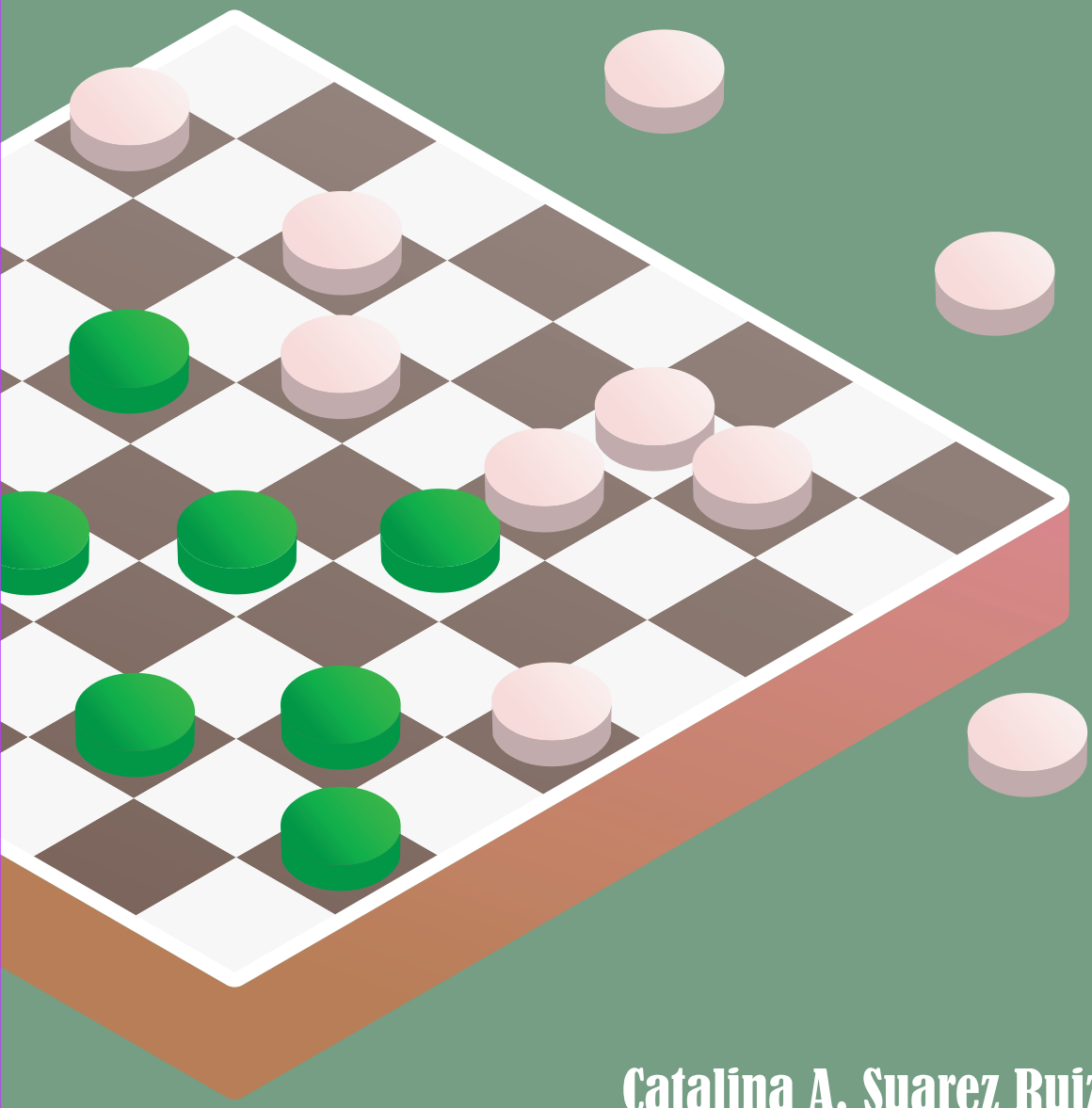
Aqueous two phase systems

for the purification of microalgae biomolecules



Catalina A. Suarez Ruiz

Propositions

1. The fractionation of the main microalgal biomolecules in a single step is not realistic. (this thesis)

2. Aqueous two phase systems are the one-stop shop for microalgae biorefinery. (this thesis)

3. Green chemistry is not necessarily sustainable.

4. Quality should be the primary parameter when optimizing microbial processes.

5. The benefits of recycling are defined by the country you live in.

6. Work stress-related diseases are ignored when not affordable.

Propositions belonging to the thesis, entitled

Aqueous two phase systems for the purification of microalgae biomolecules

Catalina Andrea Suarez Ruiz
Wageningen, 14 January 2022

Aqueous two phase systems for the purification of microalgae biomolecules

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Aqueous two phase systems for the purification of microalgae biomolecules

Catalina A. Suarez Ruiz

Thesis

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Prof. Dr A.P.J. Mol,

in the presence of the

Thesis Committee appointed by the Academic Board

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Chapter 1

General introduction and thesis outline

Biomass is a renewable source of diverse biomolecules essential to everyday life. Global challenges associated with climate change, food and feed supply and depletion of fossil-based resources can be solved with the correct use of renewable resources. Microalgae, for example, do not compete for arable land; can be cultivated in marine and fresh water and contains high amount of valuable biomolecules and bioactive compounds. These characteristics make microalgae one of the most promising bio-based sources (1).

Microalgae composition varies depending on the strain and cultivation conditions. In general, they contain lipids, pigments, carbohydrates, proteins and ash. *Neochloris oleoabundans and Tetraselmis suecica,* the microalgae strains used in this work, have been highlighted as a valuable resource for the production of industrially useful materials (2). *N. oloeabundans,* particularly, has high growth rates and the capacity to accumulate large quantities of lipids (3), and valuable pigments such as lutein (4). Furthermore, it can be cultivated in freshwater, brackish and saline media (5).

Microalgae production at a commercial scale is hindered by the high costs of cultivation and biorefinery. Currently, microalgae biorefineries focus on one-single product and specific markets (e.g. high value pigments and specialty chemicals), wasting and devaluating the remaining of the biomass (Figure 1.1). By valorizing all microalgal compounds the market value of the biomass can be increased and potentially outweigh the high costs of microalgae production (6).

