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Research Article

Efficient Fractionation of Lipids in a Multiproduct Microalgal Biorefinery by Polymers and Ionic Liquid-Based Aqueous Two-Phase Systems

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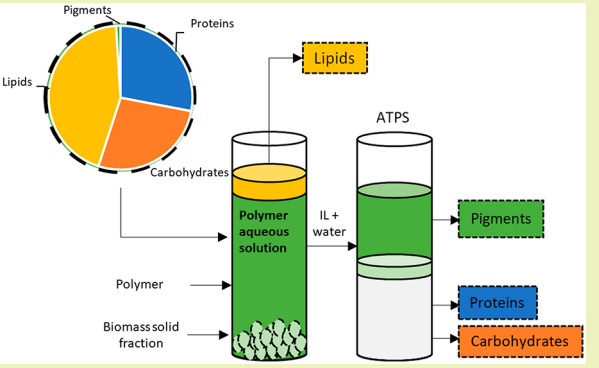
ABSTRACT: For a multiproduct microalgal biorefinery, most of the cell components should be extracted and fractionated. This work investigates the fractionation of lipids from other microalgal components (pigments, proteins, carbohydrates) using polymers and IL solutions in aqueous two-phase systems (ATPS). The microalgal lipids poorly migrated to the aqueous phases of ATPS and were recovered (97% of the total fatty acids) in a third phase (interphase) formed between the top and bottom phases. Studies with canola oil and purified phospholipids suggest that the high amount of oil, phospholipids, and other natural emulsifiers present in the microalgae mixed with the high amount of water in the ATPS form an emulsion which is difficult to fractionate. However, a solution of polypropylene glycol 400 (25% w/w) displaced 73% of lipids in an immiscible layer which was easy to recover. When combining this approach with a subsequent ATPS, most of the microalgae biomolecules (lipids, proteins, pigments, carbohydrates) could be fractionated in a three-step mild separation concept.



KEYWORDS: *Neochloris Oleoabundans, Microalgal products, Polymers, Mild separation, Downstream processing*

INTRODUCTION

Microalgal biomass has a plethora of valuable cell components, and due to resource scarcity, climate change, and other global problems, it is in great demand for many applications. Microalgae have not only potential to reduce dependence on fossil fuels and compensate green gas emissions but also to be used as a sustainable feedstock in the food, animal feed, cosmetics, pharmaceutical, biofertilizers, and nutraceutical sectors. Innovation in biorefining technologies is crucial to completely exploit microalgal biomass and mitigate microalgae production costs.[1,2]

Neochloris oleoabundans, microalgae used in this research, accumulate high amounts of neutral lipids (Triacylglycerol (TAG)) when cultivated under stress conditions such as nitrogen depletion. TAGs constitute approximately 80% of the total lipids in stressed *N. oleoabundans*, while polar lipids (PL) including glycolipids and phospholipids, aliphatic hydrocarbons, sterols, and pigments represent the remaining lipid fraction.[3] Both lipid fractions are valuable for many applications. TAGs can be used as a raw material for the production of biofuel or used in the food and feed industry (as a vegetable oil replacement) and as an ingredient in cosmetics and detergents.[4,5] PLs, namely, phospholipids are widely used for their emulsifying and structural improvement properties in food matrices.[6] Phospholipids are also used in nutritional and pharmaceutical applications.[7,8] Apart from lipids, *N. oleoabundans* contains carbohydrates, proteins, and pigments.

Aqueous two-phase systems (ATPS) gained importance in the last years due to their ability to fractionate different molecules selectively and in a mild manner. ATPS occur when two water-soluble solutes are mixed above certain concentrations and/or temperature in an aqueous solution. These solutes compete for the interaction with water, and as a result, phase separation occurs.[9] Water is the common component in ATPS, which provide mild conditions for the extraction of biomolecules. Mild conditions are crucial when working with functional compounds with applications for the food and pharmaceutical industries.[10] Furthermore, ATPS allow the replacement of volatile organic solvents, which are commonly used in liquid−liquid extraction and represent environmental and health hazards.[11] The application of ATPS was already

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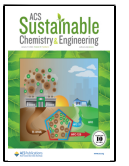

Table 1. Comparison of Conventional ATPS and Ionic Liquid-Based ATPS

Conventional ATPS	Ionic liquid-based ATPS
Strength	
Extensive research available	Flexibility (more possible combinations)
Price (relatively inexpensive)	Wider extraction capabilities
Biocompatibility (e.g., PEG)	Tunable selectivity
	Wide range of applications
	Process conditions (e.g., lower viscosity)
	Biocompatibility (e.g., Choline)
	Recycling feasible
Weakness	
Flexibility (Less combinations available)	Price
Limited range of polarity of coexisting phases	Insufficient understanding of partition behavior
Process parameters limiting industrial scale (high viscosity)	
Still a conservative approach known for years and still not implemented at manufacturing scale	
Opportunities	
Primary clarification of cell cultures	Development of Ionic liquids of low toxicity, low cost and high biodegradability
Purification of proteins and small molecules (hydrophilic compounds)	Used in different steps of downstream processing (e.g., cell disruption and fractionation)
	Purification of hydrophobic and hydrophilic compounds
Threats	
Chromatography development (for protein purification)	Development of natural biodegradable components (e.g., Deep Eutectic Solvents)

proven for many uses, including the separation of proteins, amino acids, antioxidants, pigments, colorants, and pharmaceuticals (e.g., antibiotics) from model molecules to real matrices.[12]

The interest in ATPS as a fractionation technology for microalgae biorefinery is growing. For example, phycobiliproteins from a crude enzyme extract from the red algae *Caloglossa continua* were fractionated by an ATPS formed by polyethylene glycol (PEG) and ammonium sulfate.[13] ATPS formed by aqueous solutions of surfactants and salts were used for the extraction of antioxidants (α-tocopherol, β-carotene, and gallic acid) from *Tetraselmis suecica*.[14] Phong et al.[15] integrated ultrasonication and ATPS for protein recovery from *Chlorella sorokiniana* using methanol and potassium phosphate. Due to the labile nature of proteins, denaturation will occur when certain organic solvents or other harsh conditions are used. This results in a loss of functionality reducing the value. Luo et al.[16] used an ATPS formed by PEG and potassium phosphate for the extraction of C-phycocyanin from *Spirulina maxima*. Other studies focused on the fractionation of polysaccharides,[17] lutein,[18] and B-phycoerythrin.[19] ATPS can be formed with a wide assortment of components, including polymers, salts, surfactants, ionic liquids, alcohols, and deep eutectic solvents. This feature makes ATPS highly versatile on their design and application for the purification of a wide range of biomolecules. Therefore, they are excellent bases for the development of a multiproduct biorefinery.

