

1 **Abstract**

2 Phycobiliproteins are fluorescent proteins mainly produced by red macroalgae and
3 cyanobacteria. These proteins, essential to the survival of these organisms, find
4 application in many fields of interest, from medical, pharmaceutical, and cosmetic to
5 food and textile industries. The biggest obstacle to its use is the lack of simple
6 environmental and economical sustainable methodologies to obtain these proteins with
7 a high purity.

8 In this work, a new purification process is proposed based on the induced precipitation
9 of the target proteins followed by an ultrafiltration. Purities of 89.5 % of both
10 phycobiliproteins and 87.3 % of R-phycoerythrin were achieved while keeping the
11 protein structure stability and high recovery yields. Environmental and economic
12 analyses performed to evaluate the proposed process show that the carbon footprint
13 for the proposed process is much lower than those reported for alternative processes,
14 and the economic analysis reveals the cost-effective character associated to its high
15 performance.

16

17

18 **Keywords:** *Gracilaria gracilis*, induced precipitation, purification, phycobiliproteins, R-
19 phycoerythrin.

20

21

1 Introduction

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

13 Macroalgae are an example of a biomass that could allow the development of a
14 biorefinery focusing a blue economy. Many high-value products, such as pigments,
15 phenols, lipids, and proteins, are already being explored in what should be the beginning
16 of the biorefinery cascade.⁸

17 Phycobiliproteins are a family of fluorescent and hydrophilic proteins involved in the
18 light-harvesting processes in red macroalgae. This family of proteins, in red macroalgae,
19 is mainly composed of R-phycoerythrin (R-PE) and R-phycoyanin (R-PC).⁹ R-PE has a soft
20 pink color and orange fluorescence, composed of $(\alpha\beta)_6\gamma$ complexes and with 240 kDa,
21 while R-PC has a blue color and red fluorescence, composed of $(\alpha\beta)_3$ complexes.^{9,10} Due
22 to their spectroscopic and fluorescent properties, those proteins can be applied in
23 different fields from biotechnology, biomedicine, pharmaceuticals, cosmetics, and food
24 products.¹¹ More recently, extracts rich in phycobiliproteins were also studied as natural

1 dyes to use as optical active centers for sustainable luminescent solar concentrators and
2 proving their potential towards cheap and sustainable photovoltaic energy conversion.¹²
3 Despite the efforts from several researchers on the development of new processes to
4 obtain pure phycobiliproteins, these are still far from industrialization. The purity level
5 required is defined by the application/product demands, and the process to be
6 implemented should take these requirements into account.

7 Conventionally, the purification of phycobiliproteins can be achieved by a set of unit
8 operations that may include (i) a pre-purification step commonly applying ammonium
9 sulfate precipitation, (ii) one or more purification steps applying membrane separation
10 processes (*i.e.* ultrafiltration (UF) and cross-flow ultrafiltration) and/or chromatographic
11 processes which are usually column chromatography (*i.e.* size exclusion-, ion exchange-
12 , hydrophobic interaction-, and affinity-chromatography), and iii) a last step of dialysis
13 to completely remove, replace, or decrease the concentration of salts or solvents from
14 the purified extracts.¹³⁻¹⁹ Recently, alternative methodologies of protein purification
15 have been proposed, such as membrane chromatography,²⁰ centrifugal precipitation
16 chromatography,²¹ electrophoretic elution,²² vortex flow reactor in an adsorption
17 experiment,²³ and aqueous micellar two-phase systems.²⁴ However, most of them have
18 disadvantages related to complexity, difficulty to scale-up and high associated costs,
19 limiting the applicability of these processes at an industrial scale. This is also true for the
20 process we have previously proposed based on the use of aqueous micellar two-phase
21 systems.²⁴ Despite the good results achieved for the purification of phycobiliproteins,
22 and R-PE in particular, the process included 5 main steps, comprising a first solid-liquid
23 extraction, two units of purification applying aqueous micellar two-phase systems
24 followed by two units of operation to separate the target proteins from the main

1 solvents used. In this context, the present work will attempt at the development of a
2 simpler process to purify phycobiliproteins, and also R-PE. The first approach to be used
3 was the elimination of the fourth and fifth steps of our previous process involving the
4 separation of the target proteins after purification from the extraction solvents. For that,
5 the use of induced precipitation seems to be a good strategy. The recovery and
6 purification of proteins by precipitation is one of the most important operations in
7 protein purification, recurrently used in laboratories and also industry.²⁵ This is achieved
8 by the destabilization of a protein solution that is then separated from the
9 liquid/supernatant by gravity settling, centrifugation, or filtration. The precipitation can
10 be driven by the ionic strength of the medium, but also by size exclusion, pH and
11 temperature variations.^{26,27} Much work has been done regarding the use of ammonium
12 sulfate, which is a classic salting-out agent and usually the first choice in protein
13 precipitation.²⁷ However, and despite its high efficiency promoting precipitation, it is not
14 selective, which means that it will precipitate all the proteins in the solution. It is also
15 known that many other compounds can act as precipitation agents, such as polymers,
16 copolymers, and polyelectrolytes by different phenomena such as crowding or by direct
17 interaction between the protein and the precipitation agent that can tune the solubility
18 decrease of the target protein from a crude extract, thus leading to a selective
19 precipitation.²⁶⁻³⁰

20 Precipitation is normally used as a pre-treatment^{18,31}, meaning that it is complemented
21 by a set of other purification steps. However, in this work, the main objective was to
22 decrease the number of steps required to obtain pure phycobiliproteins from *Gracilaria*
23 *gracilis*, in particular, R-PE, thus avoiding the application of other purification steps. The
24 screening of various potential precipitating agents was studied from a large set of

1 polymers, copolymers, and polyelectrolytes. After selecting the best precipitating agent,
2 the process variables were optimized, and a final step of ultrafiltration was used. The
3 structural integrity of the fluorescent proteins was assessed by circular dichroism. In the
4 end, and considering that the main objective of this work was the development of a
5 simple and efficient process to obtain the phycobiliproteins (and particularly, R-PE), a
6 life cycle analysis was done to compare this process with the one previously proposed
7 by us using aqueous micellar two-phase systems,²⁴ followed by an economic analysis,
8 based on which the viability and sustainability of this process is discussed.

9

10 **Experimental**

11 *Biomass*

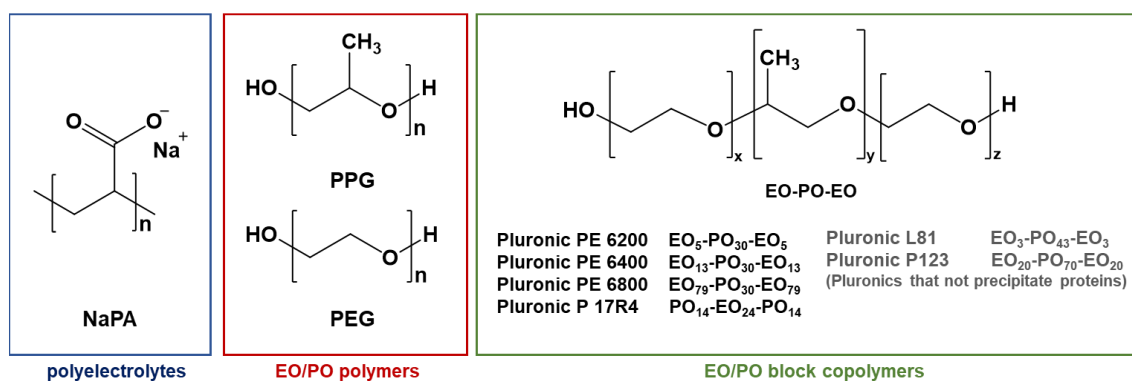
12 The biomass used in this work, fresh *Gracilaria gracilis*, was kindly provided by ALGAplus
13 (Ílhavo, Portugal). ALGAplus farms the macroalgae at Ria de Aveiro lagoon (40°36'44.7"
14 N, 8°40'27.0" W) in coastal Portugal under the EU organic aquaculture standards
15 (EC710/2009). This aquaculture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

20

21 *Chemicals*

22 Ammonium sulfate ((NH₄)₂SO₄, 99.5 %) was acquired from Merck. Poly(acrylic acid)
23 sodium salts with average molecular weight of 1200 g.mol⁻¹ (NaPA 1200, 45 wt % in
24 water solution) and 8000 g.mol⁻¹ (NaPA 8000, 45 wt% in water solution), poly(ethylene

1 glycol) (PEG) with average molecular weight of 8000 g.mol⁻¹ (PEG 8000, pure) and
 2 poly(propylene glycol) (PPG) polymer with average molecular weight of 400 g.mol⁻¹
 3 (PPG 400, pure) were purchased from Sigma-Aldrich. PEG with average molecular
 4 weight of 10000 g.mol⁻¹ (PEG 10000, pure) was supplied from Fluka.
 5 Nonionic copolymers composed of PEG and PPG blocks were also used (Figure 1). Their
 6 commercial names were adopted along this work. Pluronic PE 6800 (PPG-PEG-*blocks*
 7 with approx. 8000 g.mol⁻¹, composed of 80 wt % PEG), Pluronic PE 6400 (PPG-PEG-
 8 *blocks* with approx. 2900 g.mol⁻¹, composed of 40 wt % PEG) and Pluronic PE 6200 (PPG-
 9 PEG-*blocks* with approx. 2450 g.mol⁻¹, composed of 20 wt % PEG) were purchased from
 10 BASF. Pluronic P 17R4 (PPG-PEG-PPG-*blocks* with approx. 2700 g.mol⁻¹, composed of
 11 40 wt % PEG), Pluronic L81 (PEG-PPG-PEG-*blocks* with approx. 2800 g.mol⁻¹, composed
 12 of 10 wt % PEG), and Pluronic P123 (PEG-PPG-PEG-*blocks* with approx. 5800 g.mol⁻¹,
 13 composed of 30 wt % PEG) were acquired from Sigma-Aldrich.
 14 As standard, commercial R-PE (CAS 11016-17-4) supplied by Sigma-Aldrich was used.



