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Recovery of phycobiliproteins from the red macroalga *Gracilaria* sp. using ionic liquid aqueous solutions†

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Bioactive compounds extracted from natural renewable sources have attracted increased interest from both industry and academia. Several biocompounds are present in red macroalgae, among which R-phycoerythrin (R-PE), which is a phycobiliprotein with a wide range of applications. The major drawback associated with it is the absence of an efficient, low cost and green extraction and purification methodology capable of recovering phycobiliproteins (and, in particular, R-phycoerythrin) from the biomass, while maintaining their structure and activity. The search for novel and higher performance extraction processes is thus of extreme relevance. In this work, aqueous solutions of ionic liquids were screened for the extraction of phycobiliproteins from *Gracilaria* sp. The most promising solvents were identified and operational conditions such as extraction time, solid–liquid ratio, solvent concentration and pH were optimized aiming to develop a new and more efficient approach to extract phycobiliproteins. The efficiency of the proposed process is demonstrated with aqueous solutions of cholinium chloride, since the extraction of phycobiliproteins was increased to 46.5% when compared with the conventional methodology, while the protein secondary structure and the chromophore conformation integrity are maintained.

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Introduction

Phycobiliproteins are the main photosynthetic pigments present in red algae, cyanobacteria, and cryptomonads, a unicellular eukaryotic alga.¹ These are light-harvesting pigment–protein complexes organized *in vivo* in supramolecular structures called phycobilisomes, located at the stroma, on the external structure of the thylakoid membrane.² The phycobiliproteins allow simultaneously the transfer of light energy to the living organism and their survival at low light intensities.² Due to their high solubility in water, stability,^{3,4} and bioactivity, phycobiliproteins⁵ have gained special significance in many different sectors, such as the food,⁶ pharmaceutical, and cosmetic industries.^{2,7} Among the phycobiliproteins, R-phycoerythrin has been singled out as an important tool in the field of medical diagnosis, and biomedical research,⁸ due to

its excellent optical and spectroscopic properties, high absorption coefficient, and high fluorescence yield. These fluorescent pigments have also shown antioxidant and antitumoral activities.⁹ More recently, phycobiliproteins have called attention by their potential application in the energy field,¹⁰ namely in the production of “dye-sensitized solar cells” (DSSCs). DSSCs are non-tracking concentrators that redirect solar radiation into simple slab waveguides to be collected by a photovoltaic cell mounted at the edge of the slab.¹⁰ Globally, there are about 50 producers of phycobiliproteins, with their market prices ranging from \$6 to \$12 per mg.¹¹ Due to their high cost, the market is still small, although with estimated yearly growths of 20%.¹¹ Taking into account the potential of phycobiliproteins and their range of applications, the development of novel efficient processes to extract and purify these fluorescent proteins from the fresh biomass is of utmost importance, albeit a challenging task. The common extraction practices currently used are based on solid–liquid extraction with buffer aqueous solutions, namely sodium phosphate,^{12,13} followed by chromatography and gel filtration,^{13,14} or enzymatic processes^{1,15} to purify R-phycoerythrin from the phycobiliproteins. While some studies describe the use of different solvents, including the buffered solutions and water to extract the phycobiliproteins [R-phycoerythrin results found between 0.666 mg g⁻¹ (for *Gracilaria verrucosa*¹⁶) and 1.73 mg g⁻¹ (for *Gracilaria lemane-*

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formis⁸]] and then use chromatography (namely DEAD-Sephacryl ion-exchange chromatography) to purify R-phycoerythrin with a maximum purity level of 5.6,¹² others use enzymatic digestion by xylanase, to promote an improved disruption of the cells by hydrolysis, thus facilitating the extraction of the fluorescent proteins. In fact, the best results show that the increase in the amount of phycobiliproteins removed after the enzymatic pretreatment is significant, since the protein content increases 16 times.¹⁵ In general, for the processes using enzymatic digestion, the protein recovery yields are between 18%–22% (expressed according to the total proteins) as reviewed by Fleurence¹ for those using alkali systems. Despite the developments achieved and the results being reported in the past few years resulting from different approaches, these strategies are expensive (mainly those based on chromatography and enzymatic hydrolysis) and time-consuming, are not easily scaled-up (low feeds being processed), and have poor selectivity not guaranteeing adequate purity of the final product. Moreover, one of the major problems associated with the extraction strategies applied to biocompounds from macroalgae is their high water content that often compromises the economic viability of the extraction process. Aiming at turning this liability into an advantage, aqueous solutions of ionic liquids are evaluated in this work, as an alternative solvent to extract phycobiliproteins from *Gracilaria* sp., one of the red macroalgae with higher phycobiliprotein content.¹⁷ The biomass used in this work, being grown under nitrogen enriched conditions (Integrated Multi-Trophic Aquaculture system at ALGApplus, Lda), is expected to have a higher protein global content (may reach up to 47% of dry weight), where these N-derived pigments are included.¹⁸

Due to their low-charge density and the low symmetry of the ions, ionic liquids are salts with melting points close to room temperature.¹⁹ In the past few years, these compounds have been extensively studied and the development of new applications for hydrophilic ionic liquids keeps increasing. They have unique characteristics namely high solvation ability, high chemical and thermal stability, high selectivity, broad liquid temperature range, and high ionic conductivity.^{19–21} These characteristics, associated with their negligible flammability and vapour pressure, make them safer to use than conventional solvents. Due to their tunable nature, which results from the ability to manipulate their properties through a range of possible combinations of their ions, they are considered as “designer solvents”. This tunable nature is also exhibited when ionic liquids are applied as extractive solvents in aqueous solution.²² Currently, the use of aqueous solutions of ionic liquids for the extraction of biomolecules from biomass is a hot topic,¹⁹ not only due to the properties already mentioned, but also because they improve and modify the properties of water, allowing one for example, to increase the solubility of more hydrophobic compounds in aqueous media, which is normally termed the hydrotropic effect.²³ When ionic liquids' aqueous solutions are used in the extraction, the interactions with the solvent can be tuned to be stronger and more target specific than those in the processes using

common organic solvents, turning the ionic liquids' aqueous solutions into privileged solvents. If there are some heuristic rules in this sense, these can be summarized as (i) their efficiency in destroying the cell walls, associated with (ii) their outstanding capacity to solvate a large range of compounds/chemicals/biomolecules.¹⁹

