Using aqueous solutions of ionic liquids as chlorophyll eluents in solid-phase extraction processes

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ABSTRACT

There is a need for handy and fast techniques to purify biomolecules, increasing their stability and value, because the separation units are current bottlenecks in downstream processes. Solid-phase extraction is a technique that enables the purification of a compound by its adsorption from a liquid matrix. The AmberLite™ HPR900 OH resin allows the separation of chlorophylls from complex extracts, however the recovery of the chlorophylls is not easy to achieve. An innovative procedure to elute the chlorophyll from AmberLite™ HPR900 OH, based on the use of aqueous solutions of surface-active ionic liquids is proposed in this work. The operational conditions were optimized, showing that the resin can be reused for at least five cycles without losing its efficiency and the chemical structures of the pigments recovered were identified.

1. Introduction

There is a growing demand for natural products associated with the increased awareness of human health and environmental concerns that may be addressed using compounds from natural sources instead of synthetic alternatives [1,2]. The concerns about climate change and the new policies to reduce the environmental impact of industrial processes and products reinforce this trend [3], particularly when integrated into a smart and low-waste chain of different products or within a circular economy approach [1,2,4,5]. Thus, natural sources like microalgae biomass arise as a renewable and sustainable feedstock [6]. Under the concept of biorefinery, where the goal is to obtain high-value added products, biofuels and energy from biomass, microalgae emerge as an important and sustainable alternative to fossil fuels, [6]. The main advantages associated to microalgae are related with their cultures in degraded land using only water and atmospheric CO2 and also with their high growth rates [6]. So, like macroalgae or cyanobacteria [7,8], microalgae may also be considered a source of valuable bioactive compounds [6].

Natural pigments are one of the most important families of compounds that can be obtained from natural sources. Their use can boost the visual appearance of a final product and act as health promoters due to their multiple biological activities, especially regarding their antioxidant and anti-inflammatory properties [6,9,10]. Two good examples are the chlorophylls and xanthophylls (oxygenated carotenoids) that are well-known pigments present in photosynthetic organisms such as plants, microorganisms, and algae [9]. Along with the sustainable use of natural resources, obtaining and processing them should also be a concern [4,5]. Several works have proposed alternative approaches to extract these pigments without compromise their stability [11,12], and studied the economic [12] and environmental [13] viability of these processes. Aqueous solutions of surface-active ionic liquids (ILs) and common surfactants have been proposed as solvents to extract chlorophylls [12,14] and carotenoids [13,15] from various natural matrices. Surface-active ILs can be very efficient in extraction processes due to their ability to create small pores in the cell membrane, allowing the release of intracellular compounds, (cell disruption step in Fig. 1 from Martins et al. (2020) [16]) and by increasing the solubility of hydrophobic compounds (like chlorophylls and carotenoids) in water through the formation of micellar structures.
Fig. 1. Percentage of the total chlorophyll present in the carotenoid extracts in several resin reuse cycles without and with NaOH treatment. Different capital letters represent statistically different values in the process without NaOH treatment (p < 0.05). Different lowercase letters represent statistically different values in the process using a NaOH treatment (p < 0.05).

[12,17] which are formed above the critical micellar concentration (Extraction step in Fig. 1 from Martins et al. (2020) [16]).

Although the extraction of these pigments is straightforward [12], since chlorophylls and carotenoids have similar polarities and are usually present in the same cellular sites [18], their co-extraction usually occurs at similar conditions. This means that an additional purification step is required to separate chlorophylls from carotenoids (Purification step in Fig. 1 from Martins et al. (2020) [16]). Additionally, the separation of chlorophylls from carotenoids allows a more correct quantification of these compounds by simple techniques such as UV–Vis spectroscopy, since carotenoids and chlorophylls present a maximum absorption at close wavelengths [19]. However, this purification step remains the bottleneck of the whole process [20], because conventional techniques such as chromatography processes or saponification reactions are not only energy/time consuming but also require specific equipment and several steps to achieve the desired result [21,22].

A simpler and reliable separation process for this purpose is the solid-phase extraction. This is based on the adsorption of chlorophylls on a resin due to intermolecular and interionic interactions (e.g., dipole–dipole, ion–dipole, hydrogen bonding, ion-ion) between chlorophyll derivatives and the tetramethylammonium functional group of Amberlite™ HPR900 OH (previously known as Ambersep 900 OH) [23–25]. Amberlite™ HPR900 OH is a strong basic anionic resin that allows an effective and fast adsorption of chlorophyll, while the carotenoids remain in solution [23–25]. Its efficiency was already demonstrated for various extracts from green vegetables such as beans, broccoli, spinach, lettuce, and peas, among others [24,25]. However, no previous work has successfully eluted the chlorophyll from the resin, allowing the recovery of the chlorophyll as a secondary product, and enhancing the resin’s lifetime through more cycles of reuse (Polishing step in Fig. 1 from Martins et al. (2020) [16]).