15

16 **Figure 1.** Structures of the precipitation agents studied in this work.

17

18 *Solid-liquid extraction*

19 The solid-liquid extraction procedure was adopted from Martins et al.³² but with some
 20 modifications. Briefly, fresh *Gracilaria gracilis* was ground in a coffee mill after being

1 frozen with liquid nitrogen for a more efficient extraction. The extraction was performed
2 using distilled water as solvent in a solid-liquid ratio of 1:2 w/v during 20 min at room
3 temperature in an orbital shaker (IKA KS 4000 ic control) at room temperature, at 250
4 rpm and protected from light. The crude extract was obtained after centrifugation at
5 13800 g, 20 min, at room temperature in a VWR microstar 17 centrifuge.

6

7 *Induced precipitation*

8 Several precipitation agents were tested at three different concentrations (10, 20 and
9 30 g.L⁻¹). Each precipitation agent was dissolved in the crude extract and left overnight
10 at 4 °C. Pellet and supernatant phases were induced by centrifugation at 900 g, for
11 15 min at room temperature in the VWR microstar 17 centrifuge using the same
12 conditions described in the section Solid-liquid extraction. After centrifugation, the
13 pellet was resuspended in the same initial volume using distilled water. When particles
14 not soluble in water are observed in the resuspended pellets (that happened in PEG
15 10000 and Pluronic PE 6200), a vigorous centrifugation at 9600 g for 5 min was applied
16 to remove these solids before further analysis.

17

18 *Ultrafiltration (UF)*

19 500 µL of sample was added in each Amicon Ultra-0.5 mL Centrifugal Filter Unit 100 K.
20 The sample was centrifuged at 14000 g during 15 min. The permeate was discarded and
21 400 µL of ultrapure water was added to the concentrate and centrifuged in the same
22 conditions, being this last step repeated twice. Lastly, 500 µL of ultrapure water was
23 added to recover the concentrated sample after a centrifugation of 2 min, at 1000 g.

24

1 *Spectroscopic methods*

2 The absorption spectra of different fractions were measured between 200 and 700 nm
3 using a UV-Vis microplate reader (Synergy HT microplate reader – BioTek). This
4 technique was used in the initial screening of precipitation agents, in which the
5 phycobiliproteins were quantified directly at 565 nm, and the total amount of proteins
6 was quantified by the Bicinchoninic Acid (BCA) method at 562 nm, considering two
7 calibration curves previously prepared ($R^2 = 0.999$ and $R^2 = 0.998$, for phycobiliproteins
8 and total proteins, respectively). The total protein concentration was determined with
9 the Pierce™ BCA Protein Assay and Micro BCA Protein Assay (Thermo Scientific,
10 Schwerte, Germany) according to the supplier recommendations. Bovine serum albumin
11 (from Fisher Scientific) was used as the standard protein.

12 Parameters as selectivity and R-PC index were calculated according to Vicente et al.²⁴ In
13 order to determine the selectivity, the partition coefficient of R-PE (K_{R-PE}) and total
14 proteins ($K_{\text{Total proteins}}$) were firstly calculated (Eqs. 1 and 2, respectively). This parameter
15 is the ratio between the concentration of R-PE (or total proteins) in the purified fraction
16 and the discarded phases along the purification steps. Knowing the partition coefficient
17 of both R-PE and total proteins, the selectivity of the proposed method was determined
18 according to Eq. 3.

19
$$K_{R-PE} = \frac{[R-PE]_{\text{purified fraction}}}{[R-PE]_{\text{discarded fraction}}} \quad \text{Eq. 1}$$

20
$$K_{\text{Total proteins}} = \frac{[\text{Total proteins}]_{\text{purified fraction}}}{[\text{Total proteins}]_{\text{discarded fraction}}} \quad \text{Eq. 2}$$

21
$$S = \frac{K_{R-PE}}{K_{\text{Total proteins}}} \quad \text{Eq. 3}$$

1 The R-PC index relates the amount of R-PC and R-PE in a sample and it was calculated
2 by the ratio between the maximum absorbance of R-PC and R-PE, *i.e.* the absorbance at
3 617 nm and 565 nm, respectively (Eq. 4).

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

5 The purity parameter was obtained as the ratio between the phycobiliproteins content
6 and the amount of (total) proteins in the resuspended pellet, these values being
7 presented as a percentage. The yield was calculated as the ratio between the
8 phycobiliproteins content in the resuspended pellet and the phycobiliproteins content
9 in the initial extract.

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

1 Circular dichroism spectra were recorded using a Jasco J-815 circular dichroism
2 spectrometer at 298.15 K in the far UV region ($\lambda = 180\text{--}260$ nm). Spectra were collected
3 in a 0.1 cm path length quartz cuvette at a scan rate of $100\text{ nm}\cdot\text{min}^{-1}$ and sensitivity of
4 100 mdeg. The response time and the bandwidth were 2 s and 0.5 nm, respectively. The
5 samples were solubilized in distilled water up to a dilution where the influence of the
6 sample interferences was negligible, being in those conditions the circular dichroism
7 spectra obtained with high tension voltage below 600 (Figure S1, ESI).

8

9 *SDS-PAGE*

10 The phycobiliprotein crude extract was analyzed through electrophoresis that was
11 prepared on polyacrylamide gel (stacking: 4% and resolving: 20 %) with a running buffer
12 consisting of $250\text{ mmol}\cdot\text{L}^{-1}$ of Tris-HCl, $1.92\text{ mol}\cdot\text{L}^{-1}$ of glycine, and 1 % of SDS. The
13 proteins were stained with the usual staining procedure [Coomassie Brilliant Blue G-250
14 0.1 % (w/v), methanol 50 % (v/v), acetic 7 % (v/v), and water 42.9 % (v/v)] in an orbital
15 shaker, at moderate speed, for 2 - 3 hours at room temperature. The gels were
16 destained in a solution containing acetic acid 7 % (v/v), methanol 20 % (v/v), and water
17 73% (v/v) in an orbital shaker at ± 60 rpm for 3 - 4 hours at room temperature. SDS-PAGE
18 molecular weight standards and marker molecular weight full-range (VWR) were used
19 as protein standards. All gels were analyzed using the Image Lab 3.0 (BIO-RAD) analysis
20 tool.

21

22 *Environmental evaluation: carbon footprint analysis*

23 The environmental profile of the two scenarios to extract and purify R-PE was evaluated
24 by life cycle assessment, according to ISO 14040 standard,³³ and covering the impacts

1 from the production of the chemicals used in the processes, water and also the
2 electricity consumption. Table S1 of ESI shows the amounts of chemicals and water
3 consumed during the experimental procedure, as well as the amounts of electricity
4 spent. These parameters were calculated for each equipment based on the time of
5 operation, nominal power and fraction of occupancy over total capacity. These amounts
6 are expressed *per* mg of R-PE obtained to allow comparison between the two scenarios:
7 the approach developed in this work and another previously published in literature.²⁴
8 The impact factors associated with the production of chemicals and electricity
9 (Portuguese mix) were taken from the Ecoinvent 3.6 database.³⁴ The impact factors for
10 distilled and ultrapure water result from tap water production³⁵ and electricity
11 consumed during the distillation and ultrafiltration.³⁴ The impact assessment method
12 was the ReCiPe 2016 Midpoint at the Hierarchist perspective,³⁶ considering the
13 following impact categories: climate change (equivalent to the carbon footprint),
14 photochemical oxidant formation, terrestrial acidification and fossil depletion. The
15 results were compared with the ones obtained by Vicente and collaborators when
16 applying aqueous micellar two-phase systems to purify phycobiliproteins.²⁴

17 *Economic evaluation*

18 To further expand this study and understand some of the potential economic constrains
19 of implementing the process optimized in this work into an industrial scenario, an
20 economic analysis was performed considering the traditional approach (using
21 $(\text{NH}_4)_2\text{SO}_4$) and the alternative precipitation method proposed in this work. In this
22 analysis, the production cost was calculated *per* mg of R-PE ($\text{CoG} \cdot \text{mg}^{-1}$).^{37,38} Briefly, three
23 areas need to be fulfilled to have a complete process: to set up a target output or

1 production scenarios, then to determine the sequence of unit operations and their
2 process parameters, and finally to collect the economic datasets to postulate the model.
3 For the process developed in this work, the production scale to be used at the industrial
4 stage ~~have~~has not been decided and for this reason, five different scales were analyzed,
5 namely 0.01 kg, 0.1 kg, 1 kg, 10 kg, and 100 kg. This will give a wide range of operation
6 ranging from the laboratory, to pilot and, finally, industrial scales. The sequence of unit
7 operations is something that will be discussed in later sections as a result of all the
8 analyses performed in this work, but briefly, it consists of a water extraction of R-PE
9 from the biomass, then a centrifugation to remove the spent biomass. For the
10 precipitation stage, it starts with the mixing of the extract with the precipitant in a tank,
11 followed by the induced precipitation using a centrifugal step, and a re-suspension of
12 the pellet. The process ended with an ultrafiltration/diafiltration step to remove the
13 non-suspended proteins, allowing also the final polishing.