The extraction of high-value bioactive compounds from biomass starts with solid–liquid extraction. In order to obtain higher extraction yields, some of these solid–liquid extraction processes may be coupled with techniques such as microwave and ultrasound.¹⁹ The extraction of many compounds with aqueous solutions of ionic liquids was previously reported for alkaloids, flavonoids, terpenoids, aromatic compounds, phenolic acids, lipids, and natural mixtures such as essential oils, suberin and saponins.¹⁹

In this work, aqueous solutions of ionic liquids were used to recover phycobiliproteins from fresh *Gracilaria* sp. Different families of ionic liquids were evaluated for the phycobiliproteins extraction, namely imidazolium, pyridinium, pyrrolidinium, piperidinium, phosphonium, quaternary ammonium and cholinium. Moreover, considering these families as a starting point, the effect of different anions and alkyl chain lengths was also investigated. Once the most promising ionic liquid was identified, the process variables for the solid–liquid extraction were optimized, particularly the pH, the solid–liquid ratio, the buffer concentration, the time of extraction and the concentration of the ionic liquid applied. The circular dichroism and fluorescence spectroscopy techniques were used to evaluate the maintenance of the integrity of the phycobiliprotein secondary structure and the chromophore conformation. The results obtained were benchmarked against the extraction achieved with the conventional approach used for the extraction of phycobiliproteins.

Results and discussion

This work is divided into two parts: the first one presents the results for the conventional approach using sodium phosphate buffer solutions as the solvent for the extraction of phycobiliproteins, and the second one addresses the development of an alternative extraction methodology using aqueous solutions of ionic liquids.

Conventional extraction

The conventional methodology used as control in this work was adapted from literature¹² and uses aqueous solutions of sodium phosphate buffer as the solvent. This methodology was further optimized considering the time of extraction and solid–liquid ratio conditions.

Time of extraction

A kinetic study was carried out for 2 hours considering a solid–liquid ratio of 0.625 (mass of fresh biomass/volume of solvent). The extraction results are depicted in Fig. A2 (ESI[†]), where the difference between the phycobiliproteins and

the total protein content is clear. These results show that the amount of total contaminant proteins is much higher than the amount of phycobiliproteins obtained under the same conditions of extraction. Moreover, it is evident that the phycobiliprotein extraction (pink circles) reaches a maximum after 20 minutes (Fig. A2 from the ESI†), remaining constant thereafter, while the yield of contaminant proteins (black squares) increases significantly with time. These results indicate that phycobiliproteins (and in particular the R-phycoerythrin) are the first proteins to be extracted due to their location in the external part of the phycobilisomes. To maximize the extraction selectivity of phycobiliproteins, an extraction time of 20 minutes was adopted in this work.

Solid–liquid ratio

Due to the importance of the solid–liquid ratio in the extraction process, its effect in the range of 0.1 to 1.0 (fresh weight of biomass/volume of solvent) was investigated for two distinct macroalgae samples harvested in October 2014 and February 2015 (Fig. A3 in the ESI†).

The results presented in Fig. A3† evidence the higher protein content of the macroalgae harvested in February in comparison with the biomass collected in October. The accumulation of pigments in algae is frequently related to their growth conditions (mainly light and nutrients availability).^{24,25} In this context, the difference observed in our results was most likely due to the lower nitrogen water content in October (derived from fish metabolism) and stronger solar radiation. Taking into account the algal biomass harvested in October, it is possible to notice an increase of the contaminant protein content until a solid–liquid ratio of 0.6 was reached, and it remains practically constant for higher solid–liquid ratios. The same behavior was not observed for the samples harvested in February since the protein content increases in the entire range of solid–liquid ratios studied. These results are good for the extraction process, because the use of small volumes of solvent makes the process less expensive. However, the use of very high solid–liquid ratios (SLRs) hinders the extraction process itself, especially for SLR above 0.7. This allowed the standardization of the solid–liquid ratio at 0.7, for which extracts with $0.28 \pm 0.08 \text{ mg}_{\text{phycobiliproteins}} \text{ g}_{\text{fresh alga}}^{-1}$ of phycobiliproteins and $36 \pm 6 \text{ mg}_{\text{phycobiliproteins}} \text{ g}_{\text{fresh alga}}^{-1}$ of total proteins were obtained (biomass collected in February).

Alternative extraction process using ionic liquid aqueous solutions

The study of an alternative extraction process using ionic liquids' aqueous solutions to remove phycobiliproteins from the fresh algal biomass was carried out. The screening of the various ionic liquids was performed under the conditions previously defined for conventional extraction: solid–liquid ratio of 0.7 (mass fresh biomass/volume of solvent) and extraction time of 20 minutes, at room temperature, and in the absence of light. The concentration of the ionic liquids' aqueous solutions used in the first screening was fixed at 1 M.

Alkyl chain length effect

The effect of the alkyl chain length was evaluated for imidazolium-based ionic liquids. A series of 1-alkyl-3-methylimidazolium chloride compounds were used for this purpose, namely [C₂mim]Cl, [C₄mim]Cl, [C₆mim]Cl, [C₁₀mim]Cl, and [C₁₂mim]Cl. The image of the extracts obtained (Fig. 1A), the absorbance spectra collected between 400 and 700 nm (Fig. 1B and C), and the yield of phycobiliproteins extracted from the biomass (Fig. 1D) were determined, whenever possible. The spectra obtained are related to the compounds preferentially recovered in each extract, which vary with the ionic liquid used. For shorter alkyl chains, namely [C₂mim]Cl and [C₄mim]Cl, the crude aqueous extracts present a pink color similar to the one obtained with the sodium phosphate buffer. For longer alkyl chains, namely [C₆mim]Cl, [C₁₀mim]Cl, and [C₁₂mim]Cl, the colors of the crude aqueous extracts obtained were yellow or green, indicating the presence of increasing amounts of chlorophylls. The absorption spectra of the shorter alkyl chains (Fig. 1B) reveal the presence of three peaks at 498 nm, 539 nm and 565 nm ascribed to the first excited states of R-phycoerythrin in its native state,²⁶ whereas for the spectra of the longer alkyl chains (Fig. 1C) absorption bands in the red spectral region ascribed to phycocyanins (620 nm) and allophycocyanins (650 nm) are also detected.^{27,28} For this reason, the determination of the phycobiliprotein yield was carried out only for systems using ionic liquids based on shorter alkyl chains (Fig. 1D). These results can be explained by the hydrophilicity of both the ionic liquids and the phycobiliproteins, under these conditions of extraction. In contrast, when hydrophobic ionic liquids are used (long alkyl chains), the most hydrophobic bioactive compounds present in the biomass, such as carotenoids and chlorophylls, are preferentially extracted. In this sense, and aiming to maintain the highest selectivity of the solid–liquid extraction process, ionic liquids with short alkyl chains were selected for further studies.