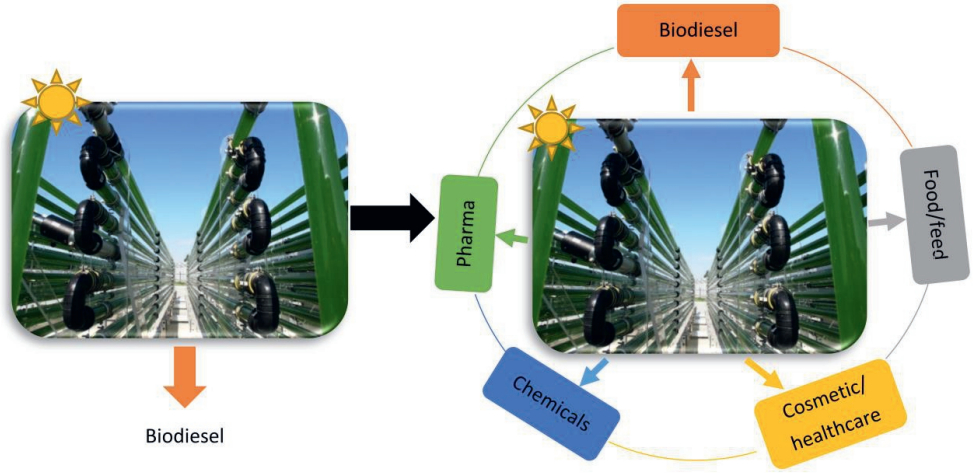

Figure 1.1. Transition from one-single product biorefinery to multi-product biorefinery.

Microalgae biorefinery

Microalgae biorefinery requires several unit operations to separate the biomass fractions (Figure 1.2). After cultivation, diluted cultures are first concentrated in the harvesting step using

technologies such as centrifugation, filtration, sedimentation and flocculation. The next step in the process is cell disruption or extraction and can involve mechanical methods (e.g. bead milling, high speed or pressure homogenization and pulse electric field) or non-mechanical methods (e.g. enzymes, chemical agents and thermolysis) (7). Volatile organic solvents and high temperatures are typically involved in the fractionation of certain microalgal products (e.g. lipids) (8). In addition to health and environmental concerns, the use of volatile organic solvents does not contribute with the development of a multiproduct biorefinery. Although they efficiently extract certain microalgae compounds (e.g. lipids), the native conformation and functionality of other compounds such as proteins can be affected (9). Therefore, the evaluation and development of mild and selective fractionation technologies is required to achieve a multiproduct biorefinery (10).

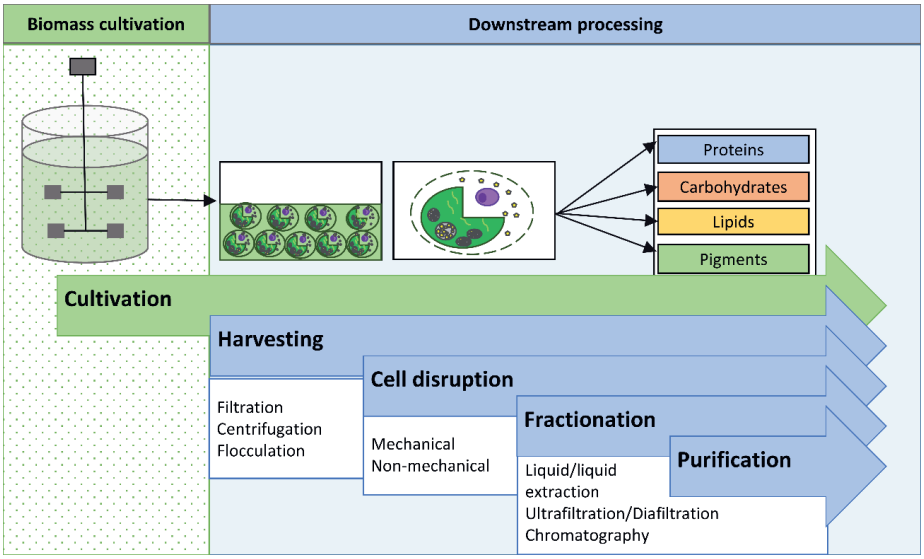

Figure 1.2. Overview of unit operations involved in microalgae biorefinery

Technologies based on aqueous solutions (e.g. aqueous two-phase systems) could help on the realization of a multiproduct biorefinery. Although these technologies have been mainly investigated for fractionation of biomolecules (e.g. proteins), they have shown applicability in the different stages of downstream processing. They are attractive platforms due to the mild conditions used for separation and their ability to integrate different unit operations in one-step (11).

Aqueous two-phase systems (ATPS)

Aqueous two-phase systems (ATPS), also called aqueous biphasic systems emerged as a promising technology to separate proteins and cells from other compounds in a mild manner (12). ATPS occurs when two hydrophilic solutes are mutually incompatible in aqueous solution above certain concentrations and conditions. These solutes compete for the interaction with

water and as a result, a phase separation occurs. Changes in temperature and pH can also trigger the phase separation of some aqueous solutions.
The high amount of water that characterizes these systems make them a mild and appropriate environment for several biomolecules, including proteins, value-added antibodies and enzymes (13). Aqueous two-phase systems are typically formed by mixing two polymers or one polymer and a salt. Besides polymers and salts, other hydrophilic compounds such as surfactants, alcohols, sugars, amino acids, ionic liquids and deep eutectic solvents can form ATPS.

ATPS are ternary systems (with three components) composed of water and two solutes that are soluble in water. To identify at which mixture points they can be used as liquid-liquid separation platforms their characterization is necessary. This is achieved by creating a phase diagram (Figure 1.3). The phase diagram delineates the potential working area for a particular ATPS under certain conditions such as pH, temperature and concentration of the phase forming components.

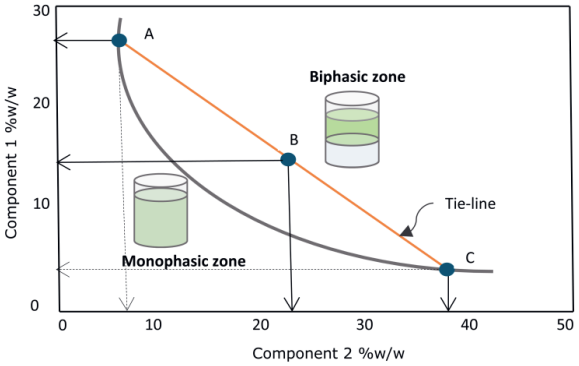

Figure 1.3 Phase diagram of hypothetical ATPS composed of aqueous solutions of component 1 and component 2.