14 The economic datasets are composed of different areas. For the capital investment
15 (mainly equipment acquisition costs), cost of equipment was obtained from the
16 database on the software Biosolve Process (Biopharm Services Ltd., Buckinghamshire,
17 UK), then different regressions were determined to interpolate the results considering
18 the different scales needed. The same strategy was employed for consumables (vessel
19 filters and ultrafiltration/diafiltration membranes). For materials costs (chemicals), as
20 this analysis comprised small and large scales, their costs were obtained from Sigma-
21 Aldrich and Alibaba, respectively. Labor has been reported to be approximately 15 % of
22 the total production costs,³⁹ so this approach was taken here. Lastly, an additional
23 economic aspect was denoted as “others”, in which utilities and maintenance costs were
24 included. This was calculated following Biosolve Process approach, which estimates

1 these costs as 4 % of the capital investment. Full data for process and economic
2 parameters employed here are included in Table S2 in ESI.

3 After the completion of the model construction, different analyses were performed to
4 understand how the CoG.mg⁻¹ of the R-PE behave. First, different production scales were
5 evaluated, for the whole range mentioned before (0.01 kg to 100 kg), following
6 incremental steps of 0.1 kg. Then, using only the discrete range of production scales
7 (0.01 kg, 0.1 kg, 1 kg, 10 kg, and 100 kg), a sensitivity analysis was performed by
8 systematically varying the values of the amount of R-PE content in the biomass (mg of
9 R-PE *per* kg of fresh biomass), the materials costs variation and the duration of the
10 process, all of them in a range from 10-fold above and below ($\pm 10X$). Additionally, the
11 impact of the overall recovery yield was included, but due to the results obtained, the
12 range was constrained, the worst-case scenario was 30 % less of what is reported in the
13 following sections and the best scenario can only increase up to 100 %. This analysis can
14 provide an insight on how each individual parameter affects the production costs and
15 help potentially to devise strategies to control their variations. As a complement to the
16 sensitivity analysis, a series of Monte Carlo simulations was performed varying the same
17 parameters, with the same ranges, but under a triangular distribution and calculating
18 their respective production costs (CoG.mg⁻¹) for each scenario. Afterwards, a multiple
19 linear regression was calculated to obtain the coefficients and *p*-value for each
20 parameter.

21 An additional approach was determined in this work, which results on the calculation of
22 the potential income, or Return (R), that the product could provide and to understand
23 how the different process parameters could affect it. Based on other reports,⁴⁰ Eq. 5 was
24 defined to calculate the R based on the results obtained from this work:

1 $R = [C_{\text{prod}} \times \$_{\text{prod}} - \$_{\text{biom}}] - [(\alpha) \times (\text{Production cost per kg of biomass})]$ Eq. 5

2

3 In Eq. 5, R stands for the Return *per* kg of processed fresh biomass, C_{prod} is the amount
4 of product *per* kg of biomass, $\$_{\text{prod}}$ is the commercial price of R-PE on the market and
5 $\$_{\text{biom}}$ is the cost associated with the acquisition of the biomass. While, in the second
6 term, the production cost *per* kg of biomass is a conversion of the $\text{CoG} \cdot \text{mg}^{-1}$ of R-PE into
7 a $\text{CoG} \cdot \text{kg}^{-1}$ of processed biomass. To obtain this, it is needed to obtain the production
8 cost *per* batch (CoG/batch) and to divide it by the amount of biomass processed in that
9 particular batch. The α is an additional term employed as a multiplier of the $\text{CoG} \cdot \text{kg}^{-1}$ in
10 order to increase or decrease its impact consequently allowing us to analyze their effect
11 in case the real production costs are higher or lower. As part of the Return analysis, a
12 sensitivity analysis was performed by varying the C_{prod} by 0.5X, 1X, or 2X (half or double
13 of the base concentration) and the α term was varied between 1X, 2X, or 5X.
14 Additionally, R-PE has a wide range of prices depending on the application, purity and
15 amount being acquired, and for this reason, the range of € 5 to € 5,000 *per* kg was
16 analyzed.

17

18 **Results**

19 *Screening of precipitation agents*

20 Various phenomena can promote protein precipitation however, substances (generally
21 in high concentration) changing the environment of the protein (e.g. some organic
22 solvents, salts, and neutral polymers); or substances (generally at low concentration)
23 interacting directly with the protein (e.g. acids, bases, polyelectrolytes and some metal
24 ions), have been reported as the most relevant.²⁵ In this work, a screening of polymers,

1 copolymers, and polyelectrolytes at different concentrations was performed, being their
 2 ability to induce protein precipitation reported in Table 1 and their performance
 3 compared with the results obtained for $(\text{NH}_4)_2\text{SO}_4$ (the conventional precipitation agent
 4 here used as control).

5
 6 **Table 1.** List of precipitation agents screened according to their ability to precipitate
 7 phycobiliproteins from the raw extract at different concentrations. The symbols ✓ and
 8 ✗ represent, respectively, the systems with and without protein precipitation occurring.

Precipitation agent	Concentration (g.L ⁻¹)		
	10	20	30
NaPA 1200	✓	✓	✓
NaPA 8000	✓	✓	✓
PEG 8000	✗	✗	✗
PEG 10000	✓	✓	✓
PPG 400	✓	✓	✓
Pluronic PE 6800	✓	✓	✓
Pluronic PE 6400	✓	✓	✓
Pluronic PE 6200	✓	✓	✓
Pluronic P 17R4	✓	✓	✓
Pluronic L81	✗	✗	✗
Pluronic P123	✗	✗	✗
$(\text{NH}_4)_2\text{SO}_4$	✓	✓	✓

9
 10 NaPA 1200 and NaPA 8000 are included in the group of precipitation agents interacting
 11 directly with the proteins, while the rest of the substances screened, *i.e.* polymers and
 12 copolymers, act by promoting changes in the environment of the initial solvent.
 13 Although according to literature,²⁵ low concentrations of precipitation agents are
 14 required when their mechanism of action involves the direct interaction with proteins,
 15 both NaPA 1200 and NaPA 8000 were found to be able to induce the precipitation of

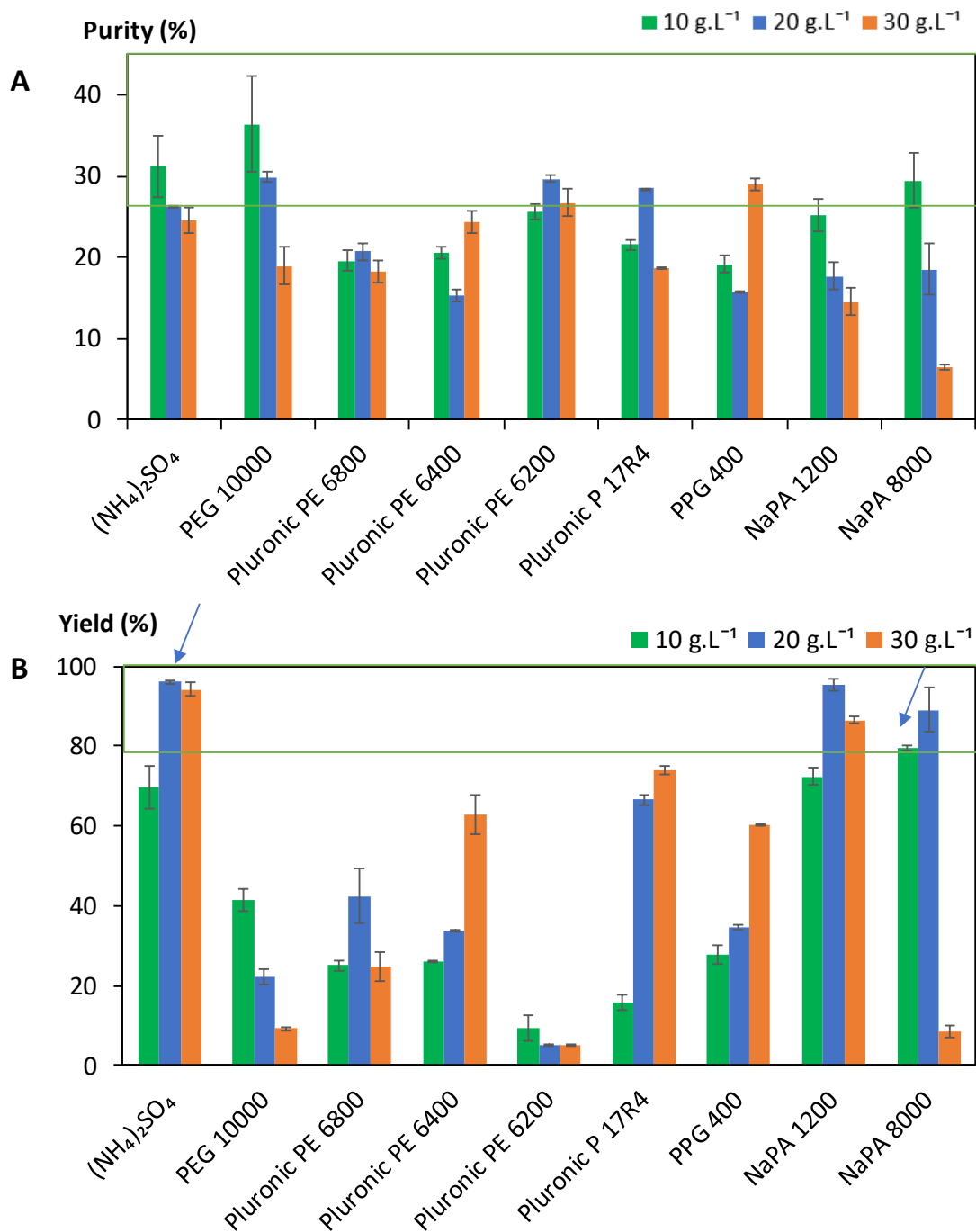
1 phycobiliproteins at all concentrations tested. The worst results, without any
2 precipitation of phycobiliproteins, were obtained for PEG 8000, Pluronic L81 and
3 Pluronic P123, independently of the concentration applied.