Cation effect

The effect of different cations with small alkyl chains (4 carbons) was investigated in the extraction of phycobiliproteins. Apart from the previously studied [C₄mim]Cl, another aromatic cation was investigated, [C₄mpyr]Cl, two non-aromatic cyclic cations, [C₄mpyr]Cl and [C₄mpip]Cl, and three non-cyclic ionic structures, namely [Ch]Cl, [N_{4,4,4,4}]Cl, and [P_{4,4,4,4}]Cl. The aqueous crude extracts obtained for the different families are shown in Fig. 2A. Looking at the results achieved with the various cations, although the absorption spectra are dominated by the R-phycoerythrin absorption peaks (498 nm, 539 nm and 565 nm), the presence of the phycocyanins and allophycocyanins in the high-wavelength region (620 nm and 650 nm, respectively) is also detected. Based on the relative absorbance, we propose the following crescent order of capacity to extract the phycobiliproteins: [P_{4,4,4,4}]Cl < [N_{4,4,4,4}]Cl < Na-phosphate buffer < [C₄mpip]Cl ≈ [C₄mpyr]Cl < [C₄mim]Cl < [Ch]Cl. The yield of phycobiliproteins extracted

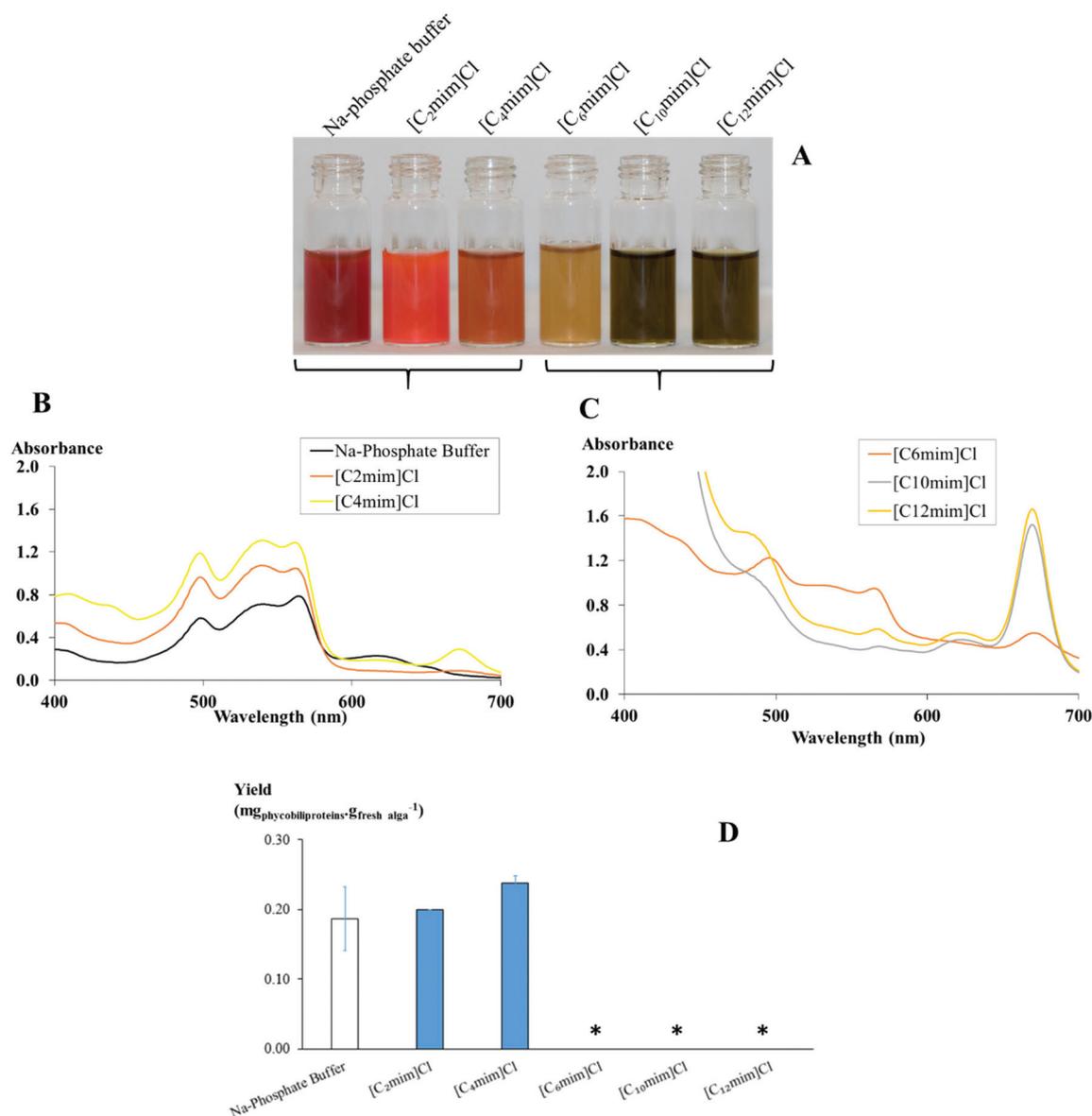


Fig. 1 Results obtained for different [C_nmim]Cl-based ionic liquids, showing: (A) a photography of the crude extracts obtained from the extraction process; (B) the absorption spectra of the crude extracts obtained by the use of the short alkyl chain-based [C_nmim]Cl ionic liquids; (C) the absorption spectra of crude extracts obtained by the use of the long alkyl chain-based [C_nmim]Cl ionic liquids; (D) the yield of extraction of phycobiliproteins using the different ionic liquids' aqueous solutions. *means that the yield of phycobiliproteins is negligible and thus impossible to be experimentally determined.

on each crude aqueous extract was determined; the results are depicted in Fig. 2C. They show [P_{4,4,4,4}]Cl and [N_{4,4,4,4}]Cl as the least effective in the extraction of phycobiliproteins due to their hydrophobic nature.²⁹ In contrast, the more hydrophilic ionic liquids are the most effective to extract phycobiliproteins from fresh biomass. However, it should be highlighted that, despite the efficiency of some ionic liquids in extracting phycobiliproteins, some also have the capacity to extract chlorophylls present in the biomass as shown by the band at 670 nm in the spectra presented in Fig. 2B. This means that, despite the high extraction ability of these compounds for the target molecules, they are not very selective.