In this work, a simple process to separate carotenoids and chlorophylls from an extract of microalgae Isochrysis galbana Parke 1949 using the resin AmberLite™ HPR900 OH is proposed, with the polishing step being accomplished by the use of aqueous solutions of surface-active ILs, to elute chlorophylls. The reutilization of the resin was also carried during five cycles in batch and in continuous regimes to show the ability of this approach to extend the lifetime of the resin envisioning its industrial application.

2. Experimental section

2.1. Biomass

The microalga used in this work is the Isochrysis galbana Parke 1949 and it was obtained in systems of photobioreactors (PhytoBloom). It was purchased at Necton S.A., a company located in Olhão (Portugal). The batch used in this work was produced in July 2018, being freeze dried and grinded in August 2018. The biomass was kept in a dry and dark environment until usage.

2.2. Chemicals

Ethanol (HPLC grade, CAS 64-17-5), methanol (HPLC grade, CAS 67-56-1), and acetone (HPLC grade, CAS 67-64-1) used on the extraction of pigments from the biomass were acquired from Fisher Scientific. AmberLite™ HPR900 OH (CAS 9017-79-2), a strong basic anionic resin, which is composed of approximately 35–55% quaternary amine styrene–divinylbenzene copolymer of the OH form and 45–65% water [25], was purchased from Sigma-Aldrich. Several organic solvents were additionally used in the attempts to elute chlorophyll from the resin. Dichloromethane (99.9 wt% of purity, CAS 75-09-2), and toluene (99.8 wt% of purity, CAS 108-88-3) were purchased from Fisher Scientific. Formic acid (99 wt% of purity, CAS 64-18-6), acetonitrile (99.99 wt% of purity, CAS 75-05-08), and petroleum ether (PA-ACS-ISO, CAS 8032-32-4) were purchased from Carlo Erba, Fisher Chemical, and Panreac, respectively. The sodium hydroxide (98.0 wt% of purity, CAS 1310-73-2) was supplied by Fisher. The ionic liquids (ILs) based on ammonium family such as dodecyltrimethylammonium bromide, [N\textsubscript{1,1,1,12}Br] (99 wt% of purity, CAS 1119-94-4) and tetradecltrimethylammonium bromide, [N\textsubscript{1,1,1,14}Br] (98 wt% of purity, CAS 1119-97-7), were purchased from Alfa Aesar while the decyltrimethylammonium bromide, [N\textsubscript{1,1,1,10}Br] (99 wt% of purity, CAS 2082-84-0), was acquired from Tokyo Chemical Industry (TCI). The tributyltetradecylphosphonium chloride, [P\textsubscript{4,4,4,14}Cl] (95 wt% of purity), was supplied by lithic. The molecular structures of the ILs used in this work are depicted in Fig. S1 in ESI. The deuterium oxide (99.9 atom % D of purity, CAS 7789-20-0) used for NMR spectroscopy was purchased from Aldrich.

2.3. Pigments’ extraction

The solid–liquid extraction step was performed using a methodology adapted from Martins et al. (2021) [12]. Pure ethanol, acetone, and methanol were screened as extraction solvents. The dry biomass was homogenised with the solvent in a shaker IKA TRAYSTER digital under constant vertical rotation (80 rpm). The extraction was performed using 0.01 g Biomass mL\textsuperscript{-1} at room temperature (20–25 °C), during 30 min and protected from light exposure [12]. The samples were centrifuged in a Thermo Scientific Heraeus Megafuge 16R centrifuge at 4700g, for 30 min at 4 °C [12]. The supernatant (initial extract) was collected and the biomass debris were discarded.
3.4. Chlorophyll adsorption and carotenoids recovery

The commercial resin AmberLite™ HPR900 OH was washed with distilled water following the procedure proposed by Larsen and Christensen (2005) [25], and dried in an oven at 50 °C for 15 min. The initial extract collected with the different solvents was further diluted in the same solvent 1:1 (v/v) to avoid the pigment saturation in the resin. Initially, 1 g of resin was put in contact with 10 mL of diluted extract under magnet stirring at room temperature (20–25 °C) for 30 min [25]. The chlorophyll content was mostly adsorbed by AmberLite™ HPR900 OH, while the carotenoid content remained in organic solution. The organic fraction was collected, being the carotenoid content analyzed for each initial solvent.

2.5. Chlorophyll elution and resin regeneration

Various solutions, reported in Table 1, were screened for their performance to elute chlorophylls from the resin, with ethanolic extracts being used in the adsorption step. Concentrations above the critical micellar concentration (CMC) were always used for the aqueous solutions of ILs (CMC values available at Table S1 in ESI). For each case, the same conditions of chlorophyll adsorption were applied [25], i.e. the resin was contacted with 10 mL of the regenerating eluent under stirring at room temperature (20–25 °C) during 30 min. After this period, the chlorophyll content of the solution was analyzed by UV–Vis spectroscopy [12]. Three replicates were made in order to decrease the error associated with each assay.