4 As previously discussed in literature,⁴¹ the main phenomena behind the protein
5 precipitation with polymers and copolymers is, in general, a result of the crowding
6 effect, which happens when high concentrations of these molecules are introduced in
7 the system, drastically reducing the volume of water molecules available for protein
8 solvation. In this context, it is well established that PEGs with high molecular weights
9 more easily precipitate proteins, which can explain the difference in the behaviours of
10 PEG 8000 and PEG 10000.

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

21 After selecting from Table 1 all the compounds able to precipitate the phycobiliproteins,
22 and considering the viscosity of the solutions, and the color intensity in the supernatants
23 (which is a proxy for the residual amounts of phycobiliproteins in solution) only

- 1 Pluronics, PPG 400 and NaPA 1200 and NaPA 8000 were retained to further evaluate the
- 2 purity and yield parameters (Figure 2).



- 3
- 4 **Figure 2.** Results obtained for the (A) purity and (B) yield (%) obtained in the
- 5 resuspended pellets after the precipitation step using different precipitation agents at
- 6 three distinct concentrations (10, 20 and 30 g.L⁻¹). These analyses were assessed by UV-
- 7 Vis absorption spectroscopy.

1 In order to find the best precipitation agent, a compromise between purity and yield of
 2 precipitation was required. The objective was to select the system providing the highest
 3 purity levels of phycobiliproteins without compromising the yields of precipitation. After
 4 the interpretation of the data presented in Figure 2 and in order to proceed with the
 5 analysis, the criteria selected was the following: to identify the precipitation agents able
 6 to simultaneously provide purities and yields higher than 25 % and 80 %, respectively.
 7 The systems fulfilling this criteria were the traditional $(\text{NH}_4)_2\text{SO}_4$ at 20 g.L^{-1} (purity =
 8 $26.2 \pm 0.1 \%$ and yield = $96.0 \pm 0.5 \%$) and the polyelectrolyte NaPA 8000 (purity = $29 \pm$
 9 3% and yield = $79.6 \pm 0.7 \%$). After choosing the best systems and respective
 10 concentrations to induce the precipitation of phycobiliproteins, the extracts obtained
 11 were further analyzed by HPLC-DAD (Table 2). This analysis identifies which
 12 phycobiliprotein (R-PE or R-PC, the two most relevant phycobiliproteins present in the
 13 initial extract^{24,42}) and in what extent, was precipitated. Moreover, it also enabled us to
 14 infer on the selectivity (capacity to separate R-PE from R-PC) of each system (*i.e.*
 15 precipitation agent and its concentration).

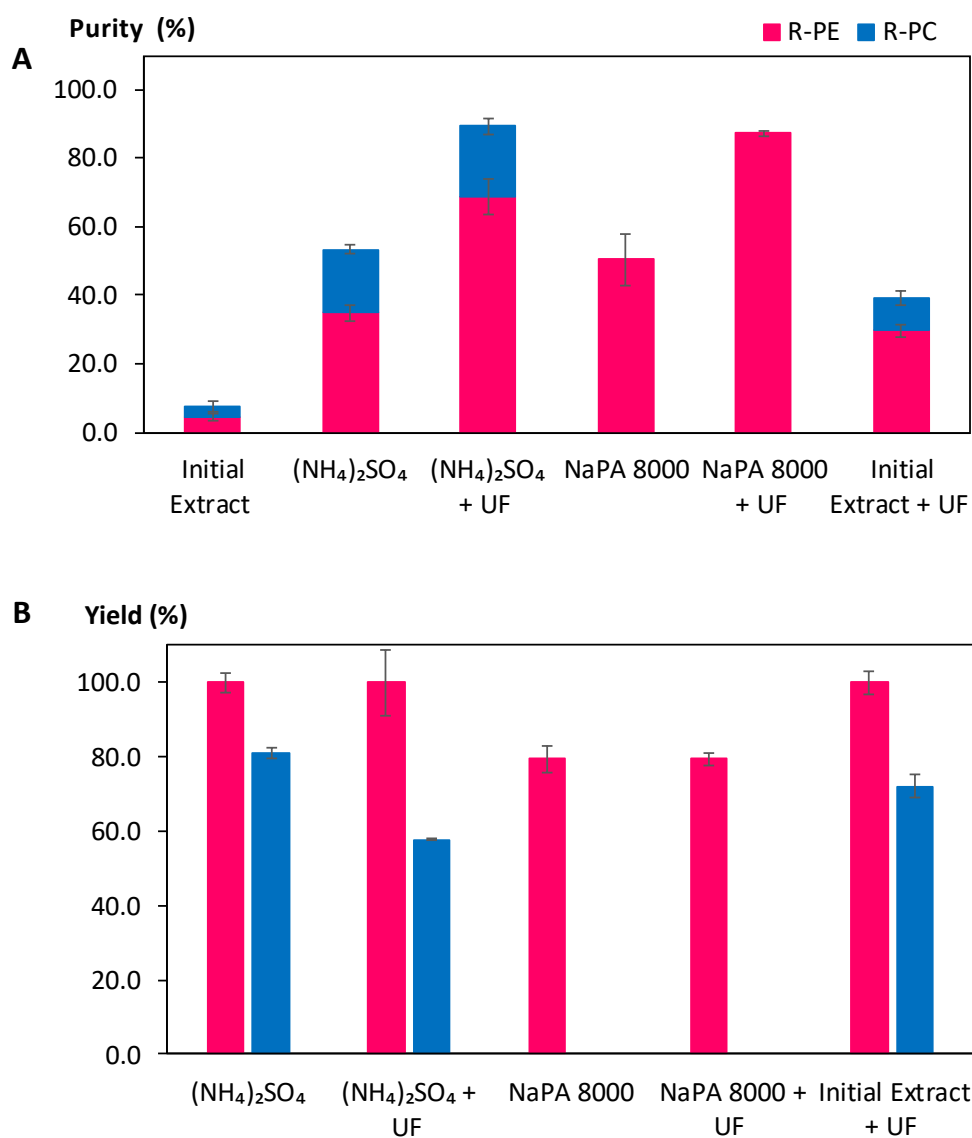
16
 17 **Table 2.** Purity and yield (%) obtained in different fractions separately for R-PE and R-PC
 18 based on HPLC-DAD analysis.

	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	-	-
$(\text{NH}_4)_2\text{SO}_4$ at 20 g.L^{-1}	35.0 ± 2.4	18.5 ± 1.2	53.4	100.0 ± 2.6	81.1 ± 1.3
NaPA 8000 at 10 g.L^{-1}	50.5 ± 7.4	-	50.5	79.5 ± 3.6	-

19

1 The results reported in Table 2 show that the $(\text{NH}_4)_2\text{SO}_4$ at 20 g.L^{-1} can precipitate both
2 R-PE and R-PC, while NaPA 8000 at 10 g.L^{-1} is selective for R-PE, *i.e.* it only causes the
3 precipitation of R-PE, while the other phycobiliproteins remain solubilized in the crude
4 extract. The results of $(\text{NH}_4)_2\text{SO}_4$ are not surprising, since it is well known that, despite
5 its high capacity to induce the precipitation of proteins, it is not selective. It is efficient
6 in precipitating the R-PE because of its very high molecular weight (240 kDa^{18}). Although
7 R-PC ($\sim 112 \text{ kDa}^{43}$) has a lower molecular weight than R-PE, due to the difference
8 between their complexes ($(\alpha\beta)_3$ for R-PC and $(\alpha\beta)_6\gamma$ for R-PE), the R-PC precipitation
9 might be induced due to the proximity between the pH of the aqueous solution of
10 $(\text{NH}_4)_2\text{SO}_4$ (5.5) and the R-PC isoelectric point, 5.7^{43} . On the other hand, NaPA 8000 at
11 10 g.L^{-1} interacts directly with R-PE, establishing soluble complexes, but not with R-PC,
12 promoting a selective precipitation. Since NaPA 8000 is a polyanion, and at the
13 conditions of the solution, R-PE is negatively charged [$\text{pH} (8.1) > \text{R-PE isoelectric point}$
14 $(4.2)^{44}$], site-specific local interactions might be happening, thus justifying the
15 establishment of soluble complexes.²⁸

16 Although the purity has increased after the precipitation step, the extracts are still not
17 very pure (maximum purity up to this point around 50 %). For that reason the
18 resuspended pellets obtained after the precipitation with NaPA 8000 and $(\text{NH}_4)_2\text{SO}_4$
19 were subjected to an additional step of purification using ultrafiltration. As previously
20 detailed in the Experimental section, filters with a cutoff of 100 kDa were applied to
21 remove the small and medium size contaminant proteins present in the macroalgae.²⁴
22 Yields and purity obtained before and after ultrafiltration are plotted in Figure 3 (with
23 more details in Table S4 of ESI).