Anion effect

The anion effect was evaluated using 1-butyl-3-methylimidazolium cation ([C₄mim]⁺) combined with the following anions: chloride, dicyanamide, tosylate, dimethylphosphate, thiocyanate, trifluoromethanesulfonate, methanesulfonate, trifluoroacetate, and acetate. Again a diversity of extracts with different colors (Fig. 3A) was obtained. The phycobiliprotein content in each extract was quantified as depicted in Fig. 3B. The anions Cl⁻, [CH₃CO₂]⁻, and [CH₃SO₃]⁻ have a higher capacity to extract phycobiliproteins, while [N(CN)₂]⁻, [SCN]⁻ and [CF₃SO₃]⁻ not only have the lowest extraction capacity for

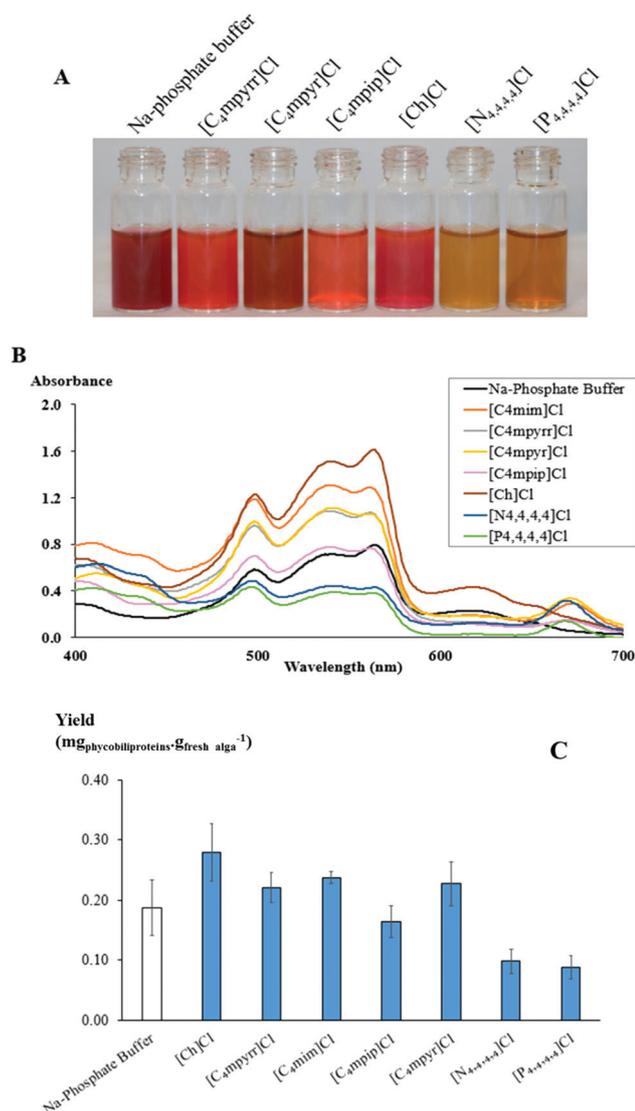


Fig. 2 Results obtained for different cation-based ionic liquids, showing: (A) a photography of the crude extracts; (B) the absorption spectra of the crude extracts; and (C) the yield of extraction of phycobiliproteins present in the crude aqueous extracts.

phycobiliproteins but are also responsible for the extraction of chlorophylls (justifying the yellow/brown color of the aqueous extracts). These results can be explained by the hydrogen-bond acceptor ability of each anion, as described by the solvatochromic parameter β ^{30,31} also presented in Fig. 3B. The correlation found for the β value and the yield of phycobiliproteins extracted with the different [C₄mim]X-based ionic liquids show that the phycobiliprotein extraction increases with β .

The output of the screening of ionic liquids was the selection of aqueous solutions of [Ch]Cl for the optimization of the phycobiliprotein extraction process. This selection is justified by its good extractive performance for phycobiliproteins and its selectivity against chlorophylls, as the amount of chlorophylls extracted with [Ch]Cl is residual. Moreover, its biocompatibility, low price and low ecotoxicity³² make this compound

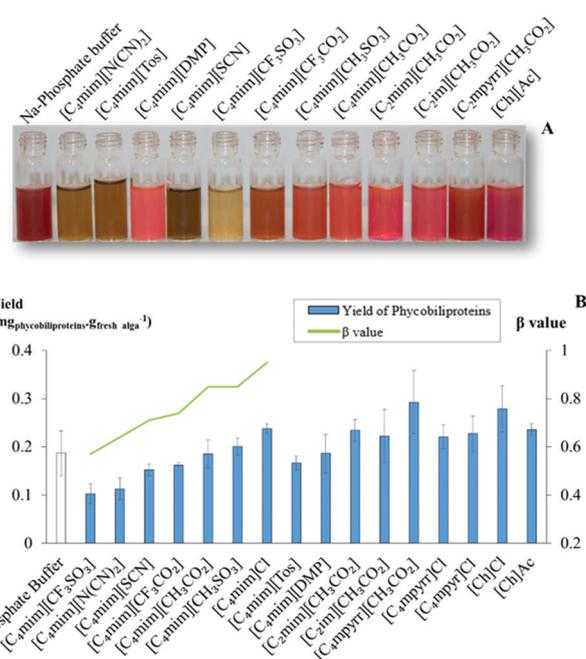


Fig. 3 Main results obtained for different anion-based ionic liquids, showing: (A) a photography of the various crude extracts; and (B) the yield of extraction of phycobiliproteins present in the crude aqueous extracts obtained. The β values^{30,31} of different [C₄mim]X-based ionic liquids are also presented in the figure (green line).

the most appealing amongst the various compounds studied for the extraction of phycobiliproteins.