2.6. Optimization of the elution of chlorophylls from the resin

In order to optimize the elution of the chlorophylls from the resin, a central composite rotatable design (CCRD – 2° with 6 central points and axial points) was applied in a total of 20 assays. This assay was done separately for the two best eluents found. The results obtained were statistically analyzed with a confidence level of 95%, using pure error as standard. Three independent variables were studied, namely the solid–liquid ratio, i.e. the ratio between the mass of resin and the volume of eluent used (SLR, g_resin mL_eluent⁻¹), time of contact (t, min), and concentration of IL (CIL, mM), being their performance analyzed in terms of the chlorophyll recovery from the resin (%). Regarding the SLR study, the volume of extract (in adsorption step) and the volume of eluent (in the elution step) were kept constant, being 5 and 15 mL, respectively. The mass of resin was variable according to the SLR along the 20 runs, however, constant from the start to the end of each run individually, i.e. the initial mass of resin used in the adsorption step was the same used in the elution step for each run. All the codified and real values used in the CCRD are shown in Table 2. The results were analyzed using the software Statistica® 12 according to the theory proposed by Dean et al. (1999) [32] and Rodrigues and Lemma (2014) [33]. After interpretation of the response surface methodology results, the optimum conditions to elute chlorophylls were determined, with further validation of the optimum conditions in triplicate by the means of relative deviation (%).

2.7. Pigments’ quantification

The UV–visible spectra of the collected samples were measured between 200 and 700 nm in a UV–Vis microplate reader (Synergy HT microplate reader – BioTek) [12]. The mass percentage of chlorophyll was determined according to calibration curves previously obtained at 667 nm (R² = 0.9389 and R² = 0.9805 for aqueous and ethanolic extracts, respectively) and comparing the chlorophyll mass loaded in the resin (present in the organic extract) and the chlorophyll mass of each fraction collected (Eq. (1)). Note that, during this work, it was used chlorophyll recovery instead of eluted chlorophyll since the chlorophyll content was analyzed not only in the elution step but also in the collected fraction of carotenoids and fraction of NaOH used in the resin regeneration. Although Isochrysis galbana has different xanthophylls, such as diadinoxanthin [34], diatoxanthin [35], and fucoxanthin [36,37] (being also the diadinoxanthin the biologic precursor of both diatoxanthin and fucoxanthin) [38], in this work the total xanthophylls content was directly related to the fucoxanthin content. The fucoxanthin quantification was done using a calibration curve in ethanol previously determined (R² = 0.998) at 450 nm, being its concentration (mFucox_{ethanolic extract}) calculated afterwards.

\[
\text{Chlorophyll recovery (\%)} = \frac{\text{Chlorophyll in the collected fraction (mg)}}{\text{Chlorophyll in organic extract loaded into the resin (mg)}} \times 100
\]  

(1)

2.8. Ultra-performance liquid chromatography coupled mass spectrometer (UHPLC-MS) analysis

The initial extract, the recovered fraction of carotenoids after the adsorption of the chlorophyll, and the eluted fraction of the chlorophylls after the polishing step were analyzed by UHPLC-MS. The UHPLC-MS analysis was performed by Thermo Scientific Ultimate 3000RSLC ( Dionex) equipped with a Dionex UltiMate 3000 RS diode array detector and coupled to a mass spectrometer adapted from Martins et al. (2021) [39]. The separation of the compounds was carried out with a gradient elution program at a flow rate of 0.3 mL min⁻¹, at 30 °C, using a Hypersil Gold C18 column (150 × 2.1 mm; 5 μm, Thermo Fisher). The injection volume in the UHPLC system was 3 μL, and the mobile phase consisted of formic acid 0.1% in water (A) and acetonitrile (7):methanol (3) (B), both...
was homogenized and kept at temperature with deuterium oxide (D$_2$O) as solvent.

2.11. Statistical analysis

One-way ANOVA (Analysis of variance) was performed followed by Bonferroni post-hoc test to compare the efficiency between batch and continuous regime along five cycles of reuse of the resin. The results were expressed as the means ± standard errors of the mean. Statistically significant differences were determined considering an α of 95% (p < 0.05).

3. Results and discussion

3.1. Carotenoids’ recovery and screening of solutions to elute chlorophyll

The use of the commercial resin AmberLite™ HPR900 OH to purify carotenoids by the adsorption of the chlorophyll content is not new. In the published works, initial extracts were obtained using acetone as solvent, extracts that were later loaded in the resin [24,25]. However, after the adsorption of chlorophylls and the recovery of the carotenoids, no procedure was found to elute the chlorophylls from the resin. The scope of the present work was to develop a protocol that allows to elute the chlorophyll derivatives from the resin, without affecting their chemical structures and biological activities, and in this way, to extend also the resin’s lifetime. Under this context, three initial extracts were prepared from Isochrysis galbana using as solvents acetone, methanol, and ethanol. These solvents were chosen due to their ability to extract carotenoids and chlorophylls [24,39]. Extracts rich in these pigments were obtained for the three organic solvents. However, when loaded in the resin, the xanthophyll rich extract collected had the lowest chlorophyll contamination when the initial ethanolic extract was used (1.8 mg.chl.L$^{-1}$) followed by the acetone and methanol initial extracts (5.9 and 8.0 mg.chl.L$^{-1}$, respectively). Based on these results, and taking into account the carbon footprint and environmental impact of the screened solvents [40], ethanol was used to progress the study.