Ionic liquids (ILs) are characterized by high design flexibility and tunability and a wide range of polarity and capacity to solubilize a wide variety of organic and inorganic compounds.[20,21] In addition, their low volatility eliminates a major concern for contamination, and a large number of ILs are already available with low toxicity, low cost, and high biodegradability.[21−23] It is preferred, however, to reuse the IL after regeneration to avoid the presence of IL in the waste and to minimize costs for the process.

In Table 1, a SWOT (strength, weakness, opportunities, threats) analysis is presented to compare the more traditional aqueous two-phase systems (ATPS) such as salt-based or polymer-based ATPS with ionic liquid-based ATPS.

IL-based ATPS demonstrated higher efficiencies to fractionate microalgal proteins compared with the commonly used ATPS that are formed using only polymers and salts.[24] Furthermore, IL-based ATPS have shown versatility to separate different components simultaneously. For example, an IL-ATPS selectively fractionated proteins and sugars in the top and bottom phases, respectively, from different microalgal extracts.[25] An ATPS formed by a polymer and a cholinium-based ionic liquid was able to separate microalgal proteins from pigments without compromising the native conformation of the proteins and causing no degradation to the pigments (chlorophylls and carotenoids).[26] Although research has shown that ILs have the capacity to solubilize different compounds, IL-ATPS has mainly been investigated for the partitioning of hydrophilic components with hydrophobic and hydrophilic ionic liquids having been used to extract proteins, pigments, and carbohydrates from microalgae.[27] However, there are only a few studies where ATPS containing ILs were used for the partitioning of lipids,[12] and no studies were found focusing on the fractionation of microalgal lipids and simultaneous separation of other components (proteins, pigments, carbohydrates). With this research, we want to understand how microalgal lipids can be separated from other components without affecting the product quality of sensitive products like proteins. A novel approach to fractionate lipids and other microalgal components (proteins, pigments, and carbohydrates) is studied here using aqueous two-phase systems. PEG 400-citrate, PEG 400-cholinium dihydrogen

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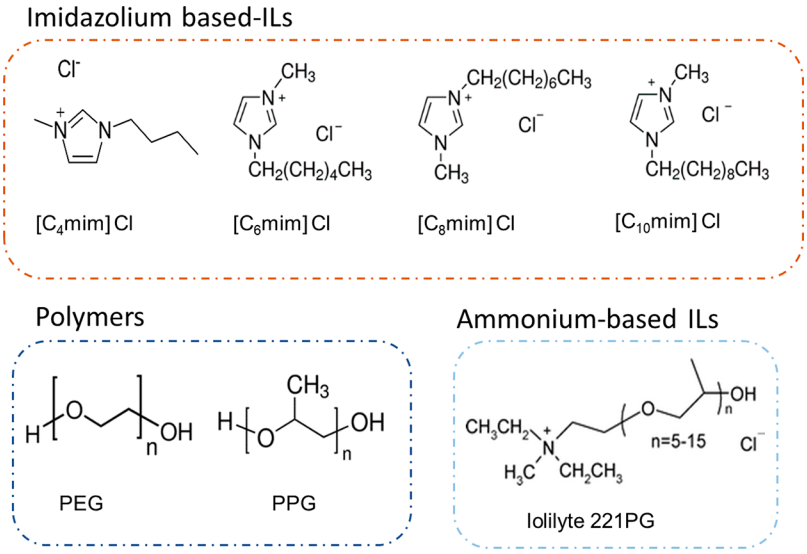

Figure 1. Structures of chemicals evaluated to understand the solubility of microalgal lipids in aqueous solutions. Imidazolium-based ionic liquids: 1-butyl-3-methylimidazolium chloride ([C4mim]Cl), 1-hexyl-3-methylimidazolium chloride ([C6mim]Cl), 1-methyl-3-octylimidazolium chloride ([C8mim]Cl), 1-decyl-3-methylimidazolium chloride ([C10mim]Cl). Polymers: polyethylene glycol (PEG) and polypropylene glycol (PPG) with average molecular weights of 400 g mol^{-1}. Ammonium-based ionic liquid: Iolilyte 221PG.

citrate, and Iolilyte 221PG-citrate ATPS were selected based on our previous research.[28] The partitioning of commercial canola oil (representing TAGs) and partially purified yeast polar lipids (representing PL) was initially used to understand their behavior in ATPS without the influence of other biomolecules present in the microalgal cells. The partitioning behaviors of these model lipids were compared with the partitioning behaviors of lipids from disrupted *N. oleoabundans*. To this end, the top phase main component concentration (% w/w), bottom phase main component concentration (% w/w), and temperature (°C) were studied. Moreover, the solubility of microalgal lipids in various aqueous solutions was explored. Finally, based on the findings of this research, we propose a multiproduct fractionation process using aqueous two-phase systems.

MATERIALS AND METHODS

Materials. The ionic liquids Iolilyte 221PG, ≥95%, and choline dihydrogen phosphate (Ch DHp, ≥98%) were both supplied by Iolitec. The following imidazolium-based ionic liquids were provided by Sigma-Aldrich: 1-butyl-3-methylimidazolium chloride ([C_4mim]-Cl, ≥98%), 1-hexyl-3-methylimidazolium chloride ([C_6mim] Cl, ≥97%), 1-methyl-3-octylimidazolium chloride ([C_8mim]Cl, ≥97%), 1-decyl-3-methylimidazolium chloride ([C_{10}mim]Cl, ≥96%). Polyethylene glycol (PEG) and polypropylene glycol (PPG) with average molecular weights of 400 g mol^{-1} and potassium citrate and citric acid were obtained from Sigma-Aldrich. Figure 1 presents the structures of the imidazolium- and ammonium-based ionic liquids and polymers. Commercial canola oil (Hollands Goud, The Netherlands) was used as a model for TAGs, and yeast (*Saccharomyces cerevisiae*) polar lipid extract powder obtained from Avanti Polar Lipids, Inc. (USA) was used as a model for polar lipids (PL). Canola oil analyses show that TAGs constitute 94.4% to 99.1% of the total lipid.[29] Canola oil, representing TAGs, was selected in this study because it has a similar fatty acid profile as *N. oleoabundans* (total fatty acid profile is provided in Table S1 of the Supporting Information).