Optimization of the operational conditions: response surface methodology

Since one of the most important parameters when dealing with the recovery of proteins is the pH, this variable was also optimized. To better assess the pH effect, the McIlvaine buffer was selected since it allows a wider range of pH values than the phosphate buffer used in the conventional method. The McIlvaine buffer was evaluated in the extraction of phycobiliproteins and a comparison between the efficiencies of both buffers was performed, using the conditions previously fixed for the solid-liquid ratio (0.7 in fresh weight of biomass/volume of solvent), pH (7), and time of extraction (20 minutes). The results obtained for the phosphate (0.25 ± 0.02 mg_{phycobiliproteins} g_{fresh alga}⁻¹) and McIlvaine (0.38 ± 0.02 mg_{phycobiliproteins} g_{fresh alga}⁻¹) buffers show that the McIlvaine buffer allows a more efficient extraction of phycobiliproteins than the sodium phosphate buffer. The McIlvaine buffer was then adopted for the optimization studies. The optimization of the process variables (SLR, pH and concentration of the McIlvaine buffer ([salt])) was performed by applying a Response Surface Methodology (RSM). This methodology allows the simultaneous analysis of different parameters and the determination of the relationship between the yield of phycobiliproteins extracted from the biomass (response variable) and the operational conditions (independent variables). For the RSM, a 2³ factorial planning study (3 factors and 2 levels) was

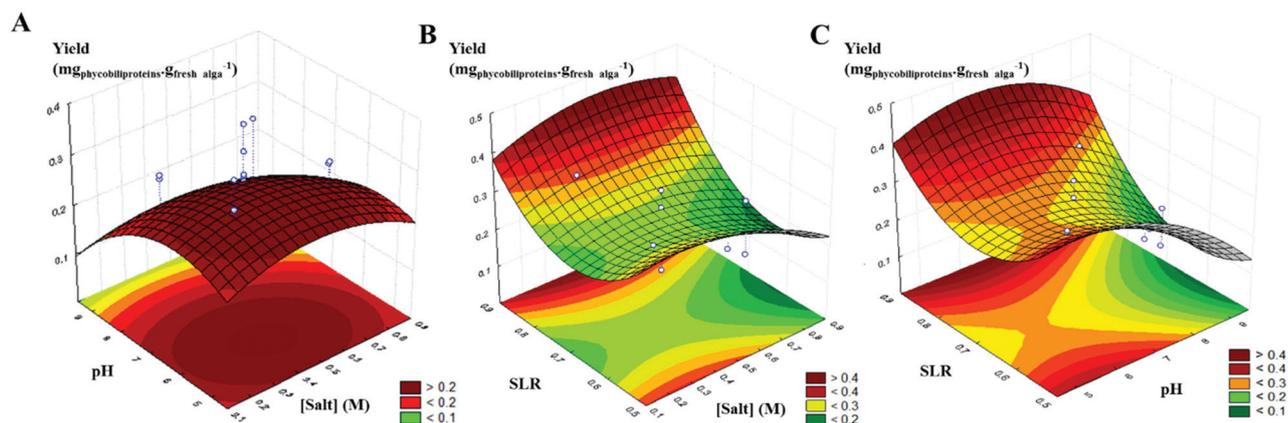


Fig. 4 Response surface plots showing the yield of extraction of phycobiliproteins ($\text{mg}_{\text{phycobiliproteins}} \cdot \text{g}_{\text{fresh alga}}^{-1}$) with the combined effects of (A) pH and salt concentration (M); (B) solid–liquid ratio and salt concentration (M); and (C) solid–liquid ratio and pH of the solvent, using the McIlvaine buffer.

executed. SLR, pH and [salt] were the conditions optimized (Table A1 and A2 in the ESI†). The model equations, the yield of phycobiliproteins experimentally and theoretically extracted, as well as all the statistical analyses results are also shown in the ESI.† The response surface plots are represented in Fig. 4.

The accuracy and the precision of the model equations were validated by comparison of the experimental and the predicted values of the extraction yield under the conditions previously selected. No significant differences (p -value = 0.05) were observed between the theoretical and experimental responses (Table A2–A4 in the ESI†).

Fig. 4 depicts the effect of the three variables on the phycobiliprotein extraction. The results presented in the response surface plots identify the pH and SLR as the most efficient conditions. The results indicate that the higher the SLR, the higher the extraction of phycobiliproteins (Fig. 4C). However, as previously observed for the conventional extraction, very high SLR hinders the extraction process itself, especially above 0.7, and this experimental restriction was taken into account in further studies. The pH data indicate that there is a theoretical maximum close to pH 6, while the [salt] has no significant effect (Fig. 4A). This means that the salt present in the solution and used in the McIlvaine buffer preparation is only important to maintain the buffer condition of the system, but it is not responsible for the cell disruption or the increase in the solubility of the phycobiliproteins in water. These conclusions are further supported by the Pareto diagram presented in Fig. A4 in the ESI.†

The surface response design has drawn attention to the importance of pH optimization. Since this work focuses on the extraction of fluorescent proteins, it is expectable that the phycobiliproteins may suffer from pH variations, since it may induce conformational changes that may impact the extraction process. In this context, various pH values were studied in the extraction of phycobiliproteins from *Gracilaria* sp. and the results are depicted in Fig. 5. Focusing on the phycobiliprotein

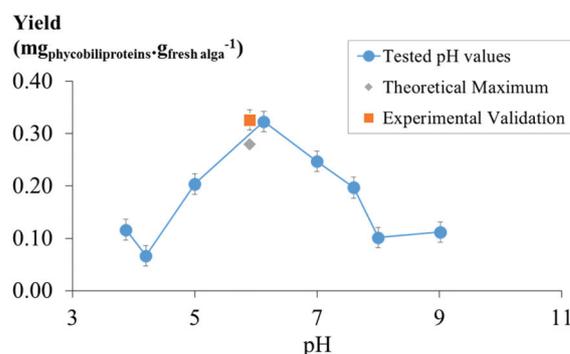


Fig. 5 Variation of the extraction yield of phycobiliproteins with the solvent pH: tested pH values (blue circles), theoretical maximum determined by the SRM (grey diamond) and experimental validation of the theoretical data (orange square).

content of the extracts, Fig. 5 presents a study of the pH effect on their extraction, and the validation of the theoretical maximum obtained from the surface response design. The results show that the best extraction yield is obtained for pH values between 5 and 7. For higher and lower pH values, the extraction of the protein decreases significantly. At pH 4.2, which corresponds to the isoelectric point of R-phycoerythrin,³³ the most abundant phycobiliprotein present in this biomass, the amount of phycobiliproteins extracted is the lowest since at this pH, R-phycoerythrin is in its neutral form and, consequently, has its lowest solubility in water.³⁴

In order to validate the optimized conditions obtained by the SRM procedure, the theoretical maximum was experimentally measured in triplicate (orange point in Fig. 5). The methodology was validated and pH 5.9 was adopted as the optimum pH for the extraction process.