The adsorption mechanism was first reported to be mediated by ion–ion interactions after the saponification of the chlorophyll and the release of phytol [25] and posteriorly to occur principally through hydrogen-bonds and dipole–dipole interactions between resin and chlorophylls polar units, leaving the carotenoid content in the ethanol [24]. In this work, a carotenoid rich-extract of orange colour was obtained after 30 min of contact, while the resin acquired a green colour degassed and filtered before use. The solvent gradient was 85% of solvent B in the first 3.9 min, followed by the increase up to 100% during 2.2 min, and maintaining 100% of solvent B for 18.9 min, returning to 85% during 6 min, and equilibrating during 7 min. The injection volume was 2 μL. UV–Vis spectral data were gathered in a range of 200 to 700 nm.

2.9. Continuous process in column

The continuous process was performed using a solid-phase extraction cartridge and a peristaltic pump to ensure a constant flow (45 μL.s$^{-1}$). In this step, the best eluent and the optimized conditions in the response surface methodology for the batch assays, up-scaled five times, were used. The time of contact adopted in the continuous process was not the same in comparison to the optimum values for the batch assays due to experimental limitations. The solid-phase extraction cartridge was prepared by packing 5.25 g of AmberLite™ HPR900 OH resin (previously washed with water and dried) between two frits into a 20 mL empty polypropylene cartridge (Bio-rad Econo-Pac), being the resin conditioned with 25 mL of sodium hydroxide aqueous solution (4% w:v) afterwards. A volume of 25 mL of the ethanolic extract was passed through the cartridge, and subsequently, the adsorbed chlorophyll content was eluted with 75 mL of 370 mM aqueous solution of [N$_{1,1,1,12}$]Br. To regenerate the OH$^-$ groups of the resin, 25 mL of an aqueous solution of sodium hydroxide (4% w:v) was passed through the column [23]. The chlorophyll content of each solution collected from the solid-phase extraction cartridge was analyzed by measuring the absorption at 667 nm and applying the correspondent calibration curve.

2.10. Chlorophyll polishing

The aqueous solution of IL containing eluted chlorophyll was freeze-dried to remove the water content of the sample. The powder obtained was dissolved in ethanol in the proportion of 10:3 ($V_{\text{ethanol}}$/$V_{\text{initial aqueous solution}}$). The ethanolic solution containing the chlorophyll content and IL was homogenized and kept at -80 °C, for three days. A viscous and colorless pellet was formed in the bottom of the flask in the liquid in the top of the flask. This procedure was adapted from Mesquita et al. (2019) [11]. In order to quantify the content of [N$_{1,1,1,12}$]Br in the ethanolic fraction, a $^1$H NMR spectroscopy was performed. The $^1$H NMR spectrum of pure IL and ethanolic fraction rich in chlorophylls was carried out using a Bruker AC 30 spectrometer (250 MHz) at room temperature.
due to the adsorption of the chlorophyll pigments (see Fig. S2B in ESI).

According to the data sheet of the resin [23], its regeneration should be done with an aqueous solution of NaOH (2–4%) to re-establish the OH groups of the resin. Several cycles of reuse of the resin with new batches of initial extract were performed with and without a step of regeneration in order to evaluate the importance of this regeneration step (Fig. 1).

The results in Fig. 1 show that the contamination of chlorophyll in the carotenoid extract increases in each cycle ($p < 0.05$), after addition of a new batch of ethanolic initial extract, when the NaOH regeneration solution was not previously used (9.1–34%). In the other hand, the contamination of chlorophyll in the carotenoid extract is much lower and evolving towards an almost constant value (12–16%) with the increase in the number of cycles of reuse, when the NaOH regeneration is carried (after cycle 4, there is no significant difference $p > 0.05$). This indicates the need of using NaOH to replace the OH$^-$ groups within the resin in a so-called regeneration process, allowing a more efficient adsorption of the chlorophyll in the subsequent cycles.

Although it was shown that the resin can be regenerated for several cycles with solutions of NaOH without compromising the quality of the carotenoid extract, this does not mean that the chlorophyll can be efficiently recovered from the resin. Given the chlorophyll market value, and to extend the lifetime of the resin, the recovery of the chlorophyll pigments adsorbed is essential. Therefore, the performance of different eluents for an efficient recovery of the chlorophyll pigments was studied being the main results shown in Fig. 2.

Although solutions of NaOH were essential to regenerate the resin and restore the terminal OH$^-$ groups, they were not able to desorb chlorophylls from the resin. Several organic solvents that are used for chlorophyll extraction from biomass were tested, as well as mixtures of solvents used in the washing procedures of reverse-phase columns. None of them were efficient in the removal of the chlorophyll content (<2.5%) from the resin as can be observed in Fig. 2.