Methods. *Microalgae Cultivation, Harvesting, and Cell Disruption.* For the partitioning studies, *N. oleoabundans* was cultivated in artificial seawater and under nitrogen depletion (N−). *N. oleoabundans* (UTEX 1185, University of Texas Culture Collection of Algae, USA) was cultivated in a 1300 L vertical stacked tubular photo bioreactor (LGem) located at AlgaePARC, The Netherlands. It was cultivated using Bold's Basal medium[30] 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: 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 microalgal biomass was harvested (80 Hz, 3000*g*, 0.75 m^3 h^{-1}) using a spiral plate centrifuge (Evodos 10, Evodos, The Netherlands), and the concentrated biomass was suspended in Milli-Q water to obtain a biomass concentration of ∼90 g L^{-1}. The algal cells were disrupted in a horizontally 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.[31] The bead-milled microalgae suspension was stored at −20 °C until later use.

Partitioning of Lipids in Aqueous Two-Phase Systems. Canola oil and partially purified yeast polar lipids were selected as model lipids to study the partitioning behavior of TAGs and PLs, respectively. A bead-milled *N. oleoabundans* suspension was used to study the partitioning of microalgae lipids (total fatty acids) from the real matrix. To understand the partitioning behavior of lipids in ATPS, three parameters were studied: top phase main component concentration (% w/w), bottom phase main component concentration (% w/w), and temperature (°C).

The biphasic systems were prepared gravimetrically $\pm 10^{-4}$ g by adding the appropriate amount of ATPS-forming components. The total concentration of canola oil used in the mixture was 0.1 g g^{-1} and of polar lipids was 5 mg g^{-1}. For the partitioning of lipids from microalgae, the total concentration of the bead-milled microalgae suspension in the mixture was 2.5 mg dw.g^{-1}. All mixtures were stirred and incubated for 1 h maintaining the desired temperature by a heating/cooling block thermostat. Then, the mixtures were centrifuged at 2500 rpm (1200*g*) for 10 min. Afterward, the phases were carefully separated, and weights and volumes were measured. Interfaces were resuspended in Milli-Q water to facilitate the quantification of lipids.

To describe the distribution of lipids in the ATPS, extraction efficiencies (EE%) were calculated with eq 1, where m_{lipids} is the lipids mass in each phase, and $m_{lipids,\ initial}$ is the initial mass of lipids quantified in the microalgae or standard molecules added to the systems.

$$EE_{lipids}\ (\%) = \frac{m_{lipids,\ phase}}{m_{lipids,\ initial}} \times 100 \quad (1)$$

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Table 2. Factors and Value Levels Used in FFD Design for PEG 400-Potassium Citrate System

Variables	Factors	Low value (−1)	Center value (0)	High value (+1)
PEG 400 concentration, % w/w	X_1	25	28.25	31.5
Potassium citrate concentration, % w/w	X_2	30	32.5	35
Temperature, °C	X_3	3	21.5	40

Table 3. Factors and Value Levels Used in FFD Design for Iolylite 221PG-Potassium Citrate System

Variables	Factors	Low value (−1)	Center value (0)	High value (+1)
Iolilyte 221PG concentration, % w/w	X_1	25	28.75	32.5
Potassium citrate concentration, % w/w	X_2	25	28.75	32.5
Temperature, °C	X_3	3	21.5	40

Table 4. Factors and Value Levels Used in FFD Design for PEG 400-Ch DHp System

Variables	Factors	Low value (−1)	Center value (0)	High value (+1)
PEG 400 concentration, % w/w	X_1	30	33.5	37
Ch DHp concentration, % w/w	X_2	30	33.5	37
Temperature, °C	X_3	3	21.5	40

Design of Experiments. Modde v.9.1 Design of experiments software (DOE) (MKS Umetrics, Sweden) was used to evaluate the effect of different parameters on the partitioning of lipids in the three ATPS. A two-level full factorial design (FFD) was used for the three systems with three independent variables (factors) each at three levels, and three replicates were taken for the center points. Tables 2, 3, and 4 show the coded values for each factor studied in the three ATPS. Phase-forming component concentrations were selected depending on the phase-forming range concentrations delimited by phase diagrams previously created.[28] Temperatures were selected in a range protecting proteins from denaturation, and an optimal pH for the fractionations of proteins in the ATPS was used.[28,32]

The studied responsive variable was the extraction efficiency of lipids (EE %) in the different phases calculated using the equations as presented in the Supporting Information (Table S4). The response surface methodology was used to understand the influence of the parameters on the fractionation of lipids. The responses were fitted in a second-order polynomial regression model, and the model validity and significance were evaluated using the analysis of variance (ANOVA).

Solubility of Microalgal Lipids in Aqueous Solutions. Lipid extraction from disrupted *N. oleoabundans* in various aqueous solutions of ILs or polymers were evaluated (Figure 1). The IL or polymer concentrations studied were 25 and 50 % w/w. Here, 1 g of disrupted microalgal suspension was added to 1 g of aqueous solution (composed of IL or polymer) at room temperature (~21 °C) and mixed in a tube rotator for 1 h. The disrupted cells were separated from the solution by centrifugation (4000 rpm, 10 min). After this treatment, chloroform−methanol was used to extract the lipids and pigments from the cells. The extraction yield (mg/g) was calculated as the total weight of fatty acids or pigments (lutein) present in the supernatant divided by the weight of biomass used.

$$\text{Extraction yield}_{\text{lipids}} = \frac{m_{\text{lipids, supernatant}}}{m_{\text{biomass}}} \quad (2)$$

Quantification of Biomolecules. *Quantification of Lipids.* Total lipids (triacylglycerides (TAG) and polar lipids (PL)) content was determined as described by Remmers et al.[33] Lipid extraction was done with a chloroform:methanol (1:1.25) solution containing two internal standards: 1,2-didecanoyl-sn-glycerol-3-phospho-(1′-rac-glycerol) and glyceryl pentadecanoate (Sigma-Aldrich) as polar lipid and TAG, respectively. 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 the solvent and helium as the carrier gas with a flow rate of 20 mL/min. The column used was a Supelco Nucol™ 25357, 30m × 530 μm × 1.0 μm. Total fatty acid (TFA) composition and content were calculated by taking the sum of all fatty acids in both fractions.