Effect of the IL concentration on the extraction process

Having optimized the main variables of the alternative extraction process, the effect of the aqueous concentration of [Ch]Cl

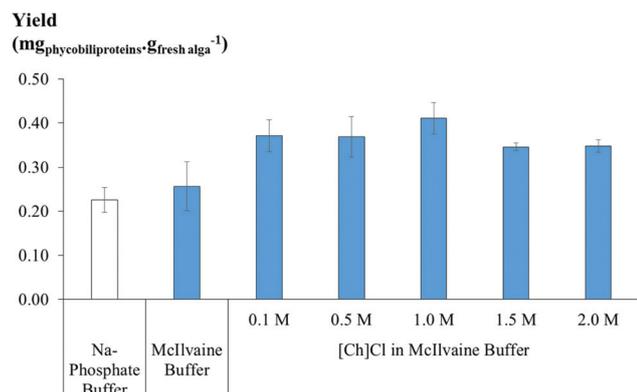


Fig. 6 Yield of extraction of phycobiliproteins by using different solvents, including [Ch]Cl in different concentrations (M).

was further evaluated. All results previously analyzed were performed with 1.0 M and, in this section, the concentrations of 0.1 M, 0.5 M, 1.5 M, and 2.0 M were tested for the conditions previously optimized of SLR (0.7), time of extraction (20 minutes), and pH (5.9) by using the McIlvaine buffer (Fig. 6).

The IL concentration effect on the extraction yield is depicted in Fig. 6. The experimental data show the increase in the yield of phycobiliproteins extracted with the addition of [Ch]Cl, even in low concentrations. This must be due to a salting-in phenomenon induced by the presence of [Ch]Cl, which even at low concentrations, allows the increase of the phycobiliprotein solubility in the aqueous bulk. The alternative extraction proposed here increases the extraction of phycobiliproteins to 46.5%, at 1.0 M of [Ch]Cl, selected here as the best concentration of ionic liquid tested. These results show the advantageous nature of aqueous solutions of ionic compounds as solvents to extract the phycobiliproteins. To evaluate the purity of the aqueous extract formed, a SDS-PAGE assay (Fig. 7) was carried out. In this experiment, the presence of R-phycoerythrin (the most abundant phycobiliprotein present in *Gracilaria* sp.) and the main contaminant proteins was proved. The presence of R-phycoerythrin was identified in the SDS-PAGE image in all extracts, which is evidenced by the presence of its subunits α , β , and γ . However, and despite the higher capacity of [Ch]Cl at 1.0 M to extract the phycobiliproteins, it seems that the aqueous extracts obtained are not pure in phycobiliproteins, as shown by the presence of other bands, from 150 kDa to 12 kDa. Moreover, the results allow us to conclude that in terms of purity of the fluorescent proteins, the alternative and conventional methodologies applied in this work provide similar results. This issue also proves the advantages of the alternative procedure here developed since the use of [Ch]Cl at 1.0 M promotes the extraction of higher contents (over 46.5%) of phycobiliproteins in a single step extraction.

Analysis of protein integrity

The protein structural integrity was evaluated regarding not only the maintenance of the secondary structure by circular

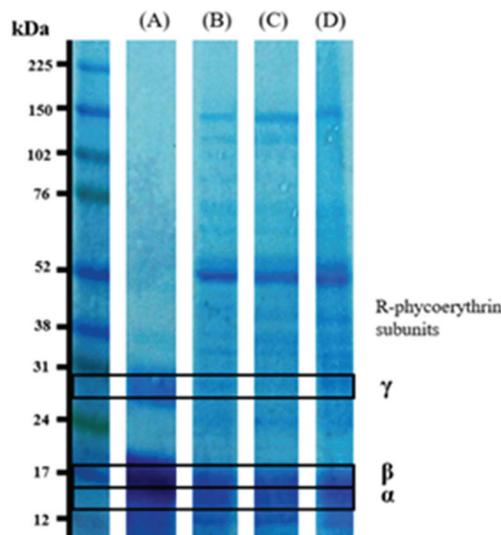


Fig. 7 SDS-PAGE of phycobiliproteins extracted from *Gracilaria* sp.: (A) pure R-phycoerythrin; aqueous extract obtained after extraction with (B) sodium phosphate buffer; (C) McIlvaine buffer; and (D) 1.0 M of [Ch]Cl and the McIlvaine buffer.

dichroism but also by the analysis of the structural integrity of the chromophores representative of the phycobiliproteins by fluorescence assays (Fig. 8). Circular dichroism has been extensively applied in the evaluation of the protein's secondary structure since the protein's circular dichroism spectrum is considered to be the sum of the spectra of the individual secondary structures found in the protein (α -helix, β -sheets, random coil, etc.).

The circular dichroism spectra measured for the conventional and the best alternative solvents identified before are depicted in Fig. 8A. The initial samples were diluted until a good spectrum could be obtained. In general, all systems are able to extract the protein without significant changes in its secondary structure. According to Fontecilla-Camps *et al.*³⁵ the secondary structure of R-phycoerythrin obtained from *Gracilaria chilensis* is mainly composed of α -helices (71%), and just 12% of β -sheets and 17% random coils. This is in good agreement with our results, since the circular dichroism spectra show the maxima of negative signals at *ca.* 222 and 210 nm, typical of proteins with a high α -helical content. Circular dichroism spectra measured in the Vis region (Fig. 8A) show the similarity between the spectra measured for the pure protein and the extracted samples.