The curve (called binodal curve) illustrates the point where the system transits from a monophasic system into a biphasic one. Each mixture point inside the biphasic zone has its own characteristic tie-line that connects two points on the curve (A and C). Once a point in the phase diagram has been chosen (e.g. B), the tie-lines can be followed to find the composition of the two phases formed. The higher point (A) of intersection with the binodal curve leads to the top phase composition, whereas the lower intersection (C) leads to the bottom phase composition. This means that along each tie-line, the final composition of each phase is the same. The volume ratio, however, does differ along the tie-line.

Since the pioneering work of Albertsson (14), **Aqueous two-phase systems (ATPS)** was used in biotechnological processes for the recovery and purification of many biological compounds. This liquid-liquid phase separation method was applied in processes dealing with proteins, nucleic acids, pharmaceuticals, pigments/colorants, and even cells and cell fragments, which were recovered for different purposes effectively and in a mild manner (15).
Since the introduction of ATPS in biotechnological processes, the development of other methods using aqueous solutions has emerged. **Aqueous micellar two-phase systems (AMTPS)/ Cloud point extraction**, for example, were developed as effective platforms to improve the recovery and purification yields biological compounds, while keeping their

biological activity (e.g. enzymes). The capacity of surfactants to form two phases by heating represent the basis of many technologies for the extraction and concentration of biological compounds (16).

Thermo-responsive polymers have been used as extraction, purification and as back extraction method in different biotechnological applications, including in the extraction and purification of proteins, DNA, antibody, enzymes and other products.(17). Polymers and surfactants that respond to different stimulus, such as temperature, pH, electromagnetic and magnetic fields are being developed for different fields, especially for biomedical applications (18, 19). In downstream processes, they became popular due to the possibility of adjusting their properties (polar/apolar, volatile/non-volatile, water miscible/water immiscible and protic/aprotic) enabling the same solvent to be used for several separation steps and facilitating the recycling of the solvent (20, 21).

Ionic liquids and IL-based ATPS

Rogers and coworkers (22) showed for the first time that aqueous solutions of hydrophilic ionic liquids and potassium phosphate form ATPS. Since this pioneering work, IL-based ATPS have been studied as an advantageous liquid-liquid platform for many separation processes. These new solvents have drastically increased the interest on the application of ATPS in various fields. Since the ions of an IL are tunable to have a strong or weak interaction with water, ILs can be designed for specific applications. Moreover, they show excellent solubility for a wide range of solutes and unusual miscibility with molecular liquids. ILs-based ATPS have a wider polarity range than traditional ATPS and therefore they are applied in the separation of a larger diversity of molecules enhancing selectivity and extraction yields. IL solutions have been named “green” solvents. One of their major advantages is their non-volatility compared to toxic volatile solvents traditionally used by industries. However, they still can be discharged into water ecosystems and therefore their toxicity, biodegradability and reuse needs to be evaluated for every application. Ionic liquids can form ATPS with organic buffers, amino acids, carbohydrates, organic salts, inorganic salts and polymers (23). IL-based ATPS can be highly dependent on temperature and pH (24). Thus, temperature and pH can be selected to fit specific requirements of the process, such as the stability of biomolecules. Moreover, these systems can integrate different unit operations in one (25).

DES-based ATPS

Although not widely studied, other class of solvents that can form ATPS are the deep eutectic solvents (DES). DES are mixtures of hydrogen bond acceptors and hydrogen bond donors. As ILs, DES have interesting solvent properties and exhibit high solubilities for a wide variety of solutes (26). These properties, in addition to their high biodegradability and easy synthesis make them very attractive for several separation processes. However, their “green” properties are often misinterpreted as in the case of ionic liquids. Some studies support that DES are more toxic than the congener ILs. Therefore, evaluation on their toxicity and biodegradability are needed before their application in industrial large-scale processes (27). DES-based ATPS have been used in the partitioning of different compounds, including proteins, amino acids, RNA, pigments, phenolic compounds and alkaloids (28-31). It was demonstrated, however, that ATPS

composed of DES can form quaternary systems instead of ternary systems, because the integrity of the solvent is destroyed in the ATPS. The nonstoichiometric partition of the DES components between the phases and the presence of volatile acids makes the system more complex and a separation process to recycle the phase formers is more difficult to design (32)

In addition, **Ionic liquid-based three phase partitioning (TTP)** and **aqueous multiphase systems (MUPs)**, appeared as promising approaches for the isolation of different compounds (two or more) present in complex mixtures, allowing their simultaneous separation amongst the different phases in a single step.(33, 34).

The ability to partition a wide plethora of compounds, along with the advances in material science and "green" solvent development, have allowed the use of technologies mediated by aqueous solutions in the different stages of microalgae downstream processing. From cell harvesting and disruption until the extraction and purification of multiple cell components. This thesis focuses on the integration and simplification of multi-product microalgae biorefineries using technologies based on aqueous solutions (mainly ATPS).

Thesis aim and outline

The aim of this thesis is to evaluate aqueous two phase systems as an alternative technology in microalgae biorefineries for the extraction and fractionation of multiple microalgae compounds. This is a novel approach envisaging an integrated and simple multiproduct biorefinery, where most of the microalgal components (proteins, pigments, carbohydrates and lipids) are valorized.

In **chapter 2,** based on a literature review and the screening of several ILs classes (different cations and anions), two hydrophilic ILs were selected considering their interaction with Rubisco (protein present in microalgae) in addition to their ability to form ATPS with organic salt/inorganic salts and polymers. The systems (PEG 400-Potassium citrate), (Iolilyte 221PG-Potassium citrate) and (PEG 400- Ch DHp) were first characterized to delineate their potential work area and the influence of different parameters was evaluated. Rubisco was used as target molecule to evaluate the performance of the three systems and the influence of TLL, pH and type of ATPS on the partitioning and integrity of the protein.

In **chapter 3,** we studied the equilibrium of an IL-based ATPS and the partitioning of crude protein extracts obtained from two green microalgae: *Neochloris oleoabundans* and *Tetraselmis suecica*. In addition, the Non Random Two Liquids (NRTL) model is used to describe equilibrium and partition coefficients. The partitioning of carbohydrates was also determined to evaluate the selectivity of the fractionation method.