1

2 **Figure 3.** Summary of the results obtained by HPLC-DAD for the (A) purity and (B) yield
 3 obtained in different fractions, namely the initial extract, the resuspended pellets after
 4 precipitation using (NH₄)₂SO₄ at 20 g.L⁻¹, (NH₄)₂SO₄ at 20 g.L⁻¹ followed by an
 5 ultrafiltration step, and NaPA 8000 at 10 g.L⁻¹, NaPA 8000 at 10 g.L⁻¹ followed by an
 6 ultrafiltration step, and lastly initial extract purified by an ultrafiltration step) separately
 7 for R-PE (pink bars with points) and R-PC (blue bars).

8

1 Summing up the results, the initial extract has a purity in phycobiliproteins around 7.4 %
2 (this representing 100 % of both R-PE and R-PC extracted from the biomass). By
3 submitting the extract to a precipitation using $(\text{NH}_4)_2\text{SO}_4$ at 20 g.L^{-1} , the purity of both
4 phycobiliproteins increased to 53.4 % without compromising the yield of precipitation.
5 By adding an ultrafiltration step, the purity increased to 89.5 % in phycobiliproteins,
6 without affecting the yield of precipitation of R-PE. On the other hand, and as previously
7 analyzed, after precipitation with NaPA 8000 at 10 g.L^{-1} only R-PE precipitated with a
8 purity of 50.4 % (R-PC remained in solution). Meanwhile, and after applying the
9 ultrafiltration step, the purity of the extract increased from 50.5 % to 87.3 % in R-PE with
10 a yield of 79.5 %.

11 The selectivity and R-PC index of the purified extract obtained from both purification
12 methodologies proposed in this work were also calculated and compared with the
13 results obtained for the process using aqueous micellar two-phase systems²⁴ for the
14 purification of R-PE (Table 3). In terms of selectivity, it was found that both processes
15 proposed in this work are superior to the systems previously reported by Vicente et al.²⁴

16 The R-PC index in the extracts purified by $(\text{NH}_4)_2\text{SO}_4$ (20 g.L^{-1}) precipitation with an
17 additional ultrafiltration step is higher than the NaPA 8000 (10 g.L^{-1}) precipitation with
18 an additional ultrafiltration, supporting the selectivity of the induced precipitation
19 process based in NaPA 8000. Moreover, the induced precipitation with $(\text{NH}_4)_2\text{SO}_4$ has a
20 higher R-PC index than those presented by Vicente et al., showing its ability in preserve
21 the R-PC content. In the other hand, systems of purification with NaPA 8000 have the
22 lowest R-PC index in comparison with all systems presented by Vicente and co-authors
23 being in the purity range of the standard R-PE sold by Sigma-Aldrich⁴⁵ (which is < 0.03)
24 showing its extremely low contamination with R-PC, as intended.

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

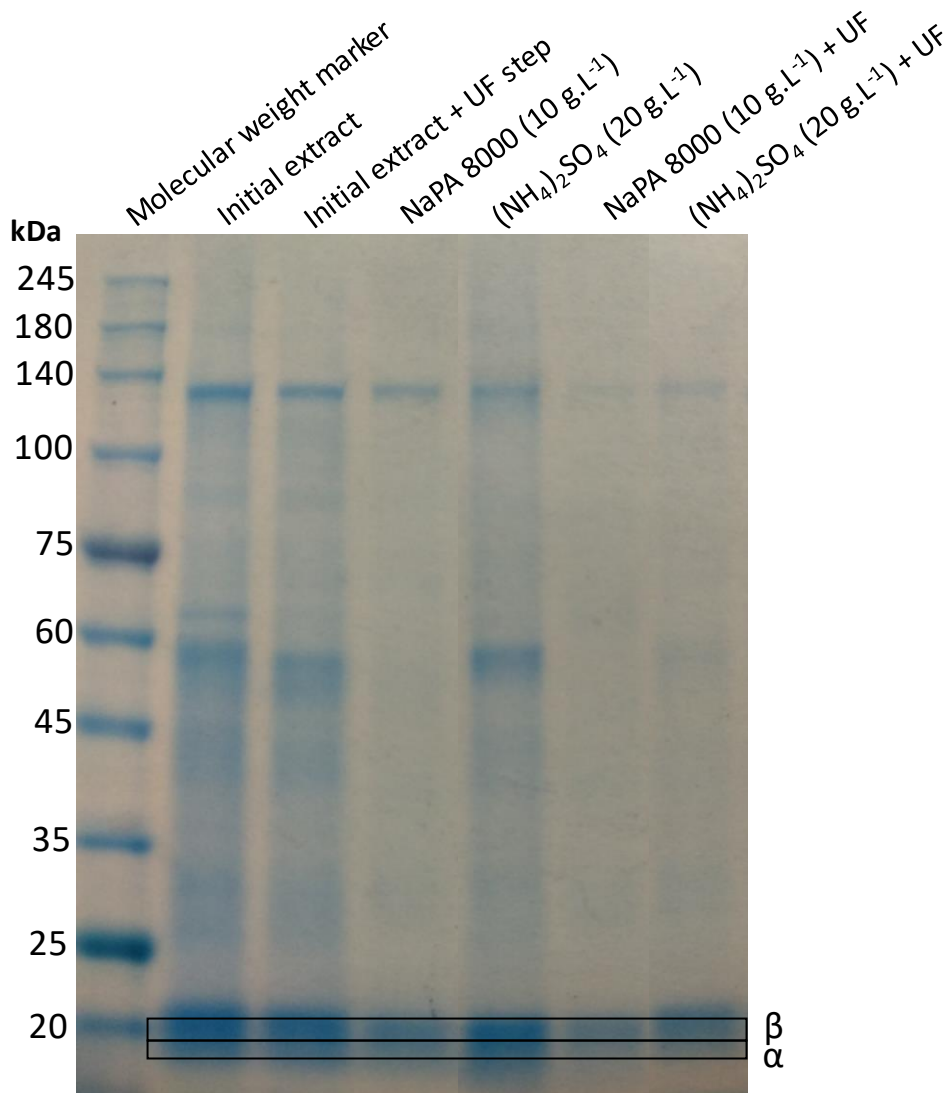
	(NH₄)₂SO₄ (20 g.L⁻¹) + ultrafiltration (this work)	NaPA 8000 (10 g.L⁻¹) + ultrafiltration (this work)	*AMTPS²⁴	Sigma- Aldrich⁴⁵
Selectivity	19.6 ± 0.1	15.3 ± 0.4	13.6 ± 0.0	---
R-PC index	0.23 ± 0.01	0.011 ± 0.001	0.047 ± 0.004	< 0.03

2 *best system proposed by Vicente and co-authors.²⁴

3

4 Lastly, an ultrafiltration step was applied to the initial extract without any previous
5 precipitation step in order to understand if the same results could be obtained by
6 skipping the precipitation procedure. At this point, the purity obtained was only of
7 39.4 % in phycobiliproteins, which represents much lower values than those discussed
8 previously with induced precipitation as a first step, thus showing the need of both steps
9 in the proposed process.

10 To confirm the results represented in Figure 3 on the increase of purity of the extracts
11 in the different scenarios tested, a SDS-PAGE electrophoresis was carried being the
12 results depicted in Figure 4.



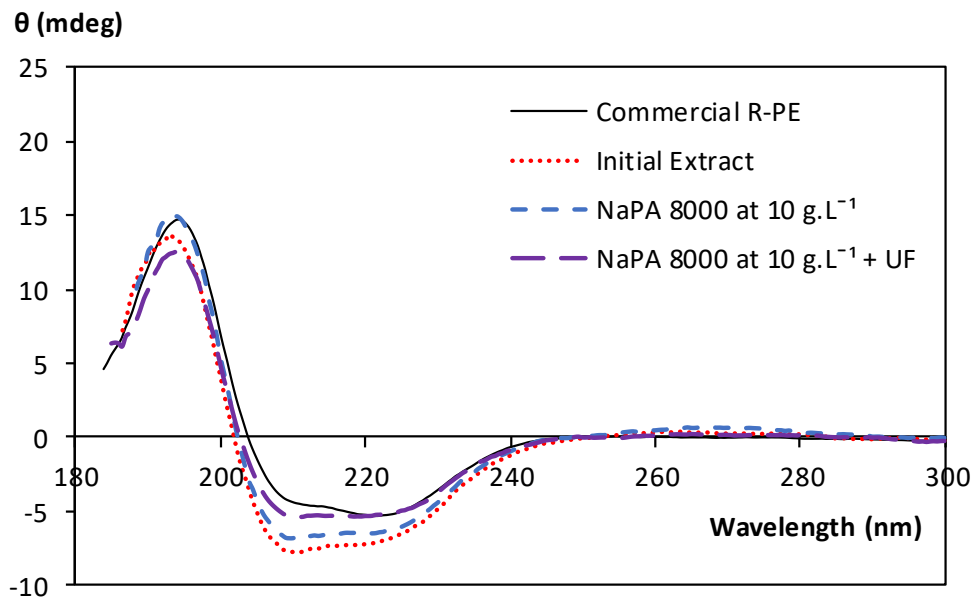
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Figure 4. SDS-PAGE analysis of different fractions obtained after testing the different scenarios under study. UF stands for ultrafiltration.