The photoluminescence features of pure R-phycoerythrin were studied. The excitation spectrum was monitored with the emission peak position around 578 nm (Fig. 8B). The spectrum resembles the absorption one revealing a series of components at 565, 539, 498, 375, 308, and 280 nm. All peaks are ascribed to the excited states of the chromophore, except the low-wavelength one around 280 nm that is due to the amino acid residues of the apoprotein.²⁶ The emission spectrum of the pure R-phycoerythrin was measured under direct excitation in the R-phycoerythrin excited states. The spectrum shows a

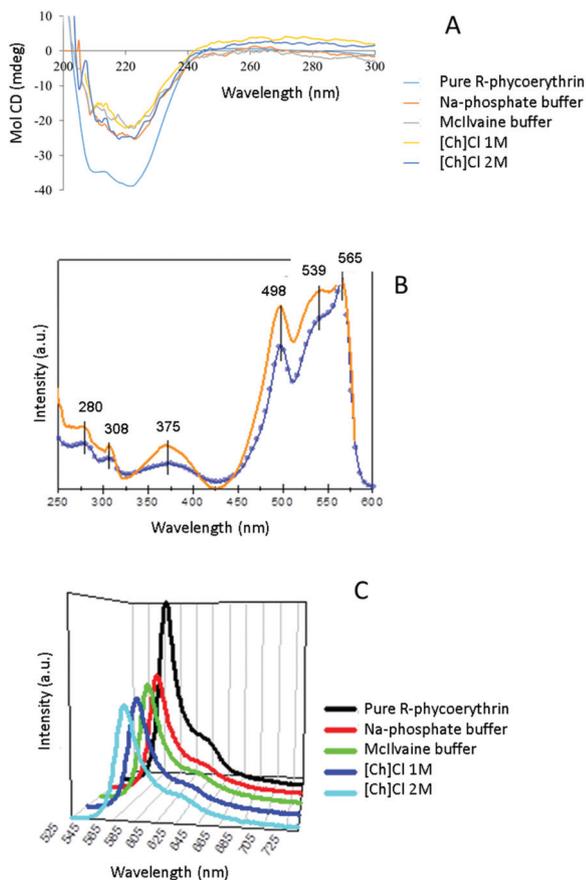


Fig. 8 (A) CD spectra measured in the far UV range at room temperature, (B) excitation spectrum of the pure R-phycoerythrin monitored at 578 nm (orange line) and the absorption one in the UV-Vis spectral range (blue line), and (C) emission spectra excited at 498 nm.

broad band with two components at 580 nm and 620 nm, typical of R-phycoerythrin fluorescence. The emission spectra of the selected samples (Fig. 8C) resemble that of pure R-phycoerythrin, which is in good agreement with the presence and maintenance of the R-phycoerythrin structure, as well as the chromophore stability, by the similarity of the spectra.

Conclusions

In this work an alternative methodology for the extraction of phycobiliproteins (namely R-phycoerythrin) was proposed and optimized. Since one of the major limitations of algae processing is their high water content, various aqueous solutions of ionic liquids were tested. The results show that it is possible to extract different classes of compounds from *Gracilaria* sp., namely phycobiliproteins and chlorophylls, using aqueous solutions of ionic liquids. The studies allowed us to select aqueous solutions of [Ch]Cl as the solvent with the best extractive performance, not only due to their capacity to extract higher amounts of fluorescent proteins, but also because it avoids the extraction of chlorophylls, considered here as con-

taminants. Taking into account the best results found for [Ch]Cl, the operational conditions for the extraction process were optimized in terms of the solid-liquid ratio, pH, salt buffer used and concentration of ionic liquid. A significant increase in the amount of phycobiliproteins extracted of around 46.5% was obtained, when compared with the conventional methodology, without compromising their conformational structure or chromophore structural integrity.

Experimental

Materials

The red macroalgae *Gracilaria* sp. used in this work was grown in a land-based integrated aquaculture system (meaning that the nitrogen input is higher due to the use of enriched water from fish production) at ALGApplus Lda, a company specialized in the production of marine macroalgae, located in Ílhavo, Portugal.

The components of the sodium phosphate buffer ($\text{Na}_2\text{HPO}_4/\text{NaH}_2\text{PO}_4$), sodium phosphate dibasic heptahydrated, $\text{Na}_2\text{HPO}_4 \cdot 7\text{H}_2\text{O}$ (≥ 98 wt%), and sodium phosphate monobasic, NaH_2PO_4 (≥ 99 wt%) were purchased from Sigma-Aldrich and Panreac, respectively. A commercial standard of R-phycoerythrin (≥ 10.0 mg mL^{-1}) was acquired from Sigma-Aldrich.

The series of 1-alkyl-3-methylimidazolium chloride ionic liquids [C_nmim]Cl, including the 1-ethyl-3-methylimidazolium chloride, [C₂mim]Cl (98 wt%), 1-butyl-3-methylimidazolium chloride, [C₄mim]Cl (99 wt%), 1-hexyl-3-methylimidazolium chloride, [C₆mim]Cl (98 wt%), 1-decyl-3-methylimidazolium chloride, [C₁₀mim]Cl (98 wt%), 1-dodecyl-3-methylimidazolium chloride, [C₁₂mim]Cl (>98 wt%), 1-ethyl-3-methylimidazolium acetate, [C₂mim][CH₃CO₂] (98 wt%), 1-butyl-3-methylimidazolium dicyanamide, [C₄mim][N(CN)₂] (98 wt%), 1-butyl-3-methylimidazolium trifluoromethanesulfonate, [C₄mim][CF₃SO₃] (99 wt%), 1-butyl-3-methylimidazolium acetate, [C₄mim][CH₃CO₂] (98 wt%), 1-butyl-3-methylimidazolium dimethylphosphate, [C₄mim][DMP] (>98 wt%), 1-butyl-3-methylimidazolium tosylate, [C₄mim][Tos] (98 wt%), 1-butyl-3-methylimidazolium methanesulfonate, [C₄mim][CH₃SO₃] (99 wt%), 1-butyl-3-methylimidazolium thiocyanate, [C₄mim][SCN] (98 wt%), 1-butyl-3-methylimidazolium trifluoroacetate, [C₄mim][CF₃CO₂] (97 wt%), 1-ethylimidazolium acetate, [C₂im][CH₃CO₂] (97 wt%), 1-butyl-1-methylpiperidinium chloride, [C₄mpip]Cl (99 wt%), 1-butyl-1-methylpyrrolidinium acetate, [C₄mpyr][CH₃CO₂] (97 wt%) and 1-butyl-1-methylpyrrolidinium chloride, [C₄mpyr]Cl (99 wt%), 1-butyl-3-methylpyridinium chloride, [C₄mpyr]Cl (98 wt%), and choline acetate, [Ch]Ac (>99 wt%) were acquired from IOLiTec (Ionic Liquids Technologies, Germany). The tetrabutylammonium chloride, [N_{4,4,4,4}]Cl (97 wt%) and the cholinium chloride, [Ch]Cl (99 wt%) were purchased from Sigma-Aldrich. Tetrabutylphosphonium chloride, [P_{4,4,4,4}]Cl, was kindly supplied by Cytec and it was dried before the experiments. The chemical structures of all compounds used in this work are depicted in Fig. A1 (ESI[†]).