Quantification of Pigments. The quantification of pigments was done by measuring the absorbance spectrum between 400 and 750 nm using a UV−vis microplate reader (Infinite M200, Tecan, Switzerland). Since lutein is the most abundant carotenoid in *N. oleoabundans*, this carotenoid was selected as a reference for the quantification of pigments. Its concentration was calculated using the average extinction coefficient for lutein of E1% = 2550 and a molecular mass of 569 g/mol as previously described by Cisneros et al.[18]

RESULTS AND DISCUSSION

An outline of the results and discussion is given below whereby initially *N. oleoabundans* is bead milled releasing the different components (proteins, carbohydrates, pigments, lipids) ready for ATPS fractionation.

At first, the lipid composition was determined in *N. oleoabundans* followed by partitioning with ATPS selecting PEG 400 and Iolilyte 221PG as ATPS components using Ch DHp and citrate as the buffer system. Second, the solubility of the lipids was determined in the different solutions containing polymers and ionic liquids. Finally, partitionings of the different products (pigments, proteins, soluble sugars and lipids) are investigated with a focus on lipids by using a multicomponent approach with a PPG 400- and Ch DHp-based ATPS system.

Lipid Composition of *N. oleoabundans*. First, the disrupted *N. oleoabundans* suspension was characterized. The microalgae suspension is composed of 44% dw total fatty acids (33.6 ± 0.2% dw TAGs and 10 ± 0.06% dw polar lipids), 27% dw total carbohydrates, and 28% dw proteins. Lutein (7.1 μg/mg dw) was detected as the most abundant pigment in this microalga, which is in agreement with other reports.[34,35]

The fatty acid profile of *N. oleoabundans* presented in Table S1 is in accordance with the profiles reported in previous studies.[36] The most abundant fatty acid (FA) in the triacyl glyceride (TAG) fraction is oleic acid (C18:1), constituting more than 50% of the total fatty acids, followed by palmitic acid (C16:0) and linoleic acid (C18:2).

Partitioning of Lipids on ATPS. Canola oil and partially purified yeast polar lipids were selected as model lipids to study their partitioning in selected ATPS. Results presented in Table 5 were obtained when using the center point values

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Table 5. Extraction Efficiency (% w/w) of Total Fatty Acids from Canola Oil Using Three Different ATPS at 21.5 °C[a]

ATPS	EE of TFA from canola oil (% w/w)	
	Immiscible phase	Aqueous top phase
Iolilyte 221PG-citrate	99.47 ± 0.078	0.41 ± 0.019
PEG 400-citrate	99.80 ± 0.003	0.20 ± 0.002
PEG 400-Ch DHp	99.93 ± 0.009	0.07 ± 0.005

[a]The mixture points studied for the systems were Iolilyte 221PG; citrate (29; 29% w/w), PEG 400; citrate (28; 32% w/w), PEG 400; and Ch DHp (33; 33% w/w). The results represent the average of three independent experiments and standard deviations.

(Tables 2, 3, and 4) of the factors evaluated (concentration of phase-forming components: Iolilyte 221PG; citrate (29; 29% w/w), PEG 400; citrate (28; 32% w/w), PEG 400; Ch DHp % w/w (33; 33% w/w) and a temperature of 21.5 °C). Canola oil, added to the ATPS, floated and formed an immiscible phase above the aqueous top phase of the ATPS (oil has a lower density than the aqueous solutions). TAGs are nonpolar molecules, which explains their poor solubility in the aqueous phases (Figure S2A, Supporting Information). Only 0.4% of the total fatty acids (TFA) were extracted in the aqueous top phase by Iolilyte 221PG-citrate, 0.2% by PEG 400-citrate, and 0.1% by PEG 400-Ch DHp. Lipids were not detected in the aqueous bottom phase of the three systems, showing a clear preference of the lipids to displace and form their own phase.

Comparing the three ATPS, Iolilyte 221PG-potassium citrate shows extraction efficiencies higher than the PEG 400-based ATPS. However, a very low amount is recovered in the aqueous phase (0.4%), from which 0.25% is oleic acid. The partitioning of TAGs in ATPS was influenced by the parameters studied: concentration of the phase-forming components and temperature. Contour plots of the effect of these parameters on the extraction efficiency of TFA from canola oil in the ATPS are presented in Figure S1 of the Supporting Information. Increasing the concentration of the phase-forming components results in the extraction of more lipids to the aqueous top phase (up to 0.75% TFA using Iolilyte 221PG-citrate). This is due to the lower water concentration in the top phase when increasing the concentration of phase-forming components. Statistical analysis showed that the temperature has no significant effect on the EE % of TFA when the temperature is increased from 4 to 40 °C. In the immiscible layer, up to 99.9% of TAGs were recovered when using the PEG 400-based ATPS.

Polar lipid extract from yeast is composed mainly of phospholipids with phosphatidylcholine (PC) as the most abundant phospholipid present. The fatty acid profile of the PL extract is provided in Table S2 of the Supporting Information. Different from canola oil (representing TAGs), PL added to the ATPS formed a solid phase between the aqueous phases (interface). The distributions of polar lipids in the three phases (top, bottom, and interface) are shown in Table 6. The results presented in this table were obtained when using the center point values (Tables 3 and 4) of the factors evaluated (concentration of phase-forming components: Iolilyte 221PG; citrate (29; 29% w/w), PEG 400; Ch DHp % w/w (33, 33% w/w), and temperature 21.5 °C).