As previously mentioned, R-PE and R-PC are composed of $(\alpha\beta)_6\gamma$ and $(\alpha\beta)_3$ complexes,¹⁰ 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.^{17,43,46} 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

1 with other proteins. Despite the removal of some impurities when applied an
2 ultrafiltration step to treat the initial extract, it is not enough to achieve a significant
3 increment in purity. The step of precipitation of phycobiliproteins by itself (53.4 %) is
4 more effective in the purification than the ultrafiltration alone (39.4 %), as proved by
5 HPLC-DAD (data depicted in Table S4 in ESI). With the application of ultrafiltration after
6 precipitation with polyelectrolyte, an extract with high purity in phycobiliproteins was
7 obtained, with just a tenuous band of contaminating protein (~ 120 kDa) present, which
8 is in agreement with the results depicted in Figure 3. It is then evident that the
9 combination of both steps is able to remove most of proteins and peptides apart from
10 α and β subunits, characteristic of phycobiliproteins.

11 After assessing the purity of the samples by SDS-PAGE electrophoresis, the structural
12 integrity of the phycobiliproteins was checked using circular dichroism. With this
13 technique, the secondary structure of the proteins along the different stages of
14 purification using NaPA 8000 were evaluated and compared with pure commercial R-
15 PE. The results are depicted in Figure 5, with the high-tension voltage graph displayed
16 in Figure S1 in ESI.



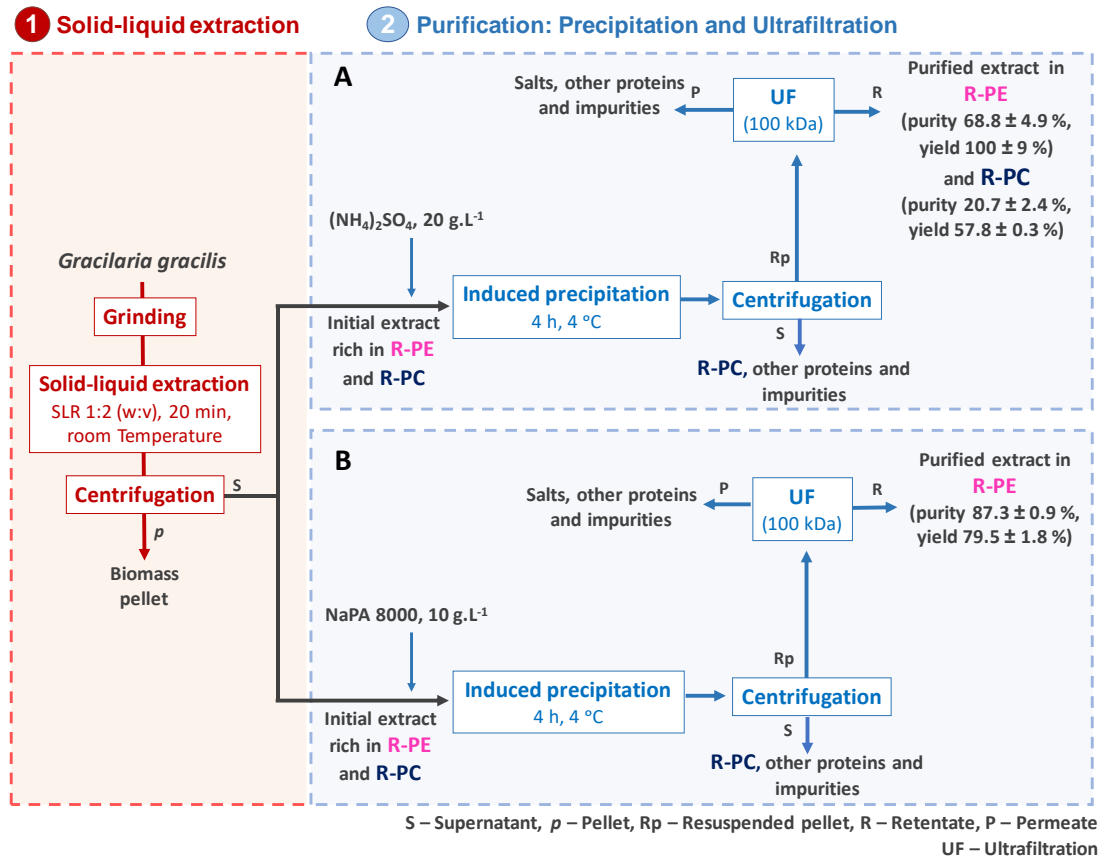
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2 **Figure 5.** Circular dichroism spectra of the initial extract (dotted line), resuspended
 3 pellet after precipitation using NaPA 8000 at 10 g.L⁻¹ (smaller dashed line), and
 4 resuspended pellet after precipitation using NaPA 8000 at 10 g.L⁻¹ followed by an
 5 ultrafiltration step (larger dashed line), and commercial R-PE from Sigma-Aldrich (solid
 6 line).

7

8 The results show that, as the purity of the extracts increases, the better the spectrum
 9 fits the commercial R-PE spectra, being indicative of the preservation of the secondary
 10 structure of R-PE after purification. The removal of contaminant proteins with different
 11 conformations allows the extract to show a spectrum more similar to the commercial R-
 12 PE. Besides, and according to literature for R-PE from *Gracilaria chilensis*, the R-PE is
 13 mainly composed of α -helixes (71 %) and a minor content in β -sheets and random coils
 14 (12 and 17 %, respectively).⁴⁷ This also suggests the preservation of the structural
 15 integrity of R-PE after precipitation, since the circular dichroism spectra shows the

- 1 maxima of negative signals at ca. 222 and 210 nm, typical of proteins with a high α -
- 2 helical content.
- 3 In conclusion, the proposed processes (A) for purification of phycobiliproteins and for
- 4 the (B) selective recovery of R-PE from *Gracilaria gracilis* are represented in Figure 6.



5

6 **Figure 6.** Final proposed processes to obtain a purified extract in phycobiliproteins (A)

7 and an extract with only R-PE (B).

8

9 *Environmental evaluation by the carbon footprint analysis*

10 Aiming to understand the potential environmental impact of the processes developed

11 in this work, and how they do compare with the process already reported using aqueous

12 micellar two-phase systems, the assessment of their carbon footprints was performed.

13 The results of the life cycle assessment, expressed *per* 1 mg of R-PE, show that the

1 impacts of the scenario where $(\text{NH}_4)_2\text{SO}_4$ is used are 23 - 25 % smaller than the impacts
 2 of the scenario with NaPA 8000 (Table 3 and Figure S2 in ESI). The main reason for this
 3 result is the higher yield when $(\text{NH}_4)_2\text{SO}_4$ is used, which leads to lower values of
 4 electricity consumption for obtaining the same amount of R-PE. Another reason is the
 5 smaller impacts associated with $(\text{NH}_4)_2\text{SO}_4$ in comparison with NaPA 8000. The
 6 purification step has the largest impacts in both scenarios, mainly due to electricity
 7 consumption during the cycles of ultrafiltration, which contributes to 70 - 73% of the
 8 total impacts (Figure 7).

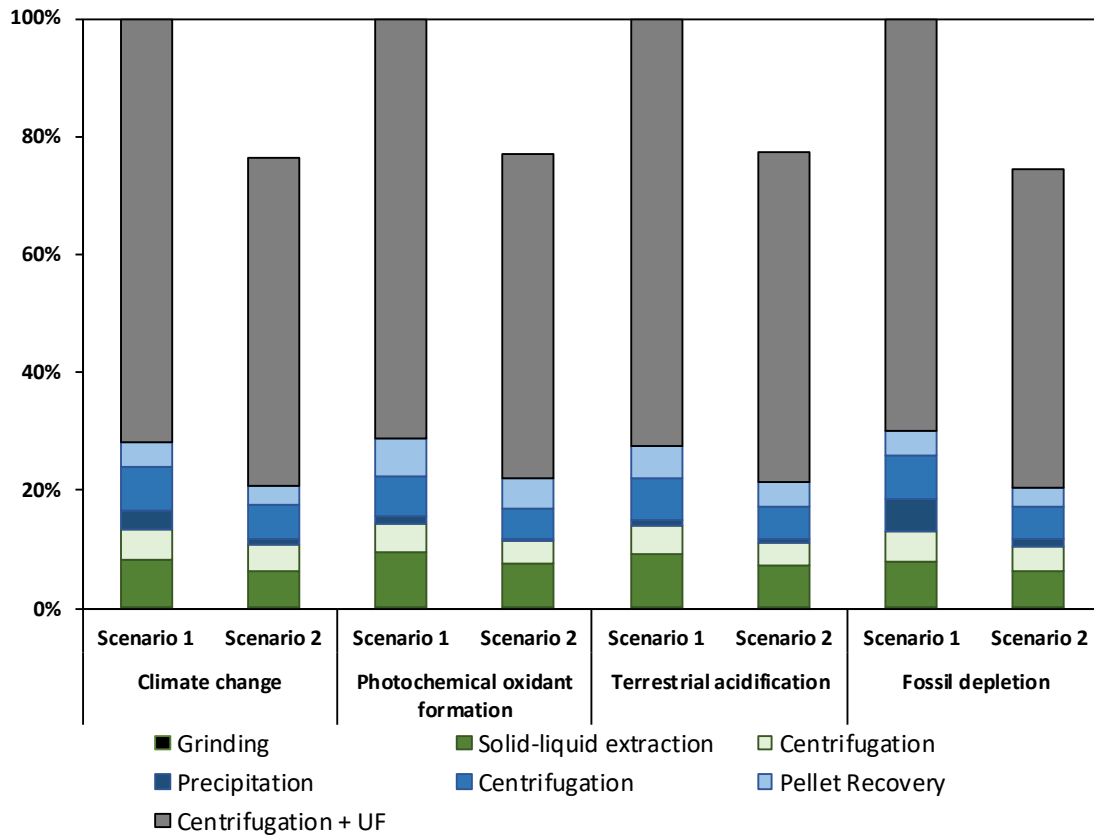
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10 **Table 3.** Life cycle assessment for 1 mg of R-PE obtained in both scenarios under study.

