impact. Using the current process results combined with the economic analysis, it was concluded that a potential real-life application can provide return dependent on the market price of the R-PE product. Some of the major factors to determine the required price are the amount of R-PE content in the biomass (or the amount extracted from it) and the price of the materials during a large-scale operation. The use of NaPA 8000 or $(NH_4)_2SO_4$ provides cost-effective results and, ultimately, the decision on their selection can be based on process-oriented results, such as the purity required of the product for the desired application, along with the possible commercial price of the product.

Supporting Information

High-tension voltage graphs representing the circular dichroism spectra, detailed values and calculations for environmental and economic analysis, hydrophilic-lipophilic balance of copolymers, and detailed data of purity and yield obtained in different fractions.

Acknowledgments

Conflicts of interest

There are no conflicts of interest to declare.

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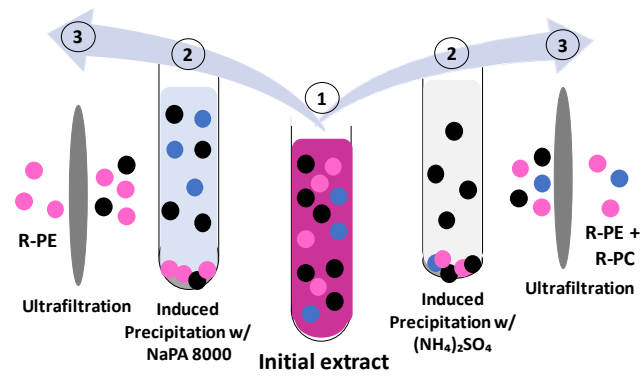
Recovery of phycobiliproteins from *Gracilaria gracilis* based on induced precipitation with NaPA 8000 or $(NH_4)_2SO_4$ conjugated with ultrafiltration.