More appealing results were achieved when aqueous solutions of various ILs (at 250 mM) were investigated as eluents (Fig. 2, right-side). The screened solutions of ILs were used before with success in the extraction of chlorophyll and carotenoids from biomass [12,13]. Unlike

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**Fig. 3.** Percentage of chlorophyll recovered in the carotenoid extract and in three successive resin elutions, using $[\text{P}_{4,4,4,14}\text{Cl}]$ as eluent.

**Fig. 4.** Response surface plots obtained for the CCRD ($2^3$) using an aqueous solution of $[\text{P}_{4,4,4,14}\text{Cl}]$ regarding: time of contact (t in min), IL concentration ($C_{\text{IL}}$ in mM), and solid–liquid ratio (SLR in $g_{\text{resin}}:\text{mL}_{\text{eluent}}^{-1}$) in terms of percentage of the chlorophyll recovery from the resin.
the other screened solutions, most of the aqueous solutions of ILs showed to be able to remove the adsorbed chlorophyll from the resin, namely the $[\text{N}_{1,1,1,1,2}]\text{Br}$, $[\text{N}_{1,1,1,1,4}]\text{Br}$ and $[\text{P}_{4,4,4,1,4}]\text{Cl}$. These aqueous solutions of ILs were previously identified as efficient solvents to extract pigments due to their ability to form micelles above a certain concentration (as it happens at 250 mM in all tested solvents, see Table S1 in ESI) providing the perfect environment to hydrophobic molecules [12]. However, an additional interaction is behind their good performance as eluents. These cationic ILs have the same positively charged head as the one present in the functional group of the resin (Fig. S3 in ESI) providing the perfect environment to hydrophobic molecules [12]. However, an additional interaction is behind their good performance as eluents. These cationic ILs have the same positively charged head as the one present in the functional group of the resin (Fig. S3 in ESI). This said, the type of interaction between the chlorophyll derivatives and the functional group of the resin can be replaced by similar interactions but now involving the cationic IL. In this work, the $[\text{N}_{1,1,1,1,2}]\text{Br}$ and the $[\text{P}_{4,4,4,1,4}]\text{Cl}$, were selected to further optimize the operational conditions.

Successive elutions of the resin, applying the same mass of resin and volume of eluent (fresh solutions of $[\text{P}_{4,4,4,1,4}]\text{Cl}$), were applied to achieve the maximum chlorophyll recovery from the resin (Fig. 3). As conclusion, chlorophyll is successively extracted during three elution steps. Given that, and to keep the elution in a single step, the volume of eluent used is always 3 times higher than the volume used in the adsorption step (with ethanolic initial extract) and in the regeneration step (with aqueous solution of NaOH), in the following proportion 1:3:1 ($V_{\text{initial extract}}:V_{\text{eluent}}:V_{\text{NaOH}}$). This analysis was just performed considering the IL $[\text{P}_{4,4,4,1,4}]\text{Cl}$, since a similar response would be obtained with $[\text{N}_{1,1,1,1,2}]\text{Br}$. After, a more detailed analysis on the SLR by the manipulation of the resin mass used, was done by applying a response surface methodology for both aqueous solutions of $[\text{N}_{1,1,1,1,2}]\text{Br}$ and $[\text{P}_{4,4,4,1,4}]\text{Cl}$.

### 3.2. Optimization of the process conditions by a response surface methodology

For the central composite rotatable design (CCRD), three variables were studied to achieve a complete optimization of the best possible chlorophyll recovery (response), namely solid–liquid ratio (SLR in $g_{\text{resin}}\text{mL}_{\text{eluent}}^{-1}$), time of contact (t in min, X2), and concentration of IL in water ($C_{\text{IL}}$ in mM, X3). A total of 20 runs was performed, including the three common levels ($-1, 0, +1$), axial ($-1.68, and +1.68$ levels), and six central points (level 0). According to the CCRD experiment using aqueous solutions of $[\text{P}_{4,4,4,1,4}]\text{Cl}$, the percentage of chlorophyll recovery from the resin varied from 26.8% (assay 13) to 80.5% (assay 14), both regarding the axial points from the variable $C_{\text{IL}}$, which demonstrates its high influence on the response (Table S2 in ESI). The predicted values were expressed by the model provided by Eq. (2).
Using aqueous solutions of \([\text{P}_{4,4,4,14}]\text{Cl}\), the main effects responsible for the recovery of chlorophylls from the resin are the time of contact and the concentration of \([\text{P}_{4,4,4,14}]\text{Cl}\) in aqueous solution with no interaction between them, as reported in Table S3 in ESI. The variables were fitted to a first-order model and examined in terms of goodness of fit. The ANOVA was used to evaluate the adequacy of the fitted model considering a 95% confidence level, with \(F_{\text{calculated}} > F_{\text{tabulated}}\), and \(R^2 = 0.72275\). Additionally, the Pareto Chart and the graph of the predicted vs. observed values (Figs. S4 and S5, respectively in ESI) show additional information regarding the influence of the independent variables in the predictive model, demonstrating the high influence of the performance of the aqueous solution of \([\text{P}_{4,4,4,14}]\text{Cl}\) in the elution performance.