11 Scenario 1 represents NaPA 8000 and scenario 2 represents the $(\text{NH}_4)_2\text{SO}_4$.

	Scenario 1	Scenario 2
Climate change ($\text{g}_{\text{CO}_2\text{eq}}$)	11.6	8.84
Photochemical oxidant formation ($\text{g}_{\text{NMVOCeq}}$)	0.0453	0.0349
Terrestrial acidification ($\text{g}_{\text{SO}_2\text{eq}}$)	0.0803	0.0622
Fossil depletion (g_{oileq})	3.66	2.73

12



1

2 **Figure 7.** Relative contribution of the operations for the results of life cycle assessment,

3 considering scenario 1 representing NaPA 8000 and scenario 2 representing $(\text{NH}_4)_2\text{SO}_4$.

4 Greenish bars are related to the recovery of phycobiliproteins from the biomass, blueish

5 bars are related to the precipitation step in the purification approach, and grey bar is

6 related to ultrafiltration.

7

8 Despite the small difference between the two scenarios; 1 (NaPA 8000) and 2

9 $(\text{NH}_4)_2\text{SO}_4$, the carbon footprint data obtained are much smaller than those reported

10 by Vicente et al.²⁴ (68.14 and 81.30 kg $\text{CO}_{2\text{eq}}\cdot\text{mg}_{\text{R-PE}}^{-1}$) as a result of a much lower

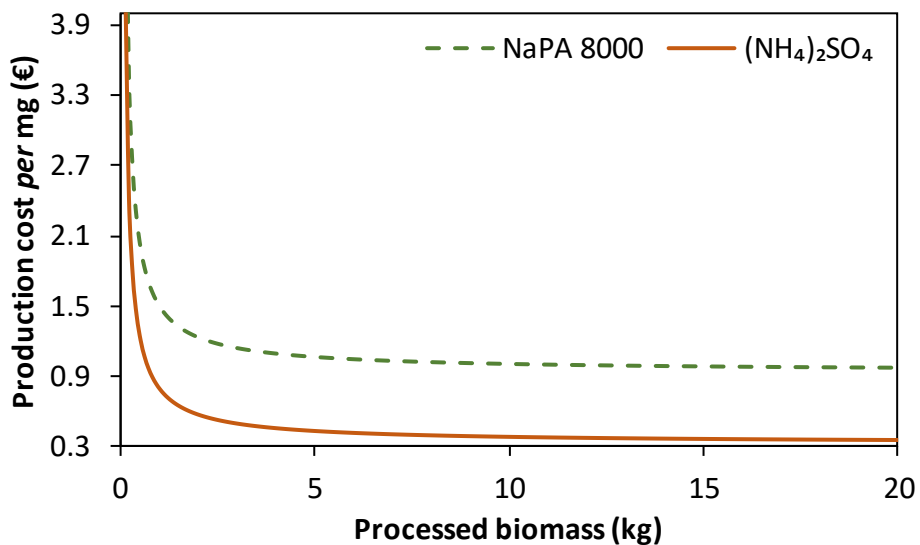
11 electricity consumption in the current process. The process developed in this work

12 proved, not only to be efficient regarding the purification of phycobiliproteins and R-PE

13 in particular, but also to have a low environmental impact.

1 *Economic evaluation*

2 Envisioning the potential industrialization of the process here developed, a detailed
3 economic analysis was performed for both systems, scenario 1 using NaPA 8000 and
4 scenario 2 using $(\text{NH}_4)_2\text{SO}_4$ as precipitating agents. The production cost *per* mg of R-PE
5 is highly variable and deeply influenced by the process scale (Figure 8). It is important to
6 mention that, as there is not a guide of when to incorporate materials prices for bulk
7 acquisitions, this analysis was performed using the laboratory-scale prices (Table S2 in
8 ESI). Depending on the precipitating agent used, the CoG. mg^{-1} tends to stabilize
9 on 0.93 € *per* mg and 0.32 € *per* mg for NaPA 8000 and $(\text{NH}_4)_2\text{SO}_4$, respectively.



10

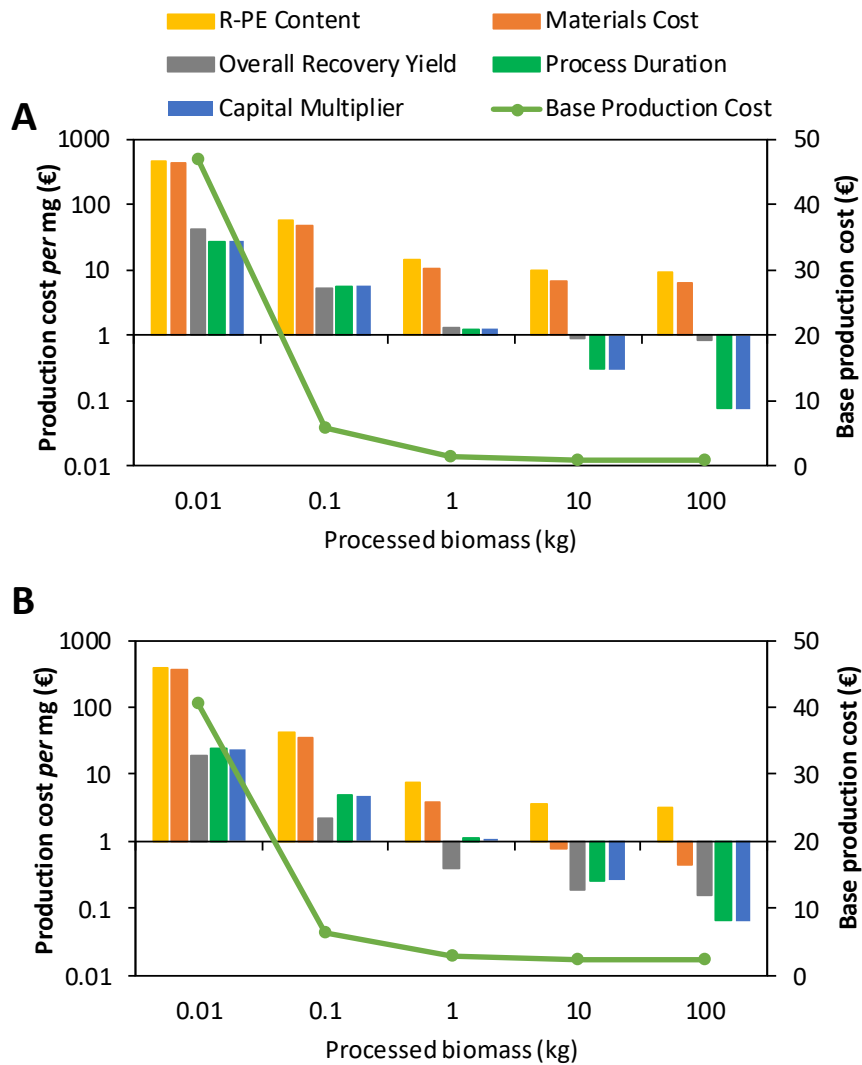
11 **Figure 8.** Results obtained from the analysis of production scale (amount of biomass
12 processed).

13

14 In practice, different aspects of the bioprocess tend to vary, and thus, a model is very
15 helpful as it is possible to create a wide range of values for different variables to
16 understand how production costs can be affected. For this reason, a sensitivity analysis
17 was performed on the amount of R-PE content in the biomass (mg of R-PE *per* kg of

1 biomass), on the materials cost variation and on the duration of the process. For these
2 three variables, the range of variation was 10-fold (either above or below the amount
3 used for the model construction). Also, the recovery yield of the process was analyzed
4 by a decrease of up to 30 % (worst case scenario), while the best scenario could not be
5 done up to 30 % because of their current level (it will result in recoveries above 100 %),
6 for this reason the optimal results were fixed at 100 %. The data collected indicated the
7 content of product in the processed biomass as the most important parameter, followed
8 by the materials costs (Figure 9). In general, the impact of all parameters decreases as
9 the production scale increases, which is related to the amount of product being
10 generated, as it dilutes the cost variations. Furthermore, the impact of the amount of
11 product being generated has been reported continuously to be one of the most
12 important parameters governing the production costs.⁴⁸⁻⁵⁰ Finally, it is critical to note
13 that for NaPA 8000, the variation on materials costs is more noticeable than for
14 $(\text{NH}_4)_2\text{SO}_4$. This is because NaPA is a much more expensive material at both laboratory
15 and large scales (Table S2 in ESI).

16



1

2 **Figure 9.** Result for the sensitivity analysis of the complete bioprocess of NaPA 8000 (A)

3 and $(\text{NH}_4)_2\text{SO}_4$ (B). Results are expressed as the difference of the highest and lowest

4 production costs calculated after varying between the worst and best scenarios.

5 Additionally, for reference, the base production cost is shown as the green line (right Y-

6 axis). The left Y-axis is presented in a logarithmic scale.

7

8 Using the same variables and ranges, a series of Monte Carlo simulations were run to

9 understand how the simultaneous variation of the main parameters affects the

10 production costs. This was done for scales of 0.01 kg and 100 kg (full data is presented

11 in Table S5 in ESI). This results in a collection of statistical data that can show the

1 significance or not of a variable. The main results confirm the importance of the product'
2 content in the biomass and of the materials cost variation, but the effect of the second
3 is almost ten times bigger for NaPA 8000 than for $(\text{NH}_4)_2\text{SO}_4$ at large-scale (Table 4).
4 Interestingly, for all the analyzed scales, the duration of the process is not statistically
5 significant, which means that, if the process is shorter or longer, it will have a negligible
6 effect on the production cost.