Comparing the ATPS, Iolilyte 221PG-citrate fractionated 12% of the polar lipids from the commercial yeast extract in the top phase (Iolilyte 221PG-rich phase). Increasing the temperature to 40 °C and the concentration of phase-forming components, the extraction efficiency of polar lipids increased to 18% when using Iolilyte 221PG-citrate.

Instead, PEG 400-based ATPS only fractionated 0.5% of polar lipids in the aqueous top phases (PEG 400-rich phase) and recovering 99.5% of the PLs in the interface. The temperature and concentration of phase-forming components (e.g., PEG 400 (30−37 % w/w) and Ch DHp (30−37 % w/w)) used in the ATPS did not significantly affect the extraction of polar lipids when using these systems (data not shown). It seems that the amphiphilic and hydrophobic natures of the Iolilyte 221PG benefit the migration of the phospholipids to the aqueous phases.[37]

Phosphate groups are polar, and therefore, when the phospholipids are suspended in aqueous solutions, they typically arrange themselves in a stable bilayer. The hydrophobic fatty acid tails will not interact with water, while the phosphate group tends to form hydrogen bonds with water and other polar molecules in both aqueous phases. Thus, the phosphate group is oriented toward the aqueous phases, and the fatty acid tails are sequestered inside the micelle due to their rejection to water.

In agreement with our results, an ATPS formed by PEG 1500 and a phosphate buffer was previously used to purify hemoglobin, concentrating cell membrane phospholipids in the interface.[38] Aqueous two-phase systems have been widely studied for the purification of proteins from fermentation broths and colloid solutions.[39] This liquid−liquid extraction method has the ability of separating cells and cell membranes in the interface from biomolecules such as proteins that are fractionated into the aqueous phases.[40] ATPS interfacial tension allows holding cells and other particles in the interface, achieving efficient cell clarifications and complex separations.[41,42] Liposomes added to ATPS tend to partition between the two-phases (interface) influenced by the phase-forming components composition and concentration and liposome surface nature and charge of lipid head groups.[43,44]

We also investigated ATPS to partition microalgae lipids (Table 7), while conserving the functionality of other biomolecules. Three phases were identified after the fractionation procedure from top to bottom: top (PEG or Iolilyte 221PG-rich), interface, and bottom (potassium citrate or Ch DHp-rich) phases. Due to the high TAG content of *N. oleoabundans* (80% of the total fatty acids), we expected that

Table 6. Distribution of Yeast Polar Lipids in Top, Bottom, and Interface at 21.5 °C[a]

ATPS	EE of yeast polar lipids (% w/w)		
	Aqueous top phase	Interface	Aqueous bottom phase
Iolilyte 221PG-citrate	11.46 ± 2.756	88.11 ± 3.478	0.42 ± 0.732
PEG 400-Ch DHp	0.48 ± 0.556	99.52 ± 0.556	0.00 ± 0.000

[a]The mixture points studied for the systems were Iolilyte 221PG; citrate (29; 29% w/w) and PEG 400; and Ch DHp (33, 33% w/w). The results represent the average of three independent experiments and standard deviations.

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Table 7. Extraction Efficiency of TFA from Disrupted *N. oleoabundans* at Room Temperature (21.5 °C)[a]

ATPS	EE of FTA from microalgae (% w/w)		
	Aqueous top phase	Interface	Aqueous bottom phase
Iolilyte 221PG-citrate	15.23 ± 0.535	84.77 ± 0.535	0.00 ± 0.000
PEG 400-citrate	1.47 ± 0.079	98.20 ± 0.350	0.32 ± 0.353
PEG 400-Ch DHp	2.22 ± 0.029	97.55 ± 0.045	0.23 ± 0.021

[a]The mixture points studied for the systems were Iolilyte 221PG; citrate (29; 29% w/w), PEG 400; citrate (28; 32% w/w), PEG 400; and Ch DHp % w/w (33, 33% w/w). The results represent the average of three independent experiments and standard deviations.