The model expressed by Eq. (2) was used to draw the response surfaces shown in Fig. 4. As described in Eq. (2), the SLR has no influence on the response. The time of contact was positively significant in the percentage of the chlorophyll recovered \((p < 0.05)\), being 48.5 min chosen as the optimum value. Even if the time of contact is not fully optimized, this variable does not cause a significant environmental and economic impact, since the homogenization method used in this work is not as energy intensive as ultrasound- and microwave-assisted extractions. The concentration of IL was completely optimized, achieving an optimum value around 350–400 mM (Fig. 4). Concentrations above 400 mM impair the performance of chlorophyll recovery, probably due a steric impediment for micelle formation, and consequently, chlorophyll recovery. Thus, the optimum operational conditions were set by using aqueous solutions of \([\text{P}_{4,4,4,14}]\text{Cl}\) at 370 mM and 48.5 min of contact at room temperature.

A model validation experiment using the optimized operational conditions was carried out in triplicate. The chlorophyll recovery from the resin obtained was around 80 ± 2%, which corresponds to a relative deviation of 2.7% (Table S4 in ESI), a very good result that suggests the high confidence and accuracy of the predictive model designed by the CCRD (2<sup>3</sup>).

Along with the aqueous solutions of \([\text{P}_{4,4,4,14}]\text{Cl}\), solutions of surfac-
active ammonium-based ILs also showed to be very promising to recover chlorophylls from the AmberLite™ HPR900 OH resin (69% of chlorophyll recovery). Therefore, the process was also optimized using [N\textsubscript{1,1,1,12}]Br as previously done for [P\textsubscript{4,4,4,14}]Cl. The maximum chlorophyll recovery was obtained in the run 8 (93.3%), using a SLR fixed at 0.070 g\textsubscript{resin} m\textsuperscript{-3}l\textsubscript{eluent} and a CI\textsubscript{H} of 370 mM, during 41 min of contact (see Table S2 in ESI). The data obtained in the Box-Behnken (Table S5 in ESI) experiment was converted into a second-order polynomial equation with three independent variables (X1, X2, and X3), as described by Eq. (3).

Chlorophyll recovery(%) = − 15.30 + 322.06(X1) − 5751.61(X1)\textsuperscript{2} + 1.17(X2) − 0.02(X2)\textsuperscript{2} + 0.30(X3) + 0.62(X1 × X3)  

The Pareto Chart shows that the most significant independent variables influencing the chlorophyll recovery are the CI\textsubscript{H}, both linear and quadratic, but the other variables also present an important role in the optimization process (Fig. S6 in ESI). The predicted and observed values were close to each other (Fig. S7 in ESI), making the model adequate. By applying ANOVA, the regression model was considered significant (\( p < 0.05 \), Table S5 in ESI), and thus useful in predicting the effects of the three different level factors in the recovery of chlorophylls. Interpreting together the response surfaces displayed in Fig. 5, it is possible to highlight an ideal recovery condition as SLR at 0.070 g\textsubscript{resin} m\textsuperscript{-3}l\textsubscript{eluent}, CI\textsubscript{H} of 370 mM, and 48.5 min of time of contact. This condition reflects in the prediction of almost 100% of recovery from the chlorophylls fixed in the resin (predicted value, see Table S6 in ESI). Although the observed recovery value under the optimum condition was 97.0 ± 0.9%, this model is considered valid and accurate, since a small relative deviation was observed (3.0%, Table S6 in ESI).

Regarding the best conditions obtained for the aqueous solutions of the two ILs (summarized in Table S7 in ESI), apart from the SLR, which has no significant value in the case of [P\textsubscript{4,4,4,14}]Cl, the best results in terms of chlorophyll recovery were found for the same concentration of [N\textsubscript{1,1,1,12}]Br, with 3.0% of relative deviation (Table S6 in ESI). The data obtained in the Box-Behnken (Table S5 in ESI) experiment was converted into a second-order polynomial equation with three independent variables (X1, X2, and X3), as described by Eq. (3).

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\text{Chlorophyll recovery(\%)} = -15.30 + 322.06(X1) - 5751.61(X1)^2 + 1.17(X2) - 0.02(X2)^2 + 0.30(X3) + 0.62(X1 \times X3)
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The results seem to suggest [N\textsubscript{1,1,1,12}]Br to be the best eluent, the aqueous solutions of both eluents were tested again, in the elution of chlorophyll, at their optimum conditions considering the reuse of the commercial resin up to five times (Fig. 6), including three stages: (i) the loading of a new ethanolic extract, (ii) the elution with aqueous solutions of IL and (iii) the regeneration with NaOH. In each cycle, three fractions were collected: the carotenoid rich extract (in ethanol), the eluted solution (aqueous solution of IL) rich in chlorophylls, and the aqueous solution of NaOH used to regenerate the resin. The xanthophyll content was analyzed in terms of fucoxanthin content (Fig. 6A). The concentration of fucoxanthin was kept constant from cycle 1 to cycle 5 (cycle 5 had the best performance), regardless the aqueous solution of IL used in the elution step, showing that the carotenoid fraction is not affected even considering five cycles reusing the resin. As depicted in Fig. 6B, the three collected fractions were also analyzed in terms of chlorophyll when compared to the initial extract (before the solid-phase extraction).