- 1 **Table 4.** Results for the Monte Carlo simulations and multiple linear regression. Input variables were in the corresponding multiplier or modifier
- 2 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
- 3 a multiplier (used for the modelling were from 0.1X to 10X), while for the recovery yield it is a modifier (± 30 %).

4

	NaPA 8000				(NH ₄) ₂ SO ₄			
	0.01 kg	<i>p</i> -value	100 kg	<i>p</i> -value	0.01 kg	<i>p</i> -value	100 kg	<i>p</i> -value ⁵
Intercept (β₀)	110.06	1.17x10 ⁻⁹	1.864961	4.19x10 ⁻¹¹	78.82741	4.67x10 ⁻⁸	0.344542	2.88x10 ⁻⁸
R-PE content (β₁)	-28.5299	1.43x10 ⁻²⁴	-0.44852	5.06x10 ⁻²⁵	-23.5171	4.22x10 ⁻³⁰	-0.06802	1.42x10 ⁻⁷¹
Overall recovery yield (β₂)	-0.94794	0.042111	-0.01485	0.04056	-0.71106	0.202417	-0.00254	0.05058
Materials cost (β₃)	22.79631	3.47x10 ⁻¹⁸	0.340471	4.68x10 ⁻¹⁷	20.58119	1.39 x10 ⁻²⁵	0.030776	1.39x10 ⁻¹² ₉
Process duration (β₄)	-1.3313	0.586571	-0.0387	0.309369	0.020603	0.991292	0.000104	0.981150

11

1 Equations have the form (Eq. 6):

2 Production Cost [€ per mg] = $\beta_0 + \beta_1 \times R - \text{PE Content} + \beta_2 \times$

3 Overall Recovery Yield + $\beta_3 \times \text{Materials Cost} + \beta_4 \times \text{Process Duration}$ **Eq. 6**

4

5 Lastly, the Return per kg of processed biomass was performed at laboratory (0.01 kg)

6 and large-scale (100 kg) using prices from Sigma-Aldrich (for 0.01 kg) and Alibaba (for

7 100 kg), the latest being considered as an example of a real-life value. Additionally, the

8 amount of product in the biomass (C_{prod}) was varied by 0.5X, 1X or 2X and the CoG.kg⁻¹

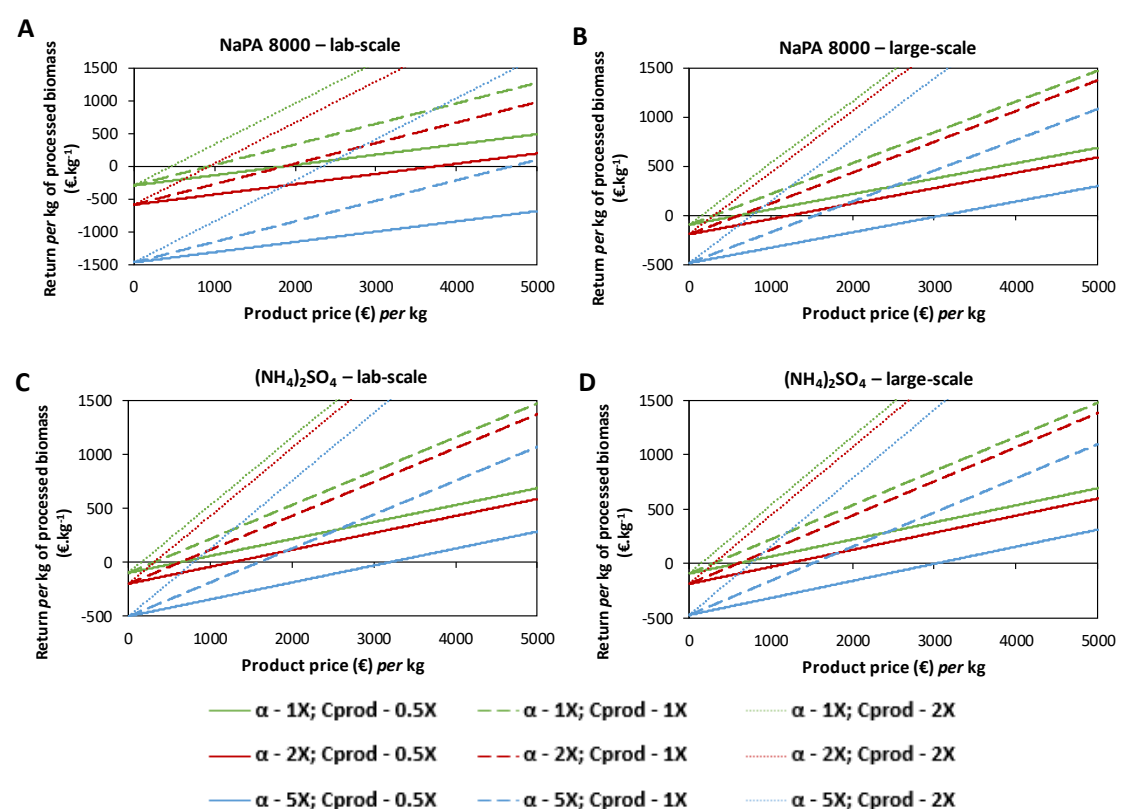
9 of processed biomass was varied by a factor of 1X, 2X and 5X. Moreover, the $\$_{\text{prod}}$ was

10 varied from € 5 per kg to € 5,000 per kg. Results provide an in-depth look into different

11 scenarios and how they can influence the potential economic return for this process

12 (Figure 10 and Figure S3 in ESI).

13



14

1 **Figure 10.** Results for the Return (R) analysis for NaPA 8000 and $(\text{NH}_4)_2\text{SO}_4$. NaPA 8000
2 results are presented in A) for laboratory-scale (0.01 kg) and B) for large-scale (100 kg),
3 while for $(\text{NH}_4)_2\text{SO}_4$ are C) laboratory-scale (0.01 kg) and D) for large-scale (100 kg).
4 Green lines are for an alpha of 1X, red for alpha of 2X and blue for alpha of 5X; solid lines
5 for a C_{prod} of 0.5X, dash lines for C_{prod} of 1X and dot lines for C_{prod} of 2X.

6

7 Results from this analysis can help to appreciate different issues considered relevant for
8 the efficiency and sustainability of the process. The slope of each line is the influence of
9 the C_{prod} on the Return: the higher the C_{prod} , the more vertical the line will be.
10 Additionally, the position where the lines intercept with the y-axis (the point where
11 $\$_{\text{prod}}$ is 0), is dictated by the CoG.kg^{-1} of biomass. The most evident result is the abrupt
12 difference on the y-axis intercept for Figure 10A and 10B, indicating the impact that the
13 change in the price of the materials has on the CoG.kg^{-1} of biomass. From the data on
14 Table S2 in ESI, the price reduction of NaPA 8000 from laboratory to large-scale is much
15 larger compared to the decrease of $(\text{NH}_4)_2\text{SO}_4$ price, which can be related to the
16 extensive use of $(\text{NH}_4)_2\text{SO}_4$. Moreover, this dramatic change becomes the critical aspect
17 for determining, for specific conditions, if there is any Return at all.

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

24

1 **Conclusions**

2 In this work, a new approach, easy to implement, using induced precipitation, is
3 proposed for the purification of phycobiliproteins, in particular R-PE. A set of polymers,
4 copolymers, and polyelectrolytes was screened correlating their ability to selective
5 precipitate proteins from a raw extract of phycobiliproteins in order to purify the
6 fluorescent proteins. It was found that the most common used precipitation agent in
7 proteins – $(\text{NH}_4)_2\text{SO}_4$ – at 20 g.L^{-1} is able to precipitate both R-PE and R-PC but it is not
8 selective, while the polyelectrolyte NaPA 8000, even at low concentrations (10 g.L^{-1}), is
9 able to selectively induce the precipitation of R-PE among the set of phycobiliproteins
10 present in the extract. By further using an ultrafiltration step, purities of 89.5 % and
11 87.3 % were achieved, respectively for the two phycobiliproteins using the strategy of
12 $(\text{NH}_4)_2\text{SO}_4$ followed by ultrafiltration and for only R-PE using NaPA 8000 followed by
13 ultrafiltration, having this last one its structural integrity preserved. Summing up, and
14 despite the regular use of $(\text{NH}_4)_2\text{SO}_4$, its use did not allow the development of a selective
15 induced precipitations, which is surpassed by the use of NaPA 8000.

16 Taking into account the results of selectivity for the system using NaPA 8000, the
17 environmental impact was determined and compared with one of the most recent
18 reports of processes optimized for the purification of R-PE using aqueous micellar two-
19 phase systems. The low carbon footprint of the process optimized by using induced
20 precipitation with NaPA 8000, shows that the process here proposed has a lower
21 environmental impact. Using the current process results combined with the economic
22 analysis, it was concluded that a potential real-life application can provide return
23 dependent on the market price of the R-PE product. Some of the major factors to
24 determine the required price are the amount of R-PE content in the biomass (or the

1 amount extracted from it) and the price of the materials during a large-scale operation.
2 The use of NaPA 8000 or $(\text{NH}_4)_2\text{SO}_4$ provide cost-effective results and, ultimately, the
3 decision on their selection can be based on process-oriented results, such as the purity
4 required of the product for the desired application, along with the possible commercial
5 price of the product.

6

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13

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