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Research Article

Multistep Fractionation of Microalgal Biomolecules Using Selective Aqueous Two-Phase Systems

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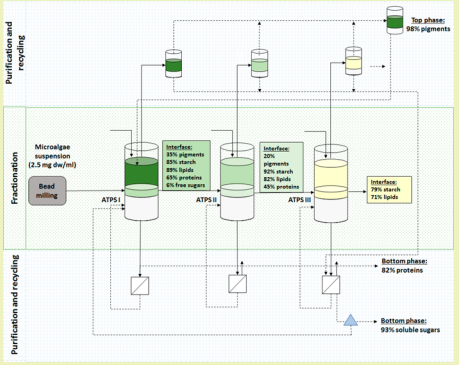
ABSTRACT: We aim to develop liquid−liquid extraction processes for the fractionation of microalgal components (proteins, pigments, lipids, and carbohydrates). The partitioning behavior of microalgal pigments and proteins in aqueous two-phase systems (ATPS) composed of the polymer polypropylene glycol with molecular weight 400 (PPG 400) + various cholinium based-ionic liquids was studied. A process for fractionation of multiple components from disrupted *Neochloris oleoabundans* was developed and evaluated. Results show that cholinium dihydrogen phosphate (Ch DHp) allows the fractionation of pigments in the PPG 400-rich phase and proteins in the Ch DHp-rich phase with high selectivity. It was demonstrated that a multiproduct approach can fractionate free glucose, and proteins in the ionic liquid-rich phase, pigments in the polymer-rich phase, while starch and lipids are recovered at the interface.



KEYWORDS: *Microalgae, Biomolecules, Cholinium-based ionic liquids, Aqueous two-phase systems, Downstream processes*

INTRODUCTION

Microalgae produce diverse lipids of nutritional and energetic importance, pigments, proteins, carbohydrates, vitamins, and antioxidants. This rich biochemical composition makes them very interesting for a range of applications including bioenergy, functional biochemical compounds, animal feed, human food, and pharmaceuticals.[1−3]

Neochloris oleoabundans has been highlighted as a valuable resource for the production of industrially useful materials.[4] It has high growth rates and the capacity to accumulate large quantities of lipids after nitrogen depletion.[5] Its high content of triglycerides (TAGs), which is 80% of the total lipid and fatty acid profiles, make it an ideal candidate for biodiesel production and as an ingredient in feed and food.[6] Besides triglycerides, *N. oleoabundans* possesses proteins, carbohydrates, and pigments. Carbohydrates from microalgae are excellent substrates for the production of biofuels (e.g., bioethanol), biopolymers,[7,8] and fermentation feedstocks.[9] Furthermore, its potential has recently gained attention for their application in other industries, including food, paper production, bioplastics, cosmetics, and pharmaceuticals.[10−12]

Although not widely investigated, *N. oleoabundans* is a promising source of high-value carotenoids that mainly accumulate lutein.[13−15] These high-value carotenoids can be used as food additives and health supplements due to their antioxidant activity.[14] Compared with conventional food proteins of great nutritional quality, proteins from *N. oleoabundans* are also of interest due to their similar amino acid profile.[16] Ribulose-1,5-biphosphate carboxylase oxygenase (RuBisCO), an enzyme present in plants and other photosynthetic organisms such as microalgae, is an attractive and sustainable source of bioactive peptides that can be applied as a functional ingredient in food applications.[17]

The valorization of all microalgae components can increase the economic feasibility of microalgae production, balancing the high costs of cultivation and downstream processes.[18] Despite the diversity of valuable biomolecules in *N. oleoabundans*, cultivation and biorefinery research has been mostly focused on it as a source of TAGs for biofuel applications.[19,20] The interest in developing more efficient and sustainable processes has been extended. Techniques like supercritical CO_2 ($scCO_2$),[21] switchable solvents,[20−22] surfactants, and ionic liquids (ILs) were investigated for the extraction of microalgae components.[23,24] Although these new technologies are promising and a step forward in microalgae biorefinery, they often target a single-product, decreasing or neglecting the value of other molecules. The development of biorefinery processes to recover and give value to all the microalgal components is needed.

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ILs are solvents that have a high solvation power for a wide range of molecules.[25,26] Their tunability is one of their most attractive features, making them adaptable to many technologies, including aqueous two-phase systems (ATPSs).[27,28] The motivation of this study is based on the high solvation ability these "designer solvents" have and their ability to form ATPSs.[29,30] This liquid−liquid extraction technology provides a milder environment for biomolecules without damaging the biomolecules present in the biomass since the two phases formed are mostly composed of water and nonvolatile organic solvents.[29,30] In this study, we aim to design a new process that is able to fractionate and valorize most of the microalgae components (lipids, pigments, proteins, and carbohydrates) using an ATPS, a greener, milder separation technology.[31−33] The downstream processing strategy is aimed at increasing the economic feasibility for microalgae-based production by maximizing the recovery and maintaining the native conformation and functionality of the biomolecules.[31−33]

ATPSs can be formed by combining a high variety of aqueous solutions, including polymers, salts, alcohols, and ILs.[29,30] Three kinds of ATPSs were previously studied to prove the suitability of this technology in terms of the fractionation of microalgae pigments and proteins: the traditional polymer-salt (PEG400-citrate) and two ionic liquid-based ATPSs, specifically ionic liquid-citrate (Iolilyte221PG-citrate) and polymer-ionic liquid (PEG400-ChDHp), as described by Suarez Ruiz et al.[32] The polymer-ionic liquid-based ATPS showed a different partitioning behavior when compared with the other ATPSs studied; it fractionates pigments from proteins with a higher selectivity. Furthermore, this ATPS was able to conserve the native form of proteins, namely RuBisCO.[34] A polymer-ionic liquid-based ATPS exhibits a wider hydrophilic−hydrophobic range than other ATPS types, and both species (polymers and ILs) are able to act as salting-out agents. This property opens possibilities for the design of an appropriate and selective ATPS.[35−37]

In the current study, due to the aforementioned advantages, a polymer-ionic liquid-based ATPS was selected to develop the fractionation of microalgae biomolecules. Polypropylene glycol with molecular weight 400 (PPG 400) was selected instead of polyethylene glycol with a molecular weight of 400 (PEG 400) due to its ability to form an ATPS with a plethora of ILs, which enables the design of the process. Cholinium-based ILs were preferred due to their biocompatibility, low toxicity,[28] and their ability to keep the native conformation of RuBisCO.[34] RuBisCo is a valuable food supplement with good techno-functional properties (e.g., gelling properties, emulsifier and foam stabilizer) when used in a native conformation and not as a degraded product.[38]

First, different cholinium-based ILs were selected based on literature reviews[39,40] and evaluated based on their phase-forming behavior and their selectivity to fractionate microalgal pigments and proteins in different phases. Second, the best combination was characterized based on the phase diagram, and the partitioning behavior of the microalgae components was studied. The influence of the concentration of the phase-forming components was an important parameter for the selection of the most favorable mixture point. Finally, a multistep fractionation process was designed with the purpose of increasing the recovery efficiency of cell components from disrupted *N. oleoabundans*. Recycling methods for the phase-forming components were proposed, and a proof of concept for their applicability was provided.