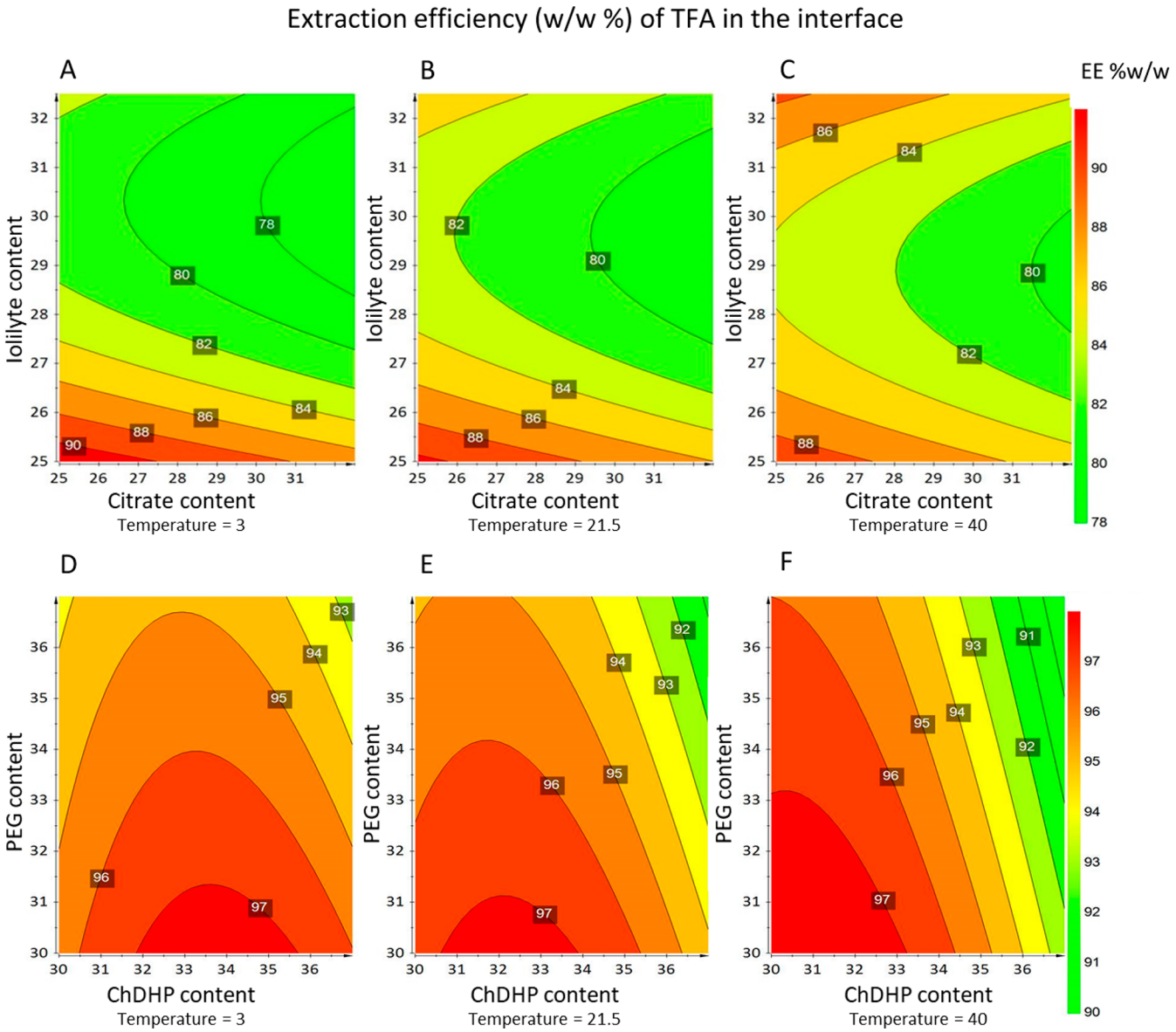

Figure 2. Contour plots showing the influence of the concentration of phase-forming components and temperature on the extraction efficiency of total fatty acids (TFA) from disrupted *N. oleoabundans* in the interface. (A, B, C) Iolilyte 221PG-citrate ATPS. (D, E, F) PEG 400-Ch DHp.

the TAGs would be displaced forming an immiscible layer on top of the aqueous phase as canola oil did. Instead, the lipids were recovered in the interface between the aqueous phases similarly as the yeast polar lipids extract.

Table 7 shows the distribution of lipids in the three phases for the three ATPS studied. These results were obtained using the center point values (Tables 2, 3, and 4) of the factors evaluated (concentration of phase-forming components: Iolilyte 221PG; citrate (29; 29% w/w), PEG 400; citrate (28; 32% w/w), PEG 400; Ch DHp % w/w (33, 33% w/w), and temperature: 21.5 °C). Most of the TFA from disrupted *N. oleoabundans* were recovered in the interface. Iolilyte 221PG-citrate ATPS fractionated 15% of the TFA in the Iolilyte 221PG-rich phase (top). PEG-based ATPS fractionated only 2% of TFA in the PEG-rich phase, recovering 98% of TFA in the interface.

The high recovery of microalgal lipids in the ATPS interface seems to be a consequence of the emulsifying properties of

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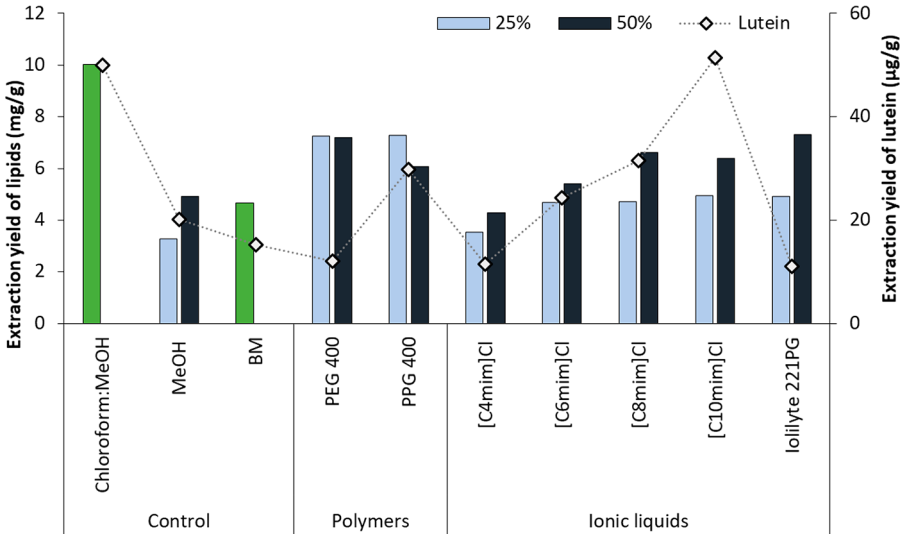

Figure 3. Extraction yield of lipids (mg lipids extracted/g microalgae suspension) from disrupted *N. oleoabundans* using aqueous solutions. Chloroform:methanol and methanol were used as control as well as bead milling (BM). The 25% and 50% mentioned in the top part of the figure refer to the IL or polymer concentrations studied (% ww). The extraction yields of lutein using the 50% solutions are also shown.

other biomolecules present in the microalgal cells, such as phospholipids and proteins.[45] Phospholipids act as a surfactant and participate in the formation of stable emulsions in the presence of water and oil. As discussed before, phospholipids tend to partition to the interface because the hydrophilic part is oriented toward the two aqueous phases (forming a bilayer), and the fatty acid tails (from the PLs and TAGs) are sequestered inside the micelle due to their rejection to water. This emulsion does not allow the TAGS to be displaced.