It is important to note that values of chlorophyll recovery > 100% in Fig. 6B can be a result of the interference of slight amounts of the solvents from the previous steps present in the fraction being analyzed, due to some experimental difficulties to completely recover the liquid extracts from the resin leaving it dry for the next step, which may consequently change the behavior of the calibration curve. However, this only means that the recovery is complete, since the chlorophyll recoveries of the cycle 1 (for both ILs) are lower compared to the other subsequent cycles (\( p < 0.05 \)), i.e. the recycling approach guarantees better efficiency regarding the recovery of both classes of pigments under study. Regarding the carotenoid rich extract, from the second cycle onwards, if the [N\textsubscript{1,1,1,12}]Br aqueous solution is used as eluent, there is a slight increase in the content of chlorophylls in the extract of carotenoids. Additionally, for each IL individually, the behavior in each cycle (and each fraction) seems to be similar after the second cycle, leading to the idea that more cycles could be done using the same resin and procedure. The chlorophyll content in the NaOH fractions has no significant differences regardless the eluent used in the elution step, being very low in all cycles. Moreover, it can be observed that, in all cases, most of the chlorophyll is in the eluted solution, i.e. in aqueous solution of IL, for both ILs. Photographs of the resin along with the adsorption and elution of chlorophyll and regeneration of the resin can be seen in Fig. S2 in ESI and the UV–Vis spectra of the collected fractions in the first cycle for both ILs are depicted in Fig. S8 in ESI. In addition to the best elution performance of chlorophyll from the AmberLite™ HPR900 OH, surface-active ammonium-based ILs are commonly used in the industry due to its low price [41], having lower associated
environmental impacts when compared with other families of ILs [42].

In the end, and after a deep analysis of all parameters optimized and results obtained, it was concluded that the ammonium-based IL provided the best results in terms of elution performance when compared with the phosphonium-based ILs. After the selection of the best eluent, the composition of the extracts at the different stages of the process was checked by UHPLC-MS. For that purpose, the initial extract (obtained after the solid–liquid extraction with ethanol from *Isochrysis galbana*), the carotenoid extract (obtained after passing the initial extract through the resin) and the chlorophyll extract (obtained after the use of [N$_{1,1,1,1,12}$]Br aqueous solution as eluent and polishing of the IL) were analyzed, being the results obtained depicted in Table 3 (see also Fig. S3 in ESI). From the data collected using UHPLC-MS it was possible to confirm the presence of the chlorophyll derivatives (pheophorbid a and pheophytin a) in the initial extracts, and also the presence of the xanthophylls (fucoxanthin and diatoxanthin). Moreover, it was confirmed the ability of the resin to preferably adsorb the chlorophyll derivatives allowing their separation from the carotenoid extract and the efficiency of the ammonium-based aqueous solution to subsequently recover the adsorbed chlorophyll derivatives. Furthermore, the UHPLC-MS analysis confirmed minor structural alterations in fucoxanthin (hydrolysis of the ester group and dehydration) affording a fucoxanthin derivative with the protonated molecular ion at m/z = 599. Concerning the chlorophyll extract, the results suggest the hydrolysis of the methyl ester in pheophorbid a and addition of water affording a pheophorbid a derivative with m/z at 597. No structural alterations were detected in diatoxanthin and in pheophytin a. Based on these results we believe that the selective removal of chlorophylls during the strong basic resin treatment can be attributed to a compromise between interionic (an anion exchange mechanism) and intermolecular forces such as hydrogen bonding and dipole–dipole interactions between the polar moieties of the resin and the chlorophyll derivatives. Similar interactions are responsible for the removal of the chlorophyll derivatives from the resin with the cationic IL (see Fig. S3 in ESI).

3.3. Continuous process in column

To study the adsorption and elution of chlorophylls, and regeneration of the resin in a continuous mode, a solid-phase extraction cartridge filled with the resin was used. The extract, eluent and regeneration solution used to perform the continuous studies were the same, although up-scaled five times, as in batch adsorption, elution, and regeneration.