MATERIALS AND METHODS

Materials. The ionic liquids cholinium dihydrogen citrate (Ch DHcit, > 98 wt % pure), cholinium chloride (Ch Cl, ≥ 99 wt % pure), cholinium bitartrate (Ch Bit, 98 wt %), and cholinium bicarbonate (Ch Bic, 80 wt % pure) were supplied by Sigma-Aldrich. Choline dihydrogen phosphate (Ch DHp, ≥ 98 wt %% pure) and cholinium acetate (Ch Ac, 98 wt % pure) were procured from Iolitec (Ionic Liquid Technologies, Germany). Polyethylene glycol (PEG) 400, polypropylene glycol (PPG) 400, the standard molecules D-(+)-glucose, bovine serum albumin (BSA), and lutein (≥97%) were purchased from Sigma-Aldrich. Starch from maize was included in the Megazyme kit (Wicklow, Ireland).

METHODS

Microalgae Cultivation, Harvesting, and Cell Disruption. *Neochloris oleoabundans* (UTEX 1185, University of Texas Culture collection of Algae, USA) was cultivated under nitrogen depletion (N-) in a fully automated 1300L vertical stacked tubular photo-bioreactor (PBR) located at AlgaePARC, The Netherlands. *N. oleoabundans* was cultivated at a pH value of 8.0, and the temperature was controlled at 30 °C. *N. oleoabundans* was cultivated under saline conditions using artificial seawater on Bold's Basal medium:[41] NaCl 24.5 g/L; $MgCl_2$ 9.8 g/L; $CaCl_2$ 0.53g/L; K_2SO_4 0.85g/L; $NaSO_4$ 3.2 g/L; $NAHCO_3$ 0.8 g/L. The microalgae were harvested (80 Hz, 3000*g*, 0.75 $m^3\ h^{-1}$) using a spiral plate centrifuge (Evodos 10, Evodos, The Netherlands), and the biomass obtained was suspended in Milli-Q water to obtain a biomass concentration of ∼90 g L^{-1}. The algal cells were disrupted in a horizontal stirred bead mill (Dyno-Mill Research Lab from Willy A. Bachofen AG Maschinenfabrik, Switzerland) using 0.5 mm ZrO_2 beads as described by Postma et al.[42] Bead-milled microalgae suspension was stored at −20 °C until further use.

Screening of an ATPS Composed of Polymer and Cholinium-Based Ionic Liquids. For a first screening, the effect of the ILs on the fractionation of pigments and proteins from disrupted microalgae was investigated. Different ATPS combinations composed of PPG 400 and cholinium-based ILs: Ch DHcit, Ch Cl, Ch Bit, Ch Bic, Ch DHp, and Ch Ac were prepared with a composition of 30% polymer, 30% IL, and 40% bead-milled microalgae suspension (2.5 mg dry weight (dw) microalgae/mL bead-milled microalgae suspension) in water. The main reason to use 2.5 mg dw/mL bead-milled microalgae suspension is first to screen different ATPS combinations, and when selection is performed, further optimization occurs by increasing the dry weight microalgae concentration. The phase-forming component concentrations were selected to guarantee that all the systems were in the biphasic region.

Partitioning Studies. *Characterization of Selected ATPS.* From the initial screening, PPG 400-Ch DHp was the most suitable ATPS for the fractionation of microalgae components. This system was characterized by creating the binodal curve and tie lines (TLs). The ternary phase diagram of PPG 400 + Ch DHp + H_2O was determined by the cloud point titration method at room temperature. Aqueous solutions of the IL and PPG 400 at concentrations of 60 (w/w %) were prepared. Repetitive dropwise addition of the aqueous solution of ionic liquid to the aqueous solution of PPG 400 was performed under constant stirring until a cloudy solution was detected, which is characteristic for a biphasic system. Followed by the dropwise addition of Milli-Q water until a clear solution was obtained. These steps were repeated to obtain a series of cloud points, corresponding to the binodal curve, which is the borderline between the monophasic and biphasic region. The compositions of the binodal curve were determined by weight quantification ($\pm 10^{-4}$ g).

TLs were determined by the gravimetric method proposed by Merchuk et al.[43] Mixtures in the biphasic system were prepared ($\pm 10^{-4}$ g), mixed, and left to equilibrate for at least 12 h to reach the complete separation and equilibration of the phases. The phases were carefully separated and weighed. The lever-arm rule was used to calculate each TL, and to calculate the tie line length (TLL), eq 1 was used.

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$$\mathrm{TLL} = \sqrt{(X_T - X_B)^2 + (Y_T - Y_B)^2} \quad (1)$$

X_T, Y_T, X_B, and Y_B are the phase compositions, where the subscript T is the top phase and B is the bottom phase.

Partitioning of Pigments, Proteins, and Free Glucose. For the partitioning studies, each system was prepared weighing the appropriate amount of phase forming components by using the TLs with a volume ratio (Vr) between top and bottom phase of 1. The total concentration of bead-milled microalgae suspension in the mixture was 2.5 mg dw/mL microalgae. The mixtures were subsequently mixed in a rotatory shaker (50 rpm, 1 h) and left to equilibrate at room temperature. To promote the separation of the phases, all systems were centrifuged for 10 min at 1200*g*. The phases (top, bottom, and interface) were carefully separated and the volume and weight were noted. The interfaces were resuspended in Milli-Q water to facilitate the quantification of the biomolecules. Possible interferences of polymer and IL on the analytical method were taken into account, and control samples were prepared using water instead of bead-milled microalgae suspension. Experiments were performed in duplicate, and the results were reported as the average of two independent assays with the respective standard deviation.

Multistep Process Design. For the multistep approach, mixtures of 40 wt % PPG 400 and 14 wt % Ch DHp were prepared and bead-milled microalgae suspension was added as described in the partitioning studies. After the first ATPS stage (ATPS I), the polymer-rich (top) and IL-rich phases (bottom) were separated from the interface (middle). The volume and weight of the phases were noted and the phases were conserved for the further quantification of the biomolecules. The obtained interface was added to a new mixture (ATPS II), consisting of 40 wt % PPG 400 and 14 wt % Ch DHp, and the procedure was repeated. In total three sequential ATPS stages were performed.

Recycling of Phase Forming Components and Further Purification of Biomolecules. **Ultrafiltration** was used to investigate the possible isolation of the proteins from the IL-rich phase and recycling of the IL. Amicon Ultra-0.5 centrifugal filtration devices (Millipore) with a 10 kDa MWCO were used. 500 μL of the IL-rich phase was added to the filters followed by centrifugation (14 000*g*, 15 min). The filtrate was collected and PBS buffer was added to the filter to dilute the partially purified protein extract and centrifuged for the second time. The proteins recovered in the concentrate were quantified with the BCA Protein Assay Reagent Kit (Thermo Fisher Scientific) as described below. Protein recovery in the concentrate was calculated using eq 2, where $m_{p,c}$ is the mass of protein in the concentrate after filtration and $m_{p,i}$ is the initial mass of protein added to the filter.

$$\text{protein recovery (\%)} = \frac{m_{p,c}}{m_{p,i}} \quad (2)$$

Conductivity was measured before and after ultrafiltration to calculate the recovery of IL. A calibration curve of conductivity vs concentration of the IL was performed and the IL recovery was calculated using eq 3. $m_{IL,p}$ is the mass if the IL in the permeate, and $m_{IL,i}$ is the initial mass of the IL added to the filter.

$$\text{IL recovery (\%)} = \frac{m_{IL,p}}{m_{IL,i}} \quad (3)$$

Thermoseparation. PPG 400 is a thermosensitive polymer that forms two phases when heated. This behavior was investigated for the further purification of the components recovered in the PPG 400-rich phase. The cloud point temperatures delimit the border between the monophasic and biphasic region. The influence of the concentration of the IL (2 and 7 wt % of Ch DHp) on the cloud point temperatures (T_c) of PPG 400 was studied. Solutions containing different concentrations of PPG 400 and Ch DHp (2 or 7 wt % of Ch DHp) were prepared and temperature controlled using a water bath. The temperature was raised within 0.5 °C until the solution became turbid, which is defined as the cloud point temperature. The determination of the cloud point temperatures where performed in duplicate to ensure good reproducibility of this procedure.

In ATPS, the concentration of the IL changes in the PPG 400-rich phase depends on the TLL. Therefore, the cloud point temperature was determined for the PPG 400-rich phase of mixtures along each TL. A mixture along each TL was prepared and bead-milled microalgae suspension was added as described in the partitioning studies. The top phase was carefully separated and temperature controlled using a water bath. The temperature at which the mixture becomes turbid and forms two-phases was noted. Proteins, pigments, and free glucose were quantified in each phase.

Analysis of Biomolecules. *Pigment Analysis.* The pigments in each phase were quantified by UV−vis spectroscopy, using a Tecan infinite M200 microplate reader. The separated phases were analyzed by measuring the absorption spectrum between 200 and 750 nm. Since lutein is the most abundant pigment in *N. oleoabundans*,[14] which was confirmed by reversed-phase high-performance liquid chromatography (information provided in the Supporting Information, Table S1), this carotenoid was selected as a reference for the quantification of the pigments. Calibration curves were prepared using commercial Lutein standard in PPG 400 and methanol. OriginPro 8.0 was used for the spectral deconvolution of the peaks at 454 and 444 nm that correspond to the maximum absorption wavelengths of Lutein in PPG 400 and methanol, respectively. The initial amount of pigments added to the partitioning experiments were calculated by the complete extraction of the pigments from bead milled microalgae with methanol, followed by immersion in an ultrasound bath (5 min) to release the pigments from microalgae biomass.[44] To recover the pigments, the samples were centrifuged at 1800*g* for 10 min. This procedure was repeated until a white pellet was obtained.

Protein Analysis. Since the phase forming components interfered in the quantification of the proteins, samples (including standards and blanks) were purified before analysis using an acetone precipitation procedure.[45] Proteins in the separated phases and in the initial biomass were quantified by the BCA Protein Assay Reagent Kit (Thermo Fisher Scientific), using Bovine Serum Albumin (BSA) as a standard. Absorbance at 562 nm was measured using a Tecan infinite M200 plate reader.

Carbohydrate Analysis. Free glucose in the ATPS was analyzed using the Megazyme kit (Wicklow, Ireland).[46] Samples (including standards and blanks) were mixed with a solution containing *p*-hydroxybenzoic acid, sodium azide (0.095% w/v), glucose oxidase plus peroxidase, and 4-aminoantipyrine (GOPOD reagent) at a ratio 0.1:3 (v/v) and incubated at 50 °C for 30 min. After cooling down to room temperature, quantification was conducted by measuring the absorbance at 510 nm using a Tecan infinite M200 plate reader. Glucose was used as a standard and a calibration curve was constructed.

The starch content was quantified by the total starch protocol of Megazyme kit (Wicklow, Ireland) adapted from the work of Dragone et al.[47] Maize starch was used as a positive control, and calibration curves were made from D-glucose. The quantification was performed by measuring the absorbance at 510 nm using a spectrophotometer (Hach Lange DR6000).

Lipid Analysis. The total fatty acid (TFA) content and composition were determined as described by Breuer et al.[48] Lipid extraction was done with a chloroform:methanol (1:1.25) solution containing the internal standard glyceryl pentadecanoate (Sigma-Aldrich). To determine the total lipids, the resulting fatty acid methyl esters (FAME) were quantified using gas chromatography (GC-FID; Agilent 1890 coupled with an autosampler). *n*-Hexane was used as solvent and helium as carrier gas with a flow rate of 20 mL/min. The column used was a Supelco Nucol 25357, 30 m × 530 μm × 1.0 μm. Total fatty acid composition and content were calculated by taking the sum of all fatty acids.

Calculations and Statistics. To describe the distribution of biomolecules in the ATPS, partition coefficients in the log scale log(K) and extraction efficiencies (EE%) were calculated using eqs 4 and 5, respectively. The concentration of certain target biomolecule $C_{biomolecule}$ (e.g., proteins) in the phase (top, bottom, or interface) and

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m the mass of the biomolecule in the phase (top, bottom, interface) or in the initial bead-milled microalgae suspension added to the system.

$$\log(K) = \log\left(\frac{C_{\text{biomolecule,top}}}{C_{\text{biomolecule,bottom}}}\right) \quad (4)$$

$$EE_{\text{biomolecule}}\% = \frac{m_{\text{biomolecule,phase}}}{m_{\text{biomolecule,initial}}} \quad (5)$$

x100%The selectivity of the systems to fractionate pigments or proteins was calculated with eq 6

$$S_{\text{pigment/protein}} = \frac{K_{\text{pigments}}}{K_{\text{proteins}}} \quad (6)$$

All experiments were conducted in duplicate, and results were reported as the average of two independent assays with the respective standard deviation. Statistical analysis was performed using Statistica 10.0 software. One-way ANOVA and Tukey HSD tests were implemented to assess significant differences among treatments.

RESULTS AND DISCUSSION

An outline of the results and discussion is given below, whereby initially, *N. oleoabundans* is bead-milled to release the different components (proteins, carbohydrates, pigments, lipids) ready for ATPS fractionation. Next, the screening of the ATPS formation with PPG 400 and cholinium-based ILs was performed after selecting PPG 400 and ChDHp as the preferred ATPS components. The partitioning of the different products (pigments, proteins, free sugar) with a PPG 400 and ChDHp-based ATPS was investigated and finalized by a multicomponent approach with three consecutive ATPS cycles including the enrichment of lipids and starch in the interface. Finally, recycling methods for the phase-forming components were proposed and a proof of concept for their applicability was provided.

Microalgae Cultivation, Harvesting, and Cell Disruption. After microalgae cultivation and harvesting, cell disruption using bead milling was implemented to break the cells and make the biomolecules accessible for ATPS fractionation. The obtained disrupted *N. oleoabundans* suspension was composed of 44% (dw of the bead-milled microalgae suspension) total fatty acids, from which 33.6 ± 0.2% dw are TAGs and 10 ± 0.06% dw are polar lipids. The total carbohydrates content was 27% dw, with glucose being the most abundant carbohydrate.[46] Glucose was present in the disrupted biomass as part of the starch content and as free glucose. The starch and free glucose contents were 14.3 ± 1.4% dw and 9.8 ± 1.3% dw, respectively. The protein content was 28% dw. Lutein (7.1 μg/mg dw) was detected as the most abundant pigment in this suspension next to chlorophyll b (Figure S1), which is in agreement with other reports.[15,32] Fatty acid composition (Table S2) and pigment chromatogram (Figure S1, S2) are shown in the Supporting Information. Since the microalgae were cultivated under stressed conditions (artificial salt water and nitrogen depletion), we expected the accumulation of a high amount of lipids, mainly TAGs and carbohydrates in the form of starch.

Screening of an ATPS Composed of Polymer and Cholinium-Based Ionic Liquids. In order to examine the partitioning behavior of microalgal biomolecules in a polymer-ionic liquid-based ATPS, the influence of the IL on the partitioning of pigments and proteins was first studied. A fixed mixture point with 30 w/w% of PPG 400 + 30 w/w% of cholinium based-ILs was adopted. The mixture point in the screening study was selected to have a common ternary mixture point were all the systems are forming ATPS. The ternary mixture composition used was selected based on literature review[39,40] and previous experiments[32,34,46] in our group. The ternary mixture (30% polymer−30% IL−40% water) was prepared with a common composition and within the biphasic region for all the systems. Figure 1 presents the partition coefficients of the pigments and proteins and the respective selectivity (pigments/proteins). Pigments prefer to migrate to the PPG 400-rich phase, which corresponds to the most hydrophobic phase. On the other hand, proteins prefer to migrate to the IL-rich phase, which is the most hydrophilic phase. Despite the preference of the biomolecules toward the PPG 400- or IL-rich phase not varying, the selectivity varied significantly between the systems.

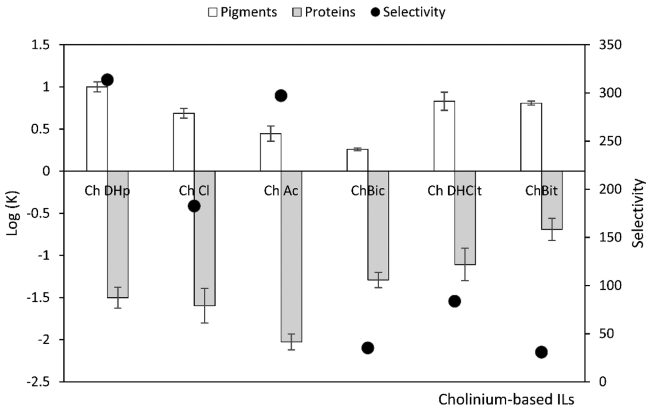

Figure 1. Partitioning of pigments and proteins from disrupted *N. oleoabundans* in an ATPS with PPG 400-cholinium based-ILs ATPS. Empty bars and full bars represent pigment and protein partition coefficients in logarithmic scale, respectively. Black dots represent the selectivity values ($S_{\text{pigment/proteins}}$) between pigments and proteins. Error bars represent standard deviation.

Being hydrophobic, the pigments were expected to partition toward the most hydrophobic phase (PPG 400). Since cholinium is a common cation for all the ILs studied, it is possible to see the influence of the IL's anion on the partitioning of the pigments in the ATPS. The partition coefficient for pigments decreases in the following order: Ch DHp > Ch DHcit > Ch Bit > Ch Cl > Ch Ac > Ch Bic. The dependency on the employed IL suggests that the hydrophobic interactions between the ILs (Table 1), and the pigments have an effect on the partitioning behavior of pigments.

In previous studies on the partitioning of lutein in an ATPS formed by polymer and salt,[49] lutein migrated to the polymer-rich phase, which is the most hydrophobic layer. The increase of the molecular weight of the polymer, which is related to an increase in hydrophobicity, resulted in a higher recovery of the pigment in the polymer-rich phase.[49] The study of another carotenoid (β-carotene) in an ATPS formed by ILs and carbohydrates demonstrated that the partition coefficient for this hydrophobic molecule depends on the ability of each carbohydrate to form an ATPS, which is related with its hydration aptitude.[50]

Referring to our results, the high partition coefficient obtained for pigments when using Ch DHp seems to be a consequence of the high hydrophilicity of this IL in comparison with the other ILs used in this study. Its hydroxyl

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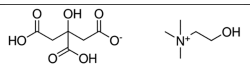
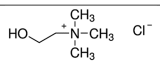
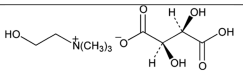
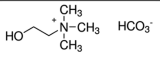
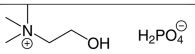
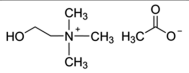
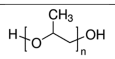
Table 1. Chemical Structures of Ionic Liquids and Polymers

Cholinium dihydrogen citrate (Ch DHcit)	
Cholinium chloride (Ch Cl)	
Cholinium bitartrate (Ch Bit)	
Cholinium bicarbonate (Ch Bic)	
Choline dihydrogen phosphate (Ch DHp)	
Cholinium acetate (Ch Ac)	
Polyethylene Glycol 400 (PEG 400)	
Polypropylene Glycol 400 (PPG 400)	

groups favor the hydrogen bonding between the IL and water. This ionic liquid was previously found to have the highest ability to form an ATPS when compared with other cholinium-based ILs, such as the ILs used in this study, as a result of its highest polar surface, i.e. affinity for water.[51]

An ATPS composed of polymer and cholinium based-ILs has been demonstrated to be an appropriate, mild environment for the partitioning of proteins since it overcomes drawbacks such as toxicity and lack of selectivity that other ATPS combinations may present.[52,53] This property is important since proteins are fragile biomolecules susceptible to denaturing by extraction processes and can have a hydrophilic or hydrophobic nature. Interestingly with this type of ATPS (polymer-ionic liquid-based), as seen in Figure 1, proteins preferentially migrate to the IL-rich phase, which is the more hydrophilic phase, while pigments prefer to migrate to the opposite phase, i.e., the PPG 400-rich phase. This characteristic makes this type of ATPS (polymer-ionic liquid-based) very interesting for the separation of pigments from proteins. The IL anion influences the partitioning coefficients obtained for proteins, increasing as follows: Ch Bit < Ch DHcit < Ch Bic < Ch DHp < Ch Cl < Ch Ac. The partitioning of proteins in an ATPS is complex since it is driven by different interactions, including electrostatic interactions, dispersive forces, and hydrogen bonding. These interactions depend on the protein surface and the phase-forming components, where steric effects may also be involved. The fact that proteins preferentially migrate to the most hydrated phase and the different partition coefficients obtained when changing the anion of the IL seem to depend on molecular interactions between the proteins and the cholinium-based ILs. This result is in agreement with the findings of other studies on the partitioning of pure proteins, namely RuBisCo, BSA, and IgG.[39,53,54] The partitioning of proteins in polymer-ILs seems to be dominated by specific interactions between the IL and the proteins. In a polymer-salt ATPS, the protein normally migrates to the polymer-rich phase, due to the dominant driving force for the partitioning of proteins being the hydrophobic interactions between the protein and the phase-forming components.[55]

All the systems studied presented precipitated material at the interface (between the aqueous phases). The interface phase was treated as a third phase and was not considered part of any of the aqueous phases (Table 1, Figure S3). The interface contains mainly cell debris generated by cell disruption method by bead-milling used in this study.[42] The same method has been tested with different microalgae species demonstrating complete cell disruption. Lipids and starch also did not migrate to the aqueous phases because of the solubility and migrate into the interface. In the case of the proteins, it seems that soluble proteins (hydrophilic) were fractionated in the first ATPS step in the Ch DHp-rich phase and the more hydrophobic proteins remained as well in the interface. The biomolecules concentrated in this interface were quantified for the calculation of a proper mass balance. The complete partitioning of pigments and proteins toward the aqueous phases may be affected by the formation of this phase, which contains insoluble material (e.g., cell debris, lipids, and starch). The ATPS formed by PPG 400 and diverse ILs showed a wide range of selectivity between 30 and 313 (Figure 1). In comparison with the other ionic liquids studied, Ch DHp and Ch Ac showed the highest selectivity, followed by Ch Cl. The systems containing Ch Cl and Ch Ac, contrarily to the other systems, presented precipitated material also in the IL-rich phase (bottom phase). The presence of insoluble microalgae material in the bottom phase hindered the separation of the phases and the calculation of the mass balance. Therefore, these ILs were not considered for further investigations.

As mentioned before, among the ILs studied, Ch DHp has the highest ability to form an ATPS due to its affinity for water, which leads to the exclusion of PPG to a second phase.[39] This property means that a smaller amount of ionic liquid is needed to form a second phase. This ability and its high selectivity to fractionate microalgal pigments and proteins make the PPG 400-Ch DHp preferable for studying the partition of microalgae molecules in more detail.

Partitioning Studies. *Characterization of Selected ATPS.* The phase diagram and respective TLs of the selected system (PPG 400-Ch DHp) were created to determine the biphasic region and which mixture points to study (Figure 2). The weight fraction data for the ternary system, which is composed of PPG 400 + Ch DHp + H_2O, and the phase-forming composition of TLs at the top and bottom phases are shown in the Supporting Information (Tables S3−S5). Figure 2 clearly shows that TL 4 is not parallel with the other TLs, a possible reason could be that obtaining a stable ATPS system with PPG 400 in the top phase higher concentrations of ChDHp and less water are needed (Table S4) in the bottom phase. This phenomenon, by increasing slightly the PPG 400 concentration in the top phase (Table S4) and the concomitant addition of much higher concentration of ChDHp in the bottom phase (Table S4), is not understood and needs further investigations.

Partitioning of Pigments, Proteins, and Free Glucose. The components able to be fractionated in the aqueous phases were identified: proteins, pigments, and free glucose. In addition, their partitioning behavior was studied. To increase the separation selectivity between the biomolecules, the influence of the phase-forming component concentrations on the partitioning of these biomolecules was explored.

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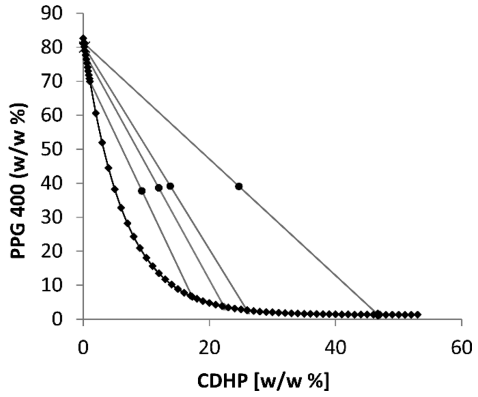

Figure 2. Phase diagram of PPG 400-Ch DHp (binodal curve and respective TLs). TLs were calculated considering mixture points at a volume ratio of 1. The mixture points studied for the system (PPG 400; Ch DHp w/w %) were (38; 9 w/w %), (39; 12 w/w %), (40; 14 w/w %), and (39; 25 w/w %).

Partition coefficients of pigments, proteins, and free glucose are presented in Figure 3. Pigments preferentially migrated to

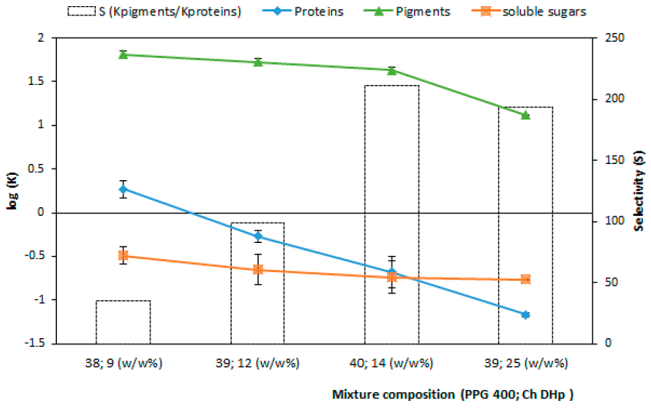

Figure 3. Partition coefficients (log(K)) of proteins, pigments, and soluble sugars from disrupted *N. oleoabundans*. The mixture points studied for the system (PPG 400; Ch DHp w/w %) were (38; 9 w/w %), (39; 12 w/w %), (40; 14 w/w %), and (39; 25 w/w %). Bars represent the selectivity between pigments and proteins. Error bars represent standard deviations.

the PPG-rich phase (most hydrophobic phase), while proteins and free glucose tended to migrate toward the Ch DHp-rich phase (most hydrophilic phase) in most of the mixtures studied.

The partition of solutes in an ATPS depends on the relative composition of the phases and the TLL can be used to represent this influence. By increasing the concentration of phase-forming components, the water content in the top phase decreased from 42% to 19%, which led to a more hydrophobic top phase. By increasing hydrophobicity, the pigments start to be excluded from the most hydrophobic phase toward the other phases; log(K) decreased from 1.8 to 1.1. This decrease was not expected since the nature of the pigments is highly hydrophobic. Thus, it seems that other interactions occurring between the phase-forming components (PPG 400, Ch DHp, and water) and other biomolecules present in microalgae influence this behavior. We observed that the concentration of pigments in the IL-rich phase does not vary with the TLL. Thus, the decrease of pigment concentration in the PPG 400-rich phase is a consequence of a higher concentration of pigments in the interface.

Contrary to polymer-salt and IL-salt ATPS, the driving mechanism for the phase formation in a polymer-ionic liquid-based ATPS is ruled by the strength of the interactions occurring between polymers, ILs, and water.[56] It seems that the decrease in the partition coefficient with the increase of the TLL is a consequence of the polymer-rich phase saturation of mixtures more rich in ILs. This effect of volume exclusion seems to affect the partitioning behavior of pigments and proteins. The partition coefficient of proteins decreases with an increase of the TLL. This partitioning behavior was also observed by Li et al., who demonstrated the preference of four proteins to partition in the IL-rich phase by using PPG 400-cholinium ILs and proposed the regulation of protein partitioning by changing the TLL.[57] The partitioning of IgG was recently studied by Ramalho et al., using PPG 400 and cholinium-based ILs.[53] IgG preferentially partitioned to the IL-rich phase, revealing that interactions between the amino acids at the antibody surface and the ILs may dominate the partitioning of this molecule.

Microalgae contain soluble sugars such as free glucose, and the process of disrupting the cells increases this amount by breaking down some polysaccharides into monosaccharides.[58] The most abundant monosaccharide present in stressed *N. oleoabundans* is glucose, which possesses many hydroxyl groups, making it easily soluble in water (133 mg/mL). As observed in Figure 3, free glucose prefers to partition to the IL-rich phase because of its high water solubility. Moreover, there is not a significant difference ($p > 0.05$) in the partition coefficient of free glucose among the mixture points studied. Thus, the concentration of phase-forming components does not seem to significantly affect the partitioning of free glucose. We hypothesized that the high affinity of this molecule for water and the absence of electrostatic interaction between glucose and the phase-forming components are responsible for a constant partitioning of sugars to the IL-rich phase.

Multistep Process Design. Cell disruption by bead milling was applied as a benchmark to release most of the intracellular material from the microalgal biomass. Mechanical treatments, such as bead-milling and high pressure homogenization, can cause the disruption of cells into small fragments, including cell wall particles (cell debris) and intracellular material.[59] This cell debris production often complicates classical downstream process technologies and/or the removal of particles (by centrifugation, filtration, or chromatography) due to the high amount of small insoluble particles present.[60] Furthermore, the mechanical disruption of microalgae with a high content of lipids can form stable emulsions which are difficult to break in order to fractionate the target components.[61] An ATPS is not affected by the cell disruption process and is able to selectively fractionate a high amount of biomolecules toward the aqueous phases. Soluble components (e.g., proteins, free glucose) could be fractionated using the ATPS without being affected by the stable emulsion (e.g., lipids, starch) that is formed by the bead-milled process. High lipid containing microalgae processed by bead-milling form stable emulsions, which normally affect the fractionation of the other components.[61] It also concentrates valuable material, such as lipids and starch, in the interface. In this regard, an ATPS is an excellent technology to design a multiproduct fractionation approach that valorizes all components of the microalgae cell.

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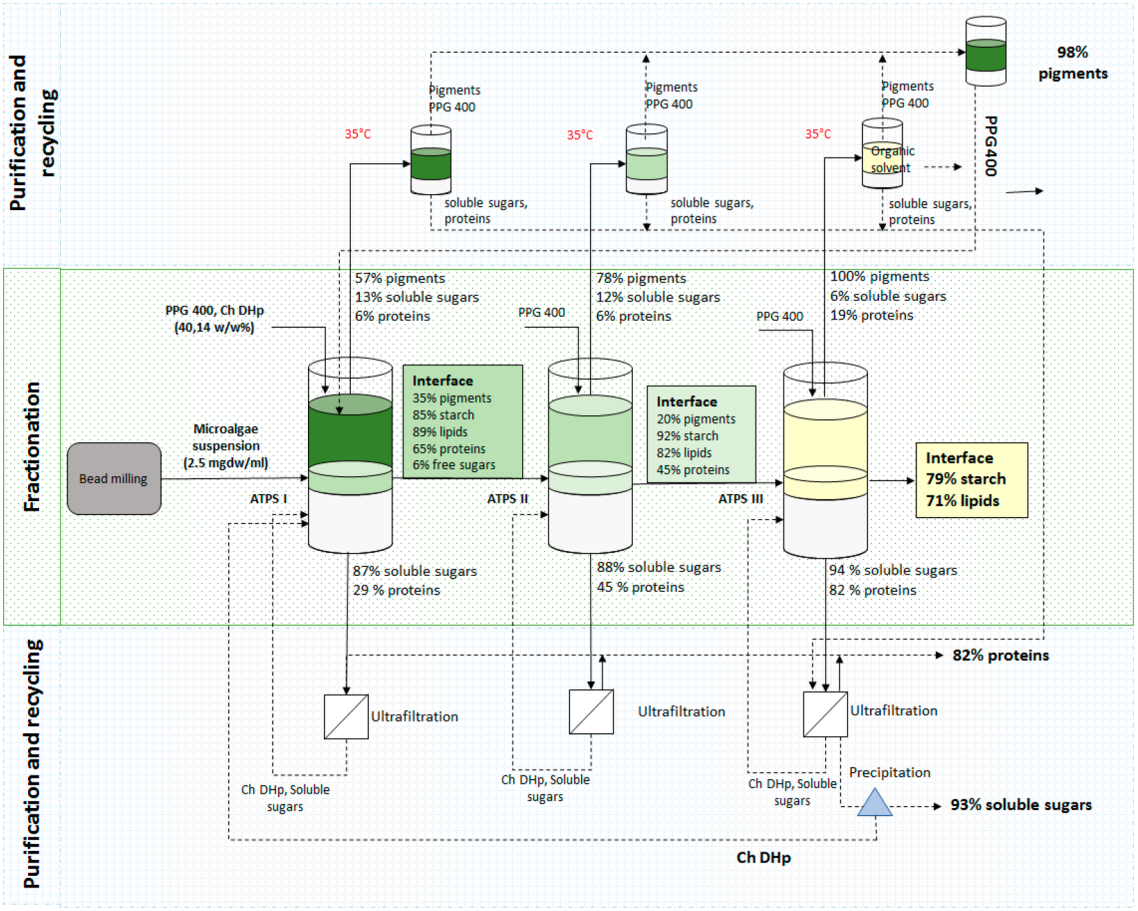

Figure 4. Diagram of the multistep approach for the selective fractionation of microalgal pigments, proteins, soluble sugars (free glucose), starches, and lipids. The approach studied includes three stages of fractionation, using an ATPS formed by 40% PPG 400 and 14% Ch DHp. Microalgal suspension is introduced in ATPS I. The interface that is formed in this ATPS is consecutively introduced in the next ATPS (ATPS II) followed by the third ATPS (ATPS III). The extraction efficiency (w/w %) of each step is provided. Dashed lines represent the proposed purification and recycling methods.

Intending to design an efficient and selective fractionation process for most of the components of microalgae, we proposed a multistep approach. Since the partitioning of free glucose is not affected by the concentration of phase-forming components, the selectivity values between pigments and proteins determined the best option for designing an advantageous fractionation process. A mixture of PPG 400-Ch DHp (40;14 w/w %) was selected for further studies due to its high selectivity between pigments and proteins (>200), as shown in Figure 3. The multistep approach aims to fractionate pigments in the polymer-rich phase, proteins and soluble sugars in the IL-rich phase, and lipids and starch in the interface. As observed in the process depicted in Figure 4, the PPG 400-rich phase and the Ch DHp phase were separated from the interface, which was used for the next ATPS stage. Three stages of an ATPS were used to selectively fractionate the different biomass molecules: ATPS I, ATPS II, and ATPS III. The distribution of the biomolecules in each phase is presented in the same figure. The potential recycling of the IL and the thermo-separation of the PPG 400-rich phase are proposed and sketched in dashed lines in Figure 4.

Tables 2 and 3 show the partitioning information, such as the extraction efficiencies (w/w %) and the partition coefficients (log(K)) of the soluble (proteins, pigments, and free glucose) and nonsoluble biomolecules (lipids and starch), respectively.

Free glucose was completely fractionated, reaching high extraction efficiencies at the first stage (ATPS I). Only 5.6 ± 0.5% of the initial amount remained in the interface stage, and 87.4 ± 3.2% was recovered in the IL-rich phase. Similarly, a high amount of pigments (57.4 ± 0.2%) was recovered in the first stage (ATPS I) in the PPG 400-rich phase, and no pigments were detected in the bottom and interface phase in the third stage (ATPS III). 98.0 ± 2.8% of the pigments were recovered after the three ATPS stages in the PPG-rich phase. The UV−vis spectra of the PPG 400 phase and its visual appearance is depicted in the Supporting Information (Figure S2).

Although, proteins preferentially partition to the bottom phase, 65.4 ± 2.3% remain in the interface in ATPS I (Table 1). Fractionation of microalgal proteins is a challenging task due to their low solubility. They are stored inside the chloroplasts of the microalgae and form complexes with pigments and polysaccharides, which are difficult to separate. After cell disruption, a soluble and nonsoluble protein fraction is often identified, and 35−50% of the proteins have been reported as water-soluble proteins released by bead milling *N. oleoabundans*.[62,63] This finding can explain the large portion of proteins still in the interface after the ATPS I stage. Furthermore, 81.5 ± 1.3% of proteins were recovered in the bottom phase (free of pigments) after the multistep approach, and only 3.4 ± 2.4% of proteins remained in the interface.

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Table 2. Extraction Efficiencies of Pigments, Proteins, and Free Glucose in the Aqueous Phases (top PPG 400-Rich Phase; bottom Ch DHp-Rich Phase) and Interface and log(K) throughout the Three ATPS Stages and the Total Recovery (w/w %) of Each Biomolecule

	pigments				proteins				free glucose			
stage	EE% top	EE% bottom	EE% interface	log(K)	EE% top	EE% bottom	EE% interface	log(K)	EE% top	EE% bottom	EE% interface	log(K)
ATPS I	57.4 ± 0.2	1.8 ± 0.2	35.3 ± 8.3	1.6 ± 0.05	6.0 ± 0.4	28.6 ± 1.9	65.4 ± 2.3	−0.6 ± 0.0	12.6 ± 0.8	87.4 ± 3.2	5.6 ± 0.5	−0.8 ± 0.0
ATPS II	78.2 ± 4.9	1.8 ± 2.5	20.1 ± 2.4	1.1 ± 0.1	5.6 ± 2.8	45.0 ± 3.7	44.8 ± 3.5	−0.9 ± 0.2	12.4 ± 1.7	88.4 ± 0.5	1.5 ± 0.3	−0.9 ± 0.0
ATPS III	100 ± 3.2				19.2 ± 1.5	82.5 ± 0.8	5.8 ± 2.6	−0.7 ± 0.0	6.4 ± 0.0	93.6 ± 0.0		−1.1 ± 0.0
total recovery (w/w %)	**98.0 ± 2.8**	2.6 ± 0.6			15.1 ± 1.1	**81.5 ± 1.3**	3.4 ± 2.4		13.3 ± 0.8	**93 ± 5.5**		

After a successive ATPS fractionation approach, the solubility of microalgae proteins increases. Cholinium-based ILs have attracted attention as a novel solvent for proteins. Several studies demonstrated the capability of these type of ILs to enhance protein solubility while maintaining the activity of enzymes.[64,65] Lee et al. recently investigated different Good's buffer ionic liquids (GBILs) in combination with ultrasonication to recover microalgae proteins. The higher capability of GBILs when compared to water to extract proteins selectively was demonstrated, specially the cholinium based-IL [Ch] [MOPSO]-HCl buffer. They concluded that the effect of the cation seems more relevant than the anion. Thus, hydrophilic cations lead to a higher extraction of proteins and less chlorophylls.[66] Similarly, another study identified Ch Cl as an excellent extraction solvent, able to extract phycobiliproteins while avoiding the extraction of chlorophylls. This study also concluded that hydrophilicity is an important characteristic for the extraction of pigment−protein complexes from a biomass.[67]

Starch and lipids showed low affinity for the aqueous phases as 85.0 ± 1.0% of starch and 88.8 ± 1.3% of lipids were recovered in the interface after the ATPS I extraction step (Table 3). The interface is a mixture of starch, lipids, pigments, free glucose, cell fragments, and hydrophobic proteins. Most, if not all, cells are broken so cell wall fragments are left. These cell wall fragments contain lipids, hydrophobic proteins that like to be in a hydrophobic environment. The lower amount of pigments in the interface can be explained by their affinity with PPG 400 which is a hydrophobic polymer.

This phenomenon has been used in the process design to concentrate these two components in the interface. The partitioning of lipids and starch was not considerably altered by the ATPS stages. Thus, the lipids and starch composition of the interface remained relatively constant through the whole process. In the end of the three-stage process, 29% of lipids were recovered in the PPG 400-rich phase, and no lipids were detected in the IL-rich phase. Due to their nonpolar nature, lipids have a higher affinity for the most hydrophobic phase. Nonetheless, PPG 400 is hydrophobic in comparison with Ch DHp, although it is completely miscible in water,[56] which explains the low extraction efficiency of lipids in the PPG-rich phase.

Although 15.3 ± 1.4% starch was recovered in the PPG-rich phase and 17.3 ± 4.3% in the Ch DHp phase after the whole process, no partitioning preference was observed for starch. Green microalgae accumulate starch granules as storage components in their cells. These granules are composed of two polysaccharides: amylose and amylopectin, both consisting of glucose monomers. The granule's structure is very complex and varies depending on the source.[68] Moreover, the solubility of starch highly depends on the amylose, lipid content, and granule organization, which explains the low solubility of starch in the aqueous phases.[69]

71.9 ± 1.0% of the starch and 70.7 ± 0.5% of the lipids were recovered in the interface after the three fractionation stages, free of pigments, proteins, and soluble sugars. This important fraction of starch and lipids may be used in the food industry and/or nonfood industries, including energy, biopolymers, cosmetics, and pharmaceuticals. Due to its lipid profile and high starch content, *N. oleoabundans* is a potential source for biodiesel, bioethanol, food, and feed production.[70] The fatty acids methyl esters composition of the lipid fraction recovered

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Table 3. Extraction Efficiencies for Starch and Total Fatty Acids in the Aqueous Phases and Interface Throughout Three ATPS Stages and the Total Recovery Obtained for Each Biomolecule

	starch			total fatty acids		
stage	EE% top	EE% bottom	EE% interface	EE% top	EE% bottom	EE% interface
ATPS I	8.1 ± 2.9	10.2 ± 2.9	85.0 ± 1.0	11.2 ± 0.1		88.8 ± 1.3
ATPS II	4.8 ± 0.0	4.8 ± 0.0	92.3 ± 1.2	17.7 ± 0.0		82.3 ± 0.3
ATPS III	3.9 ± 1.8	3.9 ± 1.8	91.7 ± 1.3	3.3 ± 0.0		96.7 ± 0.5
total recovery (w/w %)	15.3 ± 1.4	17.3 ± 4.3	**71.9 ± 1.0**	29.3 ± 2.2		**70.7 ± 0.5**

in the interface is shown in the Supporting Information (Table S2).

To date, to the best of our knowledge, no studies have reported on the possibility of the fractionation and purification of most microalgae biomolecules via a single unit operation. The most important challenge in terms of purification is, however, the complete separation of each biomolecule. Therefore, strategies for further purification and for the recyclability of the phase-forming components were studied.

Recycling of Phase Forming Components and Further Purification of Biomolecules. Although the migration of proteins and free glucose toward the PPG 400-rich phase is low (15.1 ± 1.1% and 12.6 ± 0.8% of proteins and free glucose, respectively, is recovered in this phase), the thermosensitive nature of PPG 400 can be used to further purify the pigments from the other biomolecules (proteins and free sugars). Cloud point temperatures were determined to delimit the temperature range to which this method can be applied (Figure 5).

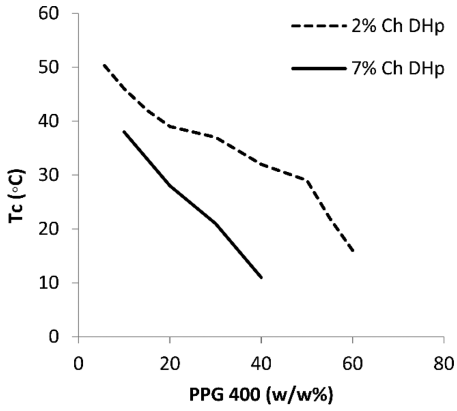

Figure 5. Cloud point temperatures of PPG 400 solutions in the presence of Ch DHp (2% and 7%).

In PPG 400-Ch DHp, the top phase is mainly composed of PPG 400 (max. 85%), Ch DHp (max. 7%), and water. Figure 5 shows two cloud point curves of PPG 400 solutions in the presence of 2% and 7% Ch DHp. The phase composition influences the formation of the phases through temperature. Thus, when increasing the concentration of PPG 400 and Ch DHp, lower temperatures are needed for the formation of two phases. This behavior is due to the high affinity of the IL for water. This is in agreement with Li et al., who studied the cloud point temperatures of PPG 400 solutions in the presence of 2% cholinium lactate, cholinium acetate, and cholinium propionate.[57] The influence of the IL on the cloud point temperature is in agreement with their ability to form an ATPS, which depends on their affinity for water.

The high concentration of ILs certainly benefits the applicability of this approach, such that lower temperatures were needed. High temperatures (>45 °C) should be avoided in the extraction of pigments and proteins in order to protect these biomolecules from denaturation and loss of their activity.[71] Therefore, mixture points with low cloud point temperatures (<45 °C) were selected for the application of this approach. Table S5 in the Supporting Information presents the composition of the mixture points used together with its respective TLL and cloud point temperature in the presence of microalgal biomolecules. To prove the applicability of this approach, the PPG 400-rich phase was incubated at 35 °C, allowing the formation of two phases. Pigments clearly migrated to the top phase ($\log(K) = 0.7$), which is composed of PPG 400. Contrastingly, proteins ($\log(K) = -0.3$) and free glucose ($\log(K) = -0.7$) preferentially migrated toward the bottom phase (composed of water and IL). Through the formation of these two phases, this step can concentrate and purify the pigments by removing proteins and soluble sugars. The recycling of PPG 400 can be performed by back extraction with organic solvents (e.g., ethyl acetate and water immiscible alcohols). This process would allow the recovery of pigments in the ethyl acetate, such that the polymer can be reused in a next ATPS stage. However, the use of heat and/or an extra solvent will increase the cost of the overall process. PPG 400 is a biodegradable and low-cost solvent that may be used in cosmetic, pharmaceutical and food applications due to its almost null toxicity level.[72] Furthermore, it is included on the FDA's GRAS (compounds generally recognized as safe) list.[39] Therefore, the carotenoids and pigments of interest may be recovered together with the PPG 400 and used subsequently for industrial applications.[73] The pigments should be further characterized and identify what impurities are present and when needed further purified.

Proteins are large biomolecules. RuBisCO has a molecular mass of ~560 kDa, in contrast to soluble sugars, for example, D-glucose and Ch DHp, which have the small molecular masses of 180.156 and 201.159 g/mol, respectively. This difference in size was used to separate the proteins from the soluble sugars and the IL. The applicability of ultrafiltration to recycle the IL was evaluated. High IL recovery in the permeate (96%) and protein recovery in the retentate (82%) was shown (Figure 4). We propose the reuse of the permeate containing the IL and soluble sugars in the next ATPS stage. In the end of the process, the sugars can be recovered by precipitation and the ionic liquid can be recycled. It is important to highlight that since it is out of the scope of this study, the recycling processes mentioned here were neither studied in detail nor optimized. However, a proof of concept of the recycling methods is provided because it is considered essential for the economic and environmental sustainability of the process.

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CONCLUSIONS

In order to avoid the need of many unit operations to separate each of the microalgae components, which results in a very expensive process, an alternative biorefinery process was proposed here. For that, the fractionation of several microalgal biomolecules was successfully achieved using consecutive purification steps. ATPS formed by polypropylene glycol (PPG 400) and several cholinium-based ionic liquids were investigated demonstrating the impact of the ionic liquid on the partitioning behavior of the biomolecules. 40 wt % PPG 400 and 14 wt % Ch DHp ATPS was selected as the best combination due to its high selectivity to fractionate pigments in the polymer-rich phase and proteins and soluble sugars in the IL-rich phase. Using this ATPS, a multistep fractionation process was designed, increasing the recovery efficiency of soluble biomolecules (pigments, proteins, and soluble sugars) in the aqueous phases, while in the interface non soluble components (lipids and starch) were concentrated.

In the developed downstream process, 98% pigments were recovered from the polymer-rich phase, 82% and 93% of proteins and soluble sugars respectively in the IL-rich phase. Furthermore, 79% and 71% of starch and lipids were recovered in the interface, respectively. Recycling and additional purification steps were proposed envisaging the application of this multiproduct process. The application of ultrafiltration to the IL-rich phase separated the 80% proteins from the soluble sugars and IL (96% IL recovery), which may lead to the purification of these two biomolecules and the recycling of the IL. The PPG 400-rich phase was thermo-separated at 35 °C into a polymer-rich phase for the recovery of purified pigments, and the water phase contained the remaining proteins and soluble sugars.

ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acssuschemeng.9b06379.

Pigment (Table S1, Figure S1) and lipid (Table S2) characterizations of the bead milled *N. oleoabundans* biomass. UV−vis spectra of the three ATPS cycles (Figure S2) and a snapshot of the interphase (Figure S3). Tables S3, S4, and S5 showing the weight fractions of the ternary complex PPG 400 + Ch DHp + H_2O (PDF)

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Notes

The authors declare no competing financial interest.

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