In **chapter 4**, *Neochloris oleoabundans* cultivated under saline and fresh water conditions was used to study the fractionation of pigments and proteins by using one conventional ATPS (polymer/salt) and two IL-based ATPSs. Mild and biocompatible phase forming components were selected based on previous chapters. The influence of the phase forming components on the partitioning selectivity and biomolecules structure was also addressed in this chapter.

In **chapter 5,** we aimed to understand the partitioning of microalgal carbohydrates (free sugars such as glucose as well as starch) using aqueous two-phase systems, and its integration in a multi-product biorefinery concept. Model molecules (D-Glucose and starch from maize) as well as disrupted *N. oleoabundans* were used to evaluate the performance of the systems.

In **chapter 6,** the partitioning behavior of microalgae lipids in the ATPS previously selected was studied. The partitioning of commercial canola oil (representing TAGs) and partially purified yeast polar lipids (representing PL) was initially used to compare their behaviour with lipids from disrupted *N. oleoabundans*. 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 based on aqueous two-phase solutions.

In **chapter 7,** due to its advantages (from previous chapters), cholinium-based ATPS was selected to develop a multistep fractionation process of multiple microalgae biomolecules. First, the most suitable IL was selected and the most selective system was characterized based on the phase diagram. The partitioning behavior of microalgae pigments, proteins, lipids and carbohydrates on the system selected was studied. Finally, a multistep fractionation process was designed with the purpose of increasing the recovery efficiency of multiple 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.

In **chapter 8,** considering their advantages, different polymeric-based systems comprising PEG 8000 + NaPA 8000 + electrolytes were characterized and investigated on the fractionation of the microalgal biomolecules. The electrolytes considered (also used as disruption agents) were selected based on their ability to disrupt the cells and to extract the biomolecules. Then, the ability of polymeric ABS to separate proteins from carbohydrates, and pigments (lutein and chlorophylls) was tested. After the appropriate electrolyte selection and operational optimization, an integrated process including cell disruption, extraction and purification of the biomolecules and recycling of the main solvents is successfully proposed.

In the general discussion (**chapter 9**), we discussed the opportunities and challenges for the integration and simplification of multiproduct microalgae biorefineries mediated by aqueous solutions such as ATPS.

Chapter 2

Rubisco separation using biocompatible aqueous two-phase systems

Published as:

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Abstract

Mild and efficient separation processes have to be developed to convert microalgal biomass into high valuable products. Aqueous two-phase system (ATPS) was adopted as a new approach in microalgae to separate hydrophilic from hydrophobic components. In this work, three biocompatible ATPSs polyethylene glycol (PEG) 400-Potassium citrate, Iolilyte 221PG-potassium citrate and PEG 400- Cholinium dihydrogen phosphate ATPS were selected based on their interaction with Ribulose-1,5-biphosphate carboxylase/oxygenase (Rubisco), a protein predominantly present in microalgae and used as ingredient in human and animal food. Binodal curves were constructed for each system and the parameters influencing phase formation were investigated. Iolilyte 221PG-potassium citrate has a stronger ability to form ATPS compared with the PEG-based systems. This stronger ability was attributed to hydrophobic and electrostatic interactions between the phase-forming components. After characterization, we investigated the performance of the ATPSs in the partitioning of Rubisco. In this study, the effect of the tie-line length (TLL), pH and type of phase-forming components on Rubisco extraction efficiency (%) was analysed. In a single step, the appropriate parameters lead to extraction efficiencies between 80-100%. Additionally, stability studies were performed to see if ATPS retain the native protein structure. Iolilyte 221PG-Citrate was found to be the most efficient ATPS in Rubisco separation. However, stability studies indicated that PEG-based ATPSs have a better performance in retaining the Rubisco integrity.

Keywords: Microalgae, proteins, ionic liquids, aqueous two phase systems, separation, Rubisco

Introduction

Microalgae can accumulate up to 50-70% oil; 60% protein and 60% carbohydrates under different conditions (35), making it excellent candidates to supply sufficient energy and raw materials without damaging the environment. In the interest of sustainability and economic competition with other sources, a microalgae biorefinery approach should be used to valorize all the compounds inside the cell instead of one specific compound (36). Hence mild techniques, able to break the cells and separate valuable biomolecules from cell debris need to be developed. Furthermore, such techniques need to be economical, scalable and low in energy consumption (37).

Aqueous two phase systems (ATPS) as partition technique may be used to separate biological materials from proteins to cells (38). The technique consists of a mixture of two polymers, one polymer and a salt, or two salts that in an aqueous medium at a certain concentration separate into two phases. These systems provide a mild environment for biomolecules such as proteins due to the high quantity of water they contain and their low interfacial tension (39). The use of traditional phase-forming components (salts/polymers) has been successfully used to separate proteins from different sources (40-42). Traditional systems (salts/polymers) have further evolved with the use of new green solvents named "Ionic liquids (IL)". These solvents are entirely composed of ions and are fluid around or below 100°C. They offer many advantages in green processes, but the interest in the ILs for ATPS lies mainly in their design flexibility. Furthermore, ILs have shown to enhance the extraction process and optimize selectivity and substrate solubility, overcoming the limited polarity range of traditional polymer based systems (43).

Yu et al. 2007 (44) were the first to demonstrate the extraction of proteins from biological fluids by using 1-butyl-3-methylimidazolium chloride (BmimCl) and K_2HPO_4 without altering the protein's natural properties. Subsequently, many studies focusing on protein extraction have used imidazolium based ILs (23, 45, 46). These ILs present certain constraints due to their strong alkaline or acidic character (47, 48). Therefore, many recent studies on ionic liquid-based ATPS have explored more biodegradable and environmentally friendly ionic liquids. Their buffering capacities have also been explored to ensure a mild medium to proteins and enzymes (49). Different phosphonium and ammonium based ILs for protein extraction were explored (49-52) with promising results. Ammonium based ILs in combination with inorganic salts were used by (53), demonstrating their potential for the biocompatible extraction of catalytically active enzymes. On the other hand, more recently the stability of Rubisco, BSA and IgG1 in aqueous solutions of two ionic liquids: Iolilyte 221PG and Cyphos 108 was investigated (54). This stability study indicated that high IL concentrations affect protein stability. Cholinium-based ionic liquids, a novel class of ILs with buffering characteristics, were proposed for extraction purposes. Some studies demonstrated that this class of ionic liquids can retain the protein structure and enzyme function (55-60).
To design an environmental and cost effective separation process for microalgae components, we investigated a novel approach using ATPS. An exhaustive literature review and screening of different chemicals was done to select the phase forming components (information reported

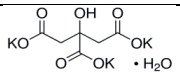
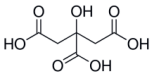
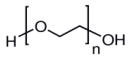
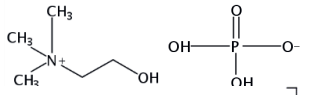
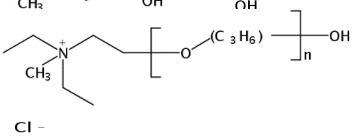
in the supplementary material). We selected two ionic liquids based on their mild interaction with proteins: Iolilyte 221PG and Cholinium dihydrogen phosphate (Ch DHp). Biocompatible components: Potassium citrate and PEG 400 were selected to replace commonly used inorganic salts. Finally, the systems are: (PEG 400-Potassium citrate), (Iolilyte 221PG-Potassium citrate) and (PEG 400- Ch DHp). These systems were first characterized to delineate their potential work area and the influence of different parameters was evaluated. Ribulose-1,5-biphosphate carboxylase/oxygenase (Rubisco) was used as target molecule to evaluate the performance of the three systems. The influence of TLL, pH and type of ATPS on the partitioning of the protein was investigated.

Experimental

Materials

Potassium citrate tribasic monohydrate, polyethylene glycol (PEG) 400 Tris (hydroxymethyl) amino methane and hydrochloric acid were purchased from Sigma-Aldrich. Citric acid was obtained from Merck. The ionic liquids Iolilyte 221PG, >95% and Choline dihydrogen phosphate, >98% were obtained from Iolitec. Table 2.1 presents the most relevant chemicals used to form the ATPS and their structure. The protein used for the research D-Ribulose 1,5-diphosphate oxygenase/carboxylase from spinach (Rubisco) was obtained from Sigma-Aldrich.

Table 2.1 Chemical structure and purity of components used to form the ATPSs. [a]N.A: data not available

Material	Purity	Chemical structure
Potassium citrate tribasic monohydrate	≥99%	
Citric acid	≥99.5%	
Polyethylene glycol 400 (PEG 400)	N.A[a]	
Cholinium dihydrogen phosphate (Ch DHp)	≥98%	
Iolylite 221PG	≥95%	

Methods

Characterization of the Aqueous two phase systems

The ionic liquid-based ATPSs (Iolilyte 221PG-Potassium citrate) and (PEG 400-Ch DHp) were selected after a strict screening based on protein stability. The ATPS (PEG 400-Potassium citrate) was selected to compare the IL-based ATPS with a more conventional system. Each ATPS was characterized creating its phase diagram which presents a binodal curve and four tie-lines. Two methods were used to obtain the binodal curves experimental data: the cloud point method and the titration method as described (61, 62). The concentrations of the phase-forming components was calculated based on weight quantification with an uncertainty of $\pm\mathbf{10^{-5}g}$. Binodal curves were constructed for each system at 4°C, 22°C and 40°C. The conductivity in each phase was measured to identify the main component.

The tie-lines (TLs) of each phase diagram were determined according to the gravimetric method proposed by Merchuk et al.1998 (63). A mixture was selected, which was at the tie-line. This mixture was prepared gravimetrically $\pm 10^{-4} g$, vigorously stirred and left to equilibrate until the phases completely separated. The phases were carefully separated with a glass Pasteur pipette and their weight was recorded. Eventually, the lever-arm rule was used to calculate each tie-line(63). To calculate the tie-line length, equation (2.1) was used:

$$TLL = \sqrt{(X_T - X_B)^2 + (Y_T - Y_B)^2} \qquad (2.1)$$

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

Rubisco partitioning: Partition coefficient and extraction efficiencies, effect of tie-lines and effect of pH

After the named systems were characterized, the partition studies were completed with a model protein (Rubisco) that was selected for being the most common biomarker protein present in microalgae and an interesting food ingredient. The total concentration of Rubisco in the mixture was 0.3 mg/mL. The mixtures were gravimetrically ($10^{-4} g$) prepared by using the four tie-lines reported with a volume ratio (V_r) between top and bottom phase of one. Blanks without protein were made to quantify accurately the protein. The mixtures were stirred for 10 minutes in a rotatory shaker and incubated at room temperature overnight to ensure phase separation. Each phase volume was then recorded and carefully separated for further analysis.

Rubisco distribution between the two phases was described by the partition coefficient Kp which in this context is the ratio of protein concentrations between top and bottom phase (Eq. 2.2). Rubisco percentage extraction efficiency $EE_{RUBISCO}\%$ expresses the ratio of protein amount between the top phase and the total mixture (Eq. 2.3).

$$Kp = \frac{C_{TOP}}{C_{BOTTOM}} \qquad (2.2)$$

$$EE_{RUBISCO}\% = \frac{C_{TOP} * V}{C_{BOTTOM} * V + C_{TOP} * V} \qquad (2.3)$$

Protein was quantified at 280 nm by using a Tecan infinite M200 plate reader. A calibration curve was determined for Rubisco showing a linear correlation. To accurately quantify protein, ATPS without protein was used as blank. Every sample was made in duplicate.

ATPS were prepared at different pH to study its effect in protein partitioning. The system pH was adjusted changing the salt ratio (Potassium citrate/Citric acid). In the case of PEG 400-Ch DHp the pH was adjusted using 10 M NAOH.

Stability studies

Size exclusion chromatography (SE-HPLC) and PAGE electrophoresis were used to analyze the effect of the two ionic liquids on the protein conformation. Top phase samples were analyzed after separation. Other experiments were performed using 2mg/mL of Rubisco stock solution and was then mixed with ILs at concentrations from 10-40%w/w.

Size exclusion chromatography: SE-HPLC was performed on Shimadzu UPLC using an Agilent Bio SEC-3, 3µ particle size, 300 A, 7.8 x300mm. 0.1M sodium phosphate buffer pH 7 and 0.3M sodium chloride (mobile phase) was run isocratically with a flow rate of 1mL/min during 25 min. The protein was detected at 280nm keeping a constant temperature of 25 °C.

Native-PAGE: The samples were diluted with native sample buffer (1:2). After mixing the samples were applied on a 4-20% Criterion TGX (Tris-Glycine eXtended) precast gel and run in 10 X Tris glycine native buffer at 125 V for 75 min. Native gels were stained with Bio-safe coomassie and with the Pierce silver stain kit. The precast gels, buffers and Bio-safe coomassie blue were procured from Bio-Rad. The Pierce silver stain kit was obtained from Thermofisher.

Results and Discussion:

Characterization of ATPS

Phase equilibrium data is required to understand the thermodynamic behaviour of the ATPSs and can be provided by the phase diagrams. These diagrams delineate the potential working area for a particular aqueous two-phase system (ATPS). The phase diagram contains a binodal curve and tie-lines. The binodal curve divides the phase diagram into two zones: below the curve is the one-phase zone and above the curve is the two-phase zone. The larger the two-phase zone is, the more easily the components can form ATPS. In other words, the closer the curve to the origin, the more easily the components can form ATPS. Figure 1 shows the three binodal curves obtained where component X is the compound that is enriched in the lower phase and component Y is the compound that is enriched in the upper phase. When compared, these curves indicated that the order in ability to form ATPS is as follows: Iolilyte 221PG-Citrate> PEG 400-Citrate > PEG 400- Ch DHp. The binodal curves fitted parameters, standard deviations (σ) and regression coefficients (R^2) are reported in the supplementary material.

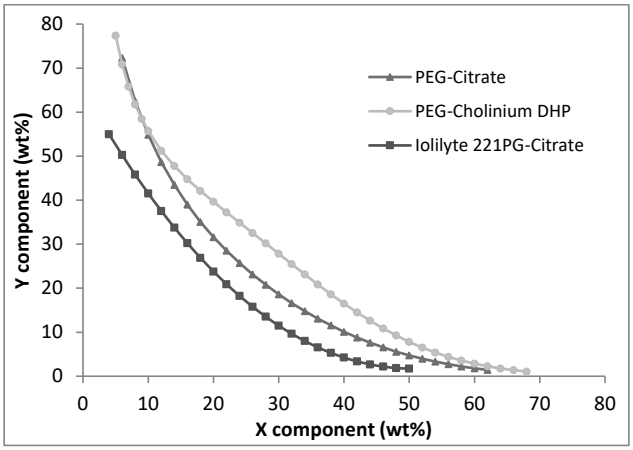


Figure 2.1 Binodal curves of ATPS at 25°C: ■, Iolilyte 221PG-Citrate; ▲, PEG400-Citrate; ●, PEG400-Ch DHp.

In the present study three different aqueous two phase systems (ATPS) were characterized to delineate their potential working area and to understand interactions behind phase separation. Phase separation depends on the type of salt involved and concentration, polymer molecular weight and concentration, phase volume ratio and equilibrium characteristics. Conventional polymer-based ATPS consist of two incompatible polymers or a polymer and a salting-out inducing salt. It is known that increasing the molecular weight of PEG also increases the working area of the ATPS (61). In other words, if the working area of the ATPS is increased, less PEG and salt are required for phase separation. PEG molecular weight is directly related to PEG hydrophobicity (64, 65). Thus, higher molecular weight PEGs are more hydrophobic and have as a consequence higher phase-forming ability. Comparing our two ATPS consisting of citrate salt (Iolilyte 221PG-citrate and PEG 400-citrate), Iolilyte 221PG (n=5-15) has a higher phase-forming ability than PEG 400 (n=9) due to its higher molecular weight.

The effect of a salt on the miscibility of a solute in an aqueous solution (salting-out effect) has received more attention in literature as an explanation for phase-forming ability. The type of salt affects phase separation depending on its salting out ability (66, 67). This salting out effect seems to be the basis of phase formation in both ATPS: Ionic liquid-salt and Polymer-salt, this effect is correlated to the hydration strength of the salt (23). In our study, the top phase corresponds to PEG-rich phase or ionic liquid-rich phase while the bottom phase is mainly composed of citrate salt. The addition of high charge density salts to aqueous solutions composed of certain ionic liquids leads to phase separation (22). This is due to a preferential hydration of the high charge density salt over the ionic liquid leading therefore to the exclusion of the ionic liquid to the ionic liquid-rich phase. In agreement with this, citrate salt has a higher effect (salting-out) over Iolilyte 221PG (more hydrophobic) than over PEG 400.

In contrast, PEG 400-Ch DHp ATPS is formed by a polymer and an ionic liquid, where the top phase is PEG-rich and the bottom phase is Ch DHp-rich. The phase forming separation of this system is more complex to explain because both components significantly contribute to phase

separation (68). Phase-forming ability in our system (PEG 400-Ch DHp) is governed mainly by the specific affinity of the ionic liquid for water. Higher affinity leads to a higher phase-forming ability. This is in agreement with PEG-salt ATPS, in which the ions with higher charge density are more able to create ion-water complexes and more repulsive interactions with PEG (69). Comparing our two ATPS using PEG 400 (Figure 2.1), both curves start and end in similar points, but the middle part of the PEG400-Ch DHp ATPS becomes linear, while PEG400-Citrate system remains curved. This shows that the phase-forming ability of PEG 400-Ch DHp ATPS depends on the amount of phase-forming components added. This was also observed with other polymer/cholinium-based ionic liquids ATPS (69, 70).

Effect of temperature on the ATPS formation

We studied the effect of temperature on the three systems by constructing the binodal curves at three different temperatures: 4, 25 and 40°C (Figure 2.2). Temperature seems to have more influence on the polymer-salt (PEG 400-Citrate) ATPS than on the ionic liquid based-ATPS (Iolilyte 221PG-Citrate, PEG400-Ch DHp). However, similarities were found in the two polymer (PEG 400) systems, where the binodal curves are closer to the origin at higher temperature (40°C). Hence, the increase of temperature enhances the phase separation of the system. On the other hand, ionic liquid-salt ATPS shows the opposite effect: lower temperature (4°C) leads to better phase separation.

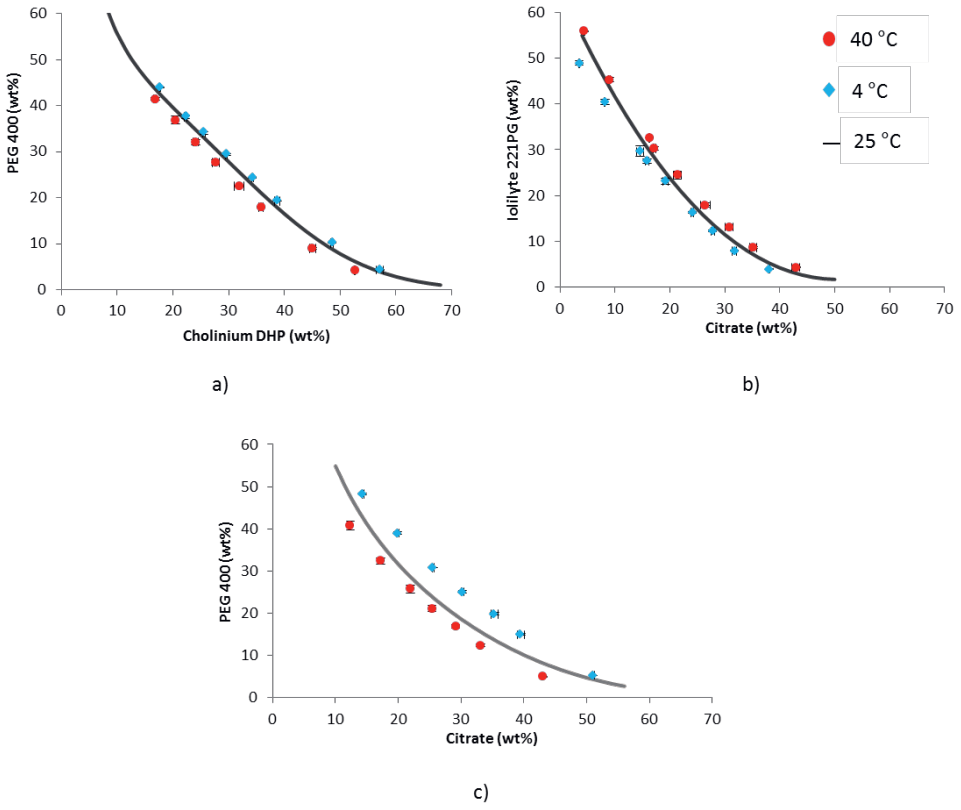


Figure 2.2. Effect of temperature on the binodal curves of the ATPS: a) PEG400-Ch DHp; b) Iolilyte 221PG-Citrate; c) PEG400-Citrate; ●, 40°C; ▬, 25°C; ◆, 4°C.

In PEG 400-citrate and PEG 400-Ch DHp ATPSs increasing temperature enhance phase-separation ability. This is in agreement with other conventional polymer-salt ATPSs and polymer-ionic liquid ATPSs previously studied (69-71). Both hydrogen-bonding and hydrophobic interactions between PEG and water are responsible for the temperature effect on phase-formation. In PEG 400-citrate and PEG 400-Ch DHp ATPS higher temperatures lead to a breakdown of the hydrogen bonds and consequently to easier phase-separation (72).

In the Iolilyte 221PG-citrate ATPS a decrease in the temperature is favorable for phase-separation, showing an opposite behavior than the other two ATPS studied. The interactions between hydrophilic ionic liquids and water are low with the presence of a salting out salt (citrate). Based on Figure 2.2, decreasing the temperature to 4°C, these interactions are lower. This leads to enhance the phase-forming ability. This behavior was found in other studies in which the effect of the temperature on phase-separation depends on the type of salt employed (53). Sadeghi et al. 2010 (71) discussed this effect based on salting-out coefficient of the salt, showing that by increasing the temperature, the salting out coefficient decreases, resulting in a better phase-formation.

Based on these results, temperature is an important parameter to understand the driving forces of phase-separation. However, looking towards the design of an effective separation process IL-based ATPS did not show an important shift in the binodal curves at different temperatures. Rubisco extraction studies were performed at room temperature (no need of heating or cooling).

Tie-lines

Figure 2.3 illustrates the binodal curve and tie-lines at 25°C of each system. Four tie-lines were determined for each system to evaluate the effect of the ATPS composition on the extraction studies. The tie-line determines the two phase composition in equilibrium. Once this is determined, we also obtained the tie-line length (TLL), an important parameter that represents the composition and thermodynamic difference of the two phases. The detailed weight fraction data and respective correlations, tie-lines, slope of the tie-line and tie-line lengths are provided in the supplementary material.

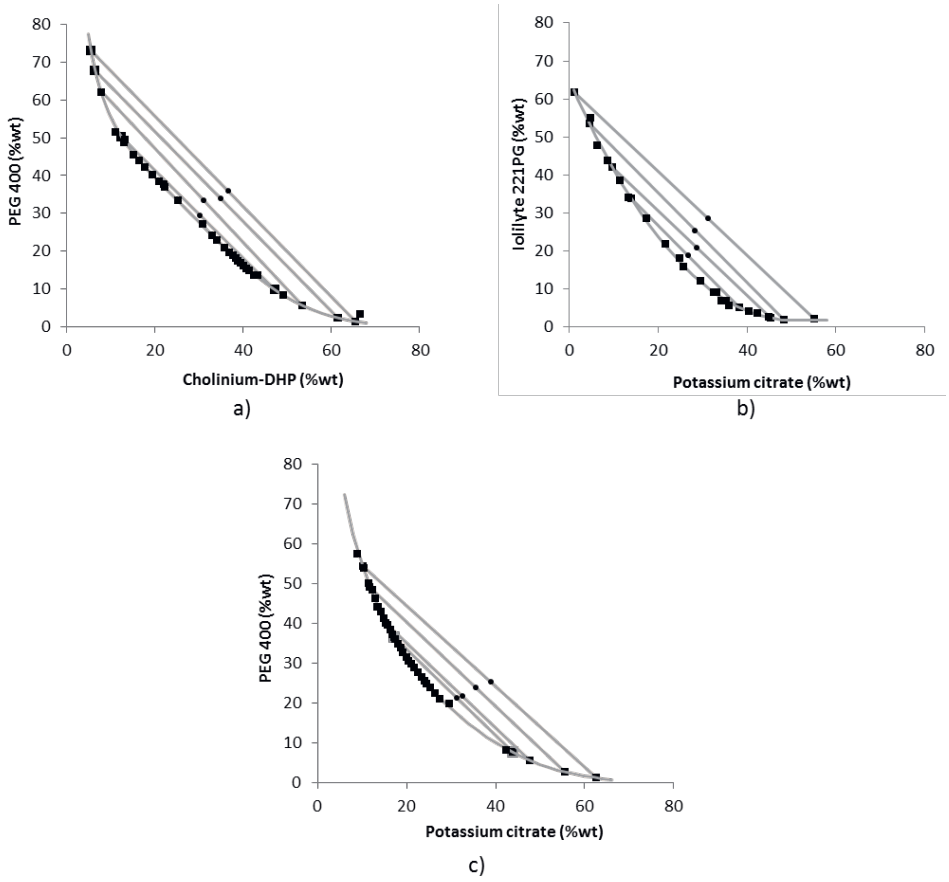


Figure 2.3 Phase diagram of the ATPS: a) PEG400-Cholinium DHp; b) Iolilyte 221PG-Citrate; c) PEG400-Citrate;♠, binodal curve; ●, total composition

Extraction of Rubisco in ATPS

Rubisco was selected as a model protein to evaluate the performance of three ATPS in a microalgae biorefinery framework. A conventional ATPS was selected to compare with two IL-based ATPS at 25°C. Table 2.2 shows the effect of the tie-line length on the Rubisco partition coefficient and extraction efficiency percentage in the three ATPS. The partition coefficient of Rubisco increases with the TLL and this effect seems to be consistent in the three systems. Increasing the salt concentration from ~30%wt to ~40%wt in the three systems will enhance the salting out effect, resulting in higher exclusion of the protein to the opposite phase.

Partition coefficient values demonstrate the preference of Rubisco for the top phase in the three cases. The best partitioning was obtained by the ionic liquid based-ATPS (Iolilyte 221PG-Citrate) with a maximal extraction efficiency of 98.8% by a single extraction step.

Table 2.2 Effect of tie-line length (TLL) on Rubisco partition at 25°C. TLL is described in Eq. 2.1. $\boldsymbol{Kp}$ is the Rubisco partition coefficient (Eq.2.2) and $\boldsymbol{EE_{RUBISCO}}\%$ stands for Rubisco percentage extraction efficiency (Eq.2.3).

ATPS	TLL	$\boldsymbol{Kp}$	$\boldsymbol{EE_{RUBISCO}}\%$
PEG 400-Citrate	39.30	7.00	88.3%
	47.25	11.28	90.7%
	63.75	17.19	94.1%
	74.63	21.50	96.6%
Iolilyte 221PG-Citrate	37.53	38.42	91.8%
	53.23	27.54	93.3%
	67.70	32.32	96.0%
	80.30	93.80	98.8%
PEG 400-Cholinium DHp	53.13	2.59	72.5%
	72.92	2.81	72.1%
	85.33	3.21	77.6%
	93.28	3.63	79.6%

The partitioning behavior of proteins is influenced by their physicochemical properties and their interactions with the system components (Polymer, salt, ionic liquid and water). Protein properties like hydrophobicity, net charge, electrostatic forces, size and solubility have been studied to understand the main driving forces for the partitioning behavior of proteins in ATPS (73, 74). Several authors agree that hydrophobic interactions between the protein and the phase-forming components are one of the main driving forces in the partitioning behavior (45, 75). These interactions are responsible for the protein partition preference for the top phase. In contrary to this, Dreyer et al.2009 found that the main driving force in protein partitioning is the electrostatic interactions between the amino acids of the protein surface and the ionic liquid cation (43). We conclude that the higher Rubisco extraction efficiency on Iolilyte 221PG-Citrate system compared to the other ATPS is a result of higher salting out in that system and higher hydrophobicity of the ionic liquid. Furthermore, electrostatic interactions between the

IL cation and negatively charged amino acid residues at the surface of Rubisco influence positively the extraction efficiency.

The maximal extraction efficiency using PEG 400-Ch DHp was 79.6 %. The protein is partitioned preferentially to the PEG-rich phase and only 20.4% of protein go to the IL-rich phase. This preference is a consequence of the salting-out effect of the cholinium- based ionic liquid over Rubisco which leads to its migration to the top phase (the more hydrophobic phase). This is in agreement with the partition preference of the protein in the PEG-salt and Ionic liquid-salt ATPSs previously discussed. On the contrary, Li et al.(55) and Quental et al. (58) found preferential partitioning of BSA protein for the IL-rich phase (less hydrophobic phase) when using PPG-cholinium-based ILs ATPS. The type of polymer influences the partition preference of the proteins. Salabat et al. 2010 (76) showed that the recovery of the proteins in the ionic liquid-rich phase was higher than in the polymer-rich phase when using PPG than when using PEG (76). This difference seems to be related with the higher hydrophobicity of PPG compared with PEG. In addition, the protein structure and its hydrophobic interactions with the ATPS components affect notably the partitioning preference (45, 75). Protein partitioning is then the result of a more complex phenomenon, where hydrogen bonding and molecular interactions between the protein and ATPS components are the main driving forces.

Effect of the pH

pH is an important parameter that influences protein charge and conformation. Three different pH conditions were studied 6, 7 and 8 to look for the optimal conditions for Rubisco partitioning in ATPS. The pH studied are higher than Rubisco isoelectric point (pI), which is between 5.5 and 5.7. This means that the protein has a net negative charge in the ATPSs. Rubisco partitioned preferentially to the top phase. This can be explained based on the interactions between the protein that is negatively charged and the compound that is enriched in the upper phase (PEG 400 and Iolilyte 221PG). Figure 2.4 shows the effect of pH on Rubisco extraction efficiency in the three systems studied. A system with pH 8 drives more protein to the top phase, showing a higher extraction efficiency. This increase is easier to observe in the PEG-based ATPSs than in the Iolilyte 221PG-citrate system, which efficiency is already close to 100% at pH 6. The effect of the system pH on Rubisco partitioning indicates that negatively charged characteristics of the protein contribute to an enrichment in the upper phase. This can be explain by stronger attraction between the charged exposed groups of the proteins and the phase forming compounds when the protein became more negatively charged (higher pH). Other investigations using PEG-salt and Iolilyte 221PG-salt indicated a similar behavior (43, 77) .