Fig. 7. Comparison of the process using an aqueous solution of [N$_{1,1,1,1,12}$]Br as eluent both in batch and in continuous regime along five cycles of reuse of the resin, considering (A) fucoxanthin concentration in the carotenoid ethanolic extract (first fraction collected from the resin); and (B) chlorophyll recovery in the different fractions collected from the resin. Results regarding the batch process are once more displayed to facilitate the comparison. Note that different initial values in (A) are due to the need of preparing new initial extracts in the beginning of each experiment. Capital letters represent statistically results in the process mediated by [N$_{1,1,1,1,12}$]Br in batch ($p < 0.05$). Lowercase letters represent statistically results in the process mediated by [N$_{1,1,1,1,12}$]Br in continuous ($p < 0.05$).
process, with the flow kept constant at 45 \( \mu \text{L.s}^{-1} \). In each cycle, and as previously defined, the three fractions were collected, namely the carotenoid rich extract after passing through the column (in ethanol), the eluted solution (aqueous solution of IL) rich in chlorophylls, and the aqueous solution of NaOH used to regenerate the resin. All fractions were analyzed, being the results depicted in Fig. 7.

As previously observed for the batch process, the concentration of fucoxanthin (Fig. 7A) was kept constant from cycle 1 to cycle 5, with no loss in efficiency in the collection of fucoxanthin (cycle 5 had better or equivalent performance compared to the previous ones). It can also be observed (Fig. 7B) that, from the second cycle onwards, the amount of chlorophyll in the different fractions analyzed remains constant for the following adsorption, elution and regeneration cycles. Nevertheless, the chlorophyll content on the ethanol extract rich in xanthophylls after contact with the resin is higher than that observed at the batch process (26.0% and 12.2%, respectively, in the 2nd cycle). This decrease in the amount of chlorophyll adsorbed by the resin may be due to a low residence time of the adsorbate (chlorophyll) in the adsorbent (resin). Due to experimental constraints, the flow rate of 45 \( \mu \text{L.s}^{-1} \) was used since this was the minimum that could be achieved in the experimental setup.

![Schematic representation of the final process proposed in this work](image)

Fig. 8. Schematic representation of the final process proposed in this work, where i) represents the solid-liquid extraction of pigments from the biomass; ii) the recovery of xanthophyll and chlorophyll through continuous process in column; and iii) the polishing of pigments and recovery of the solvents. Dashed lines were not experimentally tested, being just a proposal of what can be done.
This flow seems to be somewhat high for this adsorption process, not allowing the time for the complete adsorption of the chlorophyll to the adsorbent. Since the residence time is a relevant parameter in the adsorption of chlorophylls by this resin (as well as in the elution of chlorophyll, as seen in the response surface methodology), the flow rate should be lower to achieve better results.

3.4. Chlorophylls polishing and proposal of an integrated process

At the end of the elution, an aqueous solution of [N1,1,1,12]Br with chlorophyll was collected. A process to achieve the polishing of chlorophyll was developed in order to have the chlorophyll free of IL to be used in any application and/or to allow the reuse of the IL in new cycles of elution. As explained in the methodology section, the water content was completely removed by freeze-drying. The resulting powder (a mixture of IL and chlorophylls) was dissolved in pure ethanol in the proportion of 10:3 (Vethanol/Vinitial aqueous solution). The liquid solution was stocked at −80 °C during three days. As a result, a pellet at the bottom and a green liquid on the top of the flask were obtained corresponding to the IL and ethanolic fractions rich in chlorophylls, respectively. The 1H NMR spectrometry analysis, performed in pure IL and in the ethanolic fraction rich in chlorophylls using D2O as solvent, revealed the absence of IL (here seen as contamination) in the ethanolic fraction (see Fig. S9 in ESI), allowing the reuse of IL. An integrated process was thus designed considering not only the methodology developed in this work but also a proposal of operations for the entire process, as sketched in Fig. 8. In short, a solid–liquid extraction of chlorophylls and carotenoids from Isochrysis galbana is done using pure ethanol as solvent. The obtained initial extract is passed through the commercial resin AmberLite™ HPR900 OH in a continuous process allowing a carotenoid rich-extract to be collected by the adsorption of the chlorophylls to the resin. The chlorophylls are then eluted using an aqueous solution of [N1,1,1,12]Br at 370 mM, being that same solution passed through the column until it reaches saturation. Lastly, the resin is regenerated by the replacement of the OH− groups using a fresh solution of NaOH [4% (v:v) in water]. The resin can be reused, without any loss in efficiency, for at least five cycles (number of complete cycles tested). The chlorophylls in the aqueous solution of IL are recovered using ethanol with the procedure previously described. The chlorophylls and carotenoids present in ethanolic extracts can then be recovered in dry form, if required by the final application, allowing the reuse of the ethanol, using a vacuum drying technique carried at low pressures and temperatures to avoid the degradation of the pigments.

4. Conclusions

In this study, the fractionation of chlorophylls and carotenoids was successfully achieved. Aqueous solutions of surface-active ILS have been developed to obtain two purified fractions of chlorophylls and xanthophylls, while allowing the solvent recycling. Similar approaches could be applied to other biomolecules and biomass matrices, taking into account the economic viability of the process and associated waste production.

Declaration of Competing Interest

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

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/jcej.2021.131073.

References