The formation of stable emulsions after the mechanical disruption (e.g., bead milling) of microalgae was reported in other studies.[46−48] When using mechanical disruption, the cells are broken in small particles forming a thick emulsion due to the presence of lipids, water, and natural emulsifiers (e.g., phospholipids and starch).[45] This emulsion hinders fractionation processes, when using volatile organic solvents to extract lipids.[46]

The partitioning of lipids was influenced by the concentration of phase-forming components and temperature. Results presented in Table 7 show that increasing the concentration of both phase-forming components decreases the recovery of lipids in the interface; thus, more lipids partition to the aqueous top phase. Temperature has a different effect on the extraction of lipids depending on the system used (Figure S3, Supporting Information). Iolilyte 221PG-citrate recovered more lipids in the interface at higher temperatures. The interactions between Iolilyte 221PG and water are low in the presence of potassium citrate, and by decreasing the temperature, these interactions are further lowered, which enhances the ability of the IL to extract lipids.[49] PEG 400-based ATPS show an opposite trend where a lower temperature results in a higher lipid recovery in the interface. Higher temperatures lead to a breakdown of the hydrogen bonds between PEG and water, and consequently, the aqueous top phase becomes more hydrophobic.[50]

A second-order polynomial regression model was used to calculate the response contour plot for each response variable as shown in Figure 2. Model coefficients describe the effect of variables on the response. The model coefficients that represent this effect with less than 95% significance were removed. The reduced regression models that were obtained for the responses and the statistical analysis of variance (ANOVA) are shown in Table S4 of the Supporting Information. The models describing the extraction of lipids to the interface show a good correlation between the predicted and experimental values ($R^2 = 0.92$). From the analysis of the variance test that compares the variation in the regression model and the residuals, it can be observed that models are statistically significant ($p < 0.05$). Further, it showed that all the factors (concentration of phase-forming components and temperature) have a significant influence on the response variable (EE % of lipids in the ATPS). The lack of a fit test shows that errors are not significant for the model ($p > 0.05$).

As shown in Figure S2B from the Supporting Information, the lipids recovered in the interface are bleached, which is an advantage due the presence of chlorophylls negatively impacting further downstream processing and the oil quality.[51]

Solubility of Lipids from Microalgae in Aqueous solutions. The partition preference of microalgae lipids in ATPS seems to be a consequence of polar lipids entrapping the fatty acid tails inside the emulsion formed in between the phases. For a better understanding, a disrupted *N. oleoabundans* suspension was mixed with different aqueous solutions composed of ionic liquids or polymers.

Figure 3 shows the extraction yield of lipids and pigments in a variety of imidazolium based-ionic liquids, an ammonium-based IL (Iolilyte 221PG), and two polymers (polyethylene glycol 400 and polypropylene glycol 400). To compare the influence of water on the extraction of lipids, two solution concentrations, 25% and 50%, were studied for the ionic liquids and polymers. As the control, the extraction solutions chloroform:methanol (1:1.25) and methanol are shown as well as bead milling as an extraction method.

The extraction yields of lipids in the aqueous solutions studied followed this order: PEG 400 ≈ PPG 400 > Iolilyte 221 PG > [C8mim]Cl ≈ [C10mim]Cl > [C6mim]Cl > [C4mim]. Polymers extracted the highest amount of TFA from disrupted microalgae (up to 73%) compared to the benchmark chloroform:methanol. The lipids extracted with the polymer solutions formed an immiscible layer, and it was observed that

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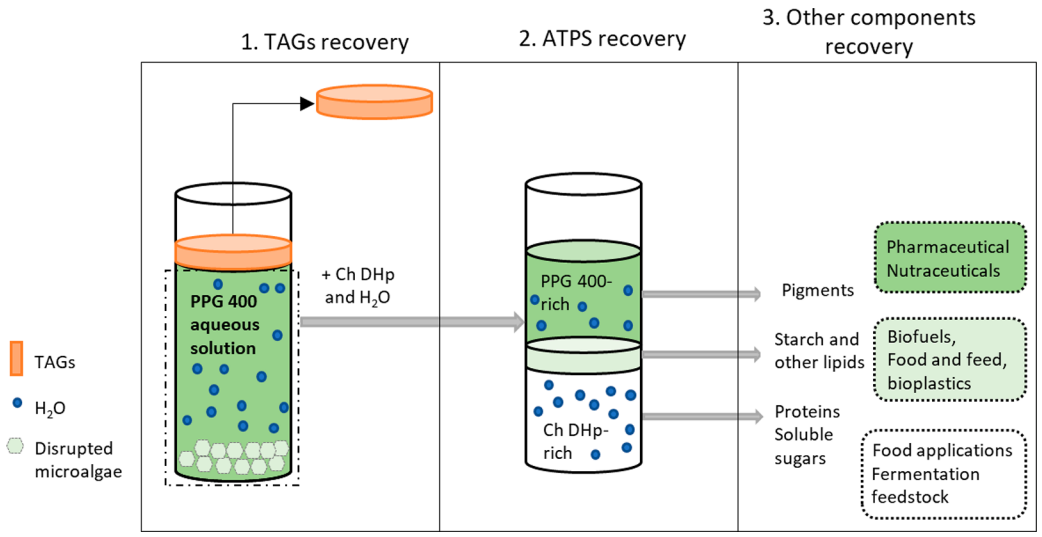

Figure 4. Overview of proposed process for fractionation of microalgal lipids and other cell components.

increasing the concentration of polymer leads to a decrease in lipid extraction yield, especially using PPG 400. Both PPG 400 and PEG 400 are highly hydrophilic and are completely miscible in water, which explains why the lipids were not solubilized in the aqueous solutions. Due to their hydrophilic nature, the polymers are preferentially hydrated, acting as salting-out agents. This leads to the exclusion of the highly hydrophobic lipids to form their own phase.[52,53] However, their polar nature is not enough to exclude the lipids completely in a separate phase. Methanol, for example, is a highly polar solvent but did not extract lipids in the same way as the polymers.

In agreement with these results, polyethylene glycols with various molecular weights were previously studied by Manic et al.[54] They compared the solubility of soybean oil in PEG with different molecular weights and in methanol. Although methanol was the most polar solvent studied, it is also the most soluble in nonpolar soybean oil. Moreover the long chain PEGs (more hydrophobic than short chain PEGs) are the least soluble in soybean oil.