Conventional solvent extraction

After the harvesting of the macroalgae, the samples were cleaned and washed with fresh and distilled water at least 3 times. The marine biomass was then stored at $-20\text{ }^{\circ}\text{C}$. The procedure adopted was based on the methodology proposed by Liu and collaborators¹² to extract phycobiliproteins (and R-phycoerythrin) from the red macroalgae, *Polysiphonia urceolata*, using a sodium phosphate buffer solution. The red macroalgae samples were frozen in liquid nitrogen and ground to increase the contact surface, thus enhancing the extraction. Then, the biomass was homogenized in a sodium phosphate buffer aqueous solution at 20 mM, pH 7.0 at room temperature, in an incubator (IKA KS 4000 ic control) protected from light exposure. At the end of the extraction, the red solution was filtered and, subsequently, the filtrate obtained was centrifuged in a Thermo Scientific Heraeus Megafuge 16 R centrifuge at 5000 rpm for 30 minutes at $4\text{ }^{\circ}\text{C}$. The resultant pellet was discarded while the phycobiliprotein-based red supernatant was collected. All the conditions were tested in duplicate. The operational conditions of the solid-liquid ratio (SLR), time of extraction and pH were also optimized in this work.

Spectroscopic methods

The absorption spectra were measured between 200 and 700 nm using a UV-Vis microplate reader (Synergy HT microplate reader – BioTek). The phycobiliproteins were quantified at 565 nm, and the total amount of contaminant proteins at 280 nm, taking into account two calibration curves previously prepared. The concentration of phycobiliproteins is expressed in $\text{mg}_{\text{phycobiliproteins}} \text{g}_{\text{fresh algae}}^{-1}$ and the content of contaminant proteins is expressed in $\text{mg}_{\text{proteins}} \text{g}_{\text{fresh algae}}^{-1}$. The potential interference of the compounds used in the preparation of the aqueous solutions (salts and ionic liquids) and other fluorescent pigments (namely chlorophylls) was analyzed and defined as null under the conditions of the process.

The photoluminescence spectra were recorded at room temperature with a modular double grating excitation spectrofluorimeter with a TRIAX 320 emission monochromator (Fluorolog-3, Horiba Scientific) coupled to a R928 Hamamatsu photomultiplier, using a right angle acquisition mode. The excitation source was a 450 W Xe arc lamp. The emission spectra were corrected for detection and optical spectral response of the spectrofluorimeter and the excitation spectra were corrected for the spectral distribution of the lamp intensity using a photodiode reference detector.

Circular dichroism spectra were recorded on a JASCO J-720 spectropolarimeter (JASCO, Hiroshima, Japan) with a 180–700 nm photomultiplier (EXEL-308). Circular dichroism spectra were recorded in the range from 200 to 700 nm with quartz Suprasil® circular dichroism cuvettes (0.1 cm) at room temperature (*ca.* $25\text{ }^{\circ}\text{C}$). Each circular dichroism spectrum is the result of three accumulations recorded in millidegrees. The following acquisition parameters were used: data pitch, 0.5 nm; bandwidth, 2.0 nm; response, 1 s; and scan speed, 200 or 500 nm min^{-1} .

Screening of ionic liquids & process conditions

Aqueous solutions of diverse ionic liquids were tested for their extractive performance in recovering phycobiliproteins. The extraction procedure firstly adopted corresponded to the optimal conditions selected in the conventional extraction methodology, particularly for the solid-liquid ratio (0.7 in fresh weight of biomass/volume of solvent) and time of extraction (20 minutes). The effect of ionic liquids with different structural features, and their concentration in aqueous solution was screened regarding their effect on the extraction of phycobiliproteins from fresh samples of *Gracilaria* sp. The solutions of ionic liquids were prepared in 20 mM of sodium phosphate buffer (pH 7) to control the pH of the solutions.

After the selection of the best ionic liquid to extract phycobiliproteins, the optimization of the process variables was performed. The conditions tested were the SLR, the pH, and the concentration of the salt used to prepare the McIlvaine buffer solution. These conditions were simultaneously analysed³⁶ by a Response Surface Methodology (RSM). This methodology allows not only the simultaneous study of different conditions, but also the assessment of the relationship between the yield of phycobiliproteins extracted from the marine biomass (response variable) and the operational conditions of interest for the solid-liquid extraction process (independent variables). A 2^3 factorial planning was carried out (see the ESI† for more details). In this work, a total of 20 extractions were performed, including 8 extractions for factorial points, 6 for axial points, and 6 repetitions of the central point, which were the various processing conditions repeated to guarantee the accuracy of the data. The results obtained were statistically analyzed considering a confidence level of 95%. The adequacy of the model was determined. The Statsoft Statistica 8.0® software Statsoft® was applied in the statistical analysis and preparation of the response surface plots.

No sequential extraction was carried out in this work. Added to the fact that the main objective was the development of a single-step extraction procedure, preliminary results have shown that sequential extractions seriously compromise the selectivity of the experimental methodology developed here. They promote aqueous extracts of lower purity, because in the second cycle of extraction, essentially, contaminant proteins are extracted (results not shown).

Sodium dodecyl sulphate–polyacrylamide gel electrophoresis, (SDS–PAGE) analysis

The electrophoresis was performed on polyacrylamide gels (stacking: 4% and resolving: 20%) with a running buffer (pH 8.3) consisting of 250 mM of Tris-HCl, 1.92 M 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 and at room temperature. The gels were destained in a solution containing acetic acid 7% (v/v), methanol 20% (v/v), and water 73% (v/v) in an orbital shaker at a moderate speed ($\pm 60\text{ rpm}$), for

3–4 hours, at room temperature. SDS-PAGE Molecular Weight Standards (VWR) were used as protein standards.

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