The water-miscible ionic liquid, [P(CH2OH)4]Cl, studied by Olkiewicz et al.[55] presented a similar behavior. The lipids did not dissolve in the ionic liquid but released the lipids from the cells, and these floated forming an immiscible layer. It was discussed that the ability of hydrophilic ionic liquids to extract lipids is caused by the low solubility of the lipids in these ionic liquids.[56] Some hydrophilic ionic liquids can solubilize other components of the microalgal cells, avoiding the formation of emulsions and promoting the exclusion of lipids that are not soluble in the aqueous solutions.[57]

Iolilyte 221PG showed the highest extraction yield among the ILs studied; however, lipids did not formed an immiscible phase. Iolilyte 221PG is a tetraammonium-based IL that contains oligoethyleneglycol units and possesses both hydrophilic (hydroxyl groups) and hydrophobic (long alkyl side chain) properties.[37,58] This ionic liquid is capable of enhancing the solubility of hydrophobic substrates from cell lysate of *E. coli* as described by Dreyer and Kragl.[59] The surfactant properties of the Iolilyte 221PG (also called Ammoeng 110 or Glensurf 42) could explain its ability to dissolve carbohydrates (cellulose), triglycerides, and amino acids,[60] and it could explain the higher solubility of microalgal lipids in this IL compared with the other solvents studied. This is in agreement with our results using ATPS. Although low extraction efficiencies were obtained, Iolilyte 221PG-citrate ATPS partitioned higher amounts of TFA in the Iolilyte 221PG-rich phase than the polymer-based ATPS.

The imidazolium-based ILs did not exclude the lipids extracted in an immiscible phase. Thus, the extracted lipids were soluble in the ionic liquid solution. As shown in Figure 3, increasing the alkyl chain length of the ILs cations resulted in the extraction of more lipids ($[C4mim]^+ > [C6mim]^+ > [C8mim]^+ \approx [C10mim]^+$). Furthermore, a higher concentration of the IL enhanced the extraction of lipids. The cation side alkyl chain lengths of ILs are directly correlated with their hydrophobicity nature; the larger the cation alkyl chain length is, the more hydrophobic the ionic liquid is, which explains the extraction trend.[61] In addition, long alkyl side chain ILs exhibit surfactant properties in water and have enhanced the extraction of different compounds.[62,63] Cheong et al.[62] found that the alkyl chain lengths of the cations of ILs have positive effects on the extraction of polyunsaturated fatty acids from fish oil (n-3 PUFA and n-3 PUFAEE).

The extraction of pigments follows a different trend than lipids (UV−vis spectra of the extracted pigments in each solvent are shown in Figure S4 of the Supporting Information). Results show that the solubility of pigments decreases in the following order: [C10mim]Cl > [C8mim]Cl > PPG400 ≈ [C6mim]Cl > Iolilyte 221PG > [C4mim]Cl ≈ PEG400. This behavior seems to be highly influenced by the hydrophobicity of the solutions used. Increasing the alkyl chain lengths of the ILs cations, increases their hydrophobicity, and thus, more pigments are extracted. Moreover, PPG 400 has an additional methyl group in the ethylene glycol repeating unit compared to PEG 400 making it more hydrophobic, which explains its better performance in the extraction of pigments.

Fractionation of Lipids in a Multiproduct Biorefinery Approach. The results above have shown that exclusion of lipids toward an immiscible phase that can be directly recovered from the solution is beneficial in the fractionation of different cell components from microalgae, due to the mild conditions (no extreme pH, no high temperatures, and no organic solvents) used. Microalgae lipids are commonly extracted by liquid−liquid extraction using organic solvents,[64]

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which can affect the native conformation of proteins. Moreover, the recovery of lipids from the extractant solution consumes extensive energy and leaves lipid-soluble contaminants in the bio-oil.[65]

We propose a fractionation process divided into three steps (Figure 4): (1) Recovery of TAGs from microalgae using PPG400. Although PEG400 and PPG400 are both hydrophilic polymers, PPG400 is more hydrophobic due to the extra methyl group in the ethylene glycol repeating unit than PEG 400 (Figure 1), which explains its better performance in the extraction of pigments. So pigments will remain in the PPG400 phase, whereas with PEG400, they will remain in the lipid phase which would be less preferred to obtain a pure lipid fraction. The lipid fraction (TAGs) can be recovered directly as a product, and the PPG 400 aqueous solution and pellet (disrupted microalgae) can be mixed with the right proportions of Ch DHp and water to form an ATPS. (2) In this system, pigments partition to the PPG 400-rich phase, proteins and soluble sugars to the Ch DHp-rich phase, and starch and phospholipids can be recovered in the interface.[26,66]

This process allows the fractionation of lipids and other microalgal components without compromising the conformation of fragile and functional components such as proteins and pigments. To enhance the purity of the components in each phase, consecutive liquid−liquid extractions can be performed, and the phase-forming components can be recycled.[66] The application of the compound and cost depends on the purity and functional state of the biomolecule. The high variety of biomolecules that can be recovered with the proposed process can substantially improve the economic viability of microalgae production. In order to scale up the envisioned process, the recycling of phase-forming components and process kinetics (determining throughput and equipment) need to be assessed first. In this way, a microalgae multiproduct biorefinery would be feasible.

CONCLUSIONS

We studied the fractionation of lipids as part of a biorefinery approach to valorize all the microalgal compounds (pigments, proteins, carbohydrates, lipids) without discarding any cell component using polymers and IL solutions in aqueous two-phase systems (ATPS). The microalgal lipids poorly migrated to the aqueous phases of ATPS and were recovered (97% of the total fatty acids) in a third phase (interphase) formed between the top and bottom phases. This behavior was influenced by the amphiphilic nature of phospholipids and other particles present in the disrupted microalgae, forming a stable emulsion which did not allow the fatty acids to freely float and form an immiscible phase. On the other hand, a polypropylene glycol 400 (25% w/w) solution displaced 73% of lipids in an immiscible layer which was easy to recover. When combining this approach with an ATPS, most of the microalgae biomolecules (lipids, proteins, pigments, carbohydrates) could be fractionated in a three-step mild separation concept.

The recycling of the phase-forming components and the further purification of the fractions are not addressed in this article but are part of our future work.

ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acssuschemeng.1c06017.

Information on lipid composition of *N. oleoabundans* (Table S1), canola oil (Table S2), and polar lipids in yeast (Table S3). Figure S1: Influence of phase-forming components on extraction efficiency of total fatty acid from canola oil. Figure S2: Real appearance of partitioning of TAGs from canola oil and *N. oleoabundans* using PEG 400-Ch DHp ATPS. Figure S3: Influence of phase-forming components on extraction efficiency of TFA from *N. oleoabundans*, statistically explained in Table S4. Figure S4: UV−vis spectra of extracted pigments using different aqueous solutions. (PDF)

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Notes

The authors declare no competing financial interest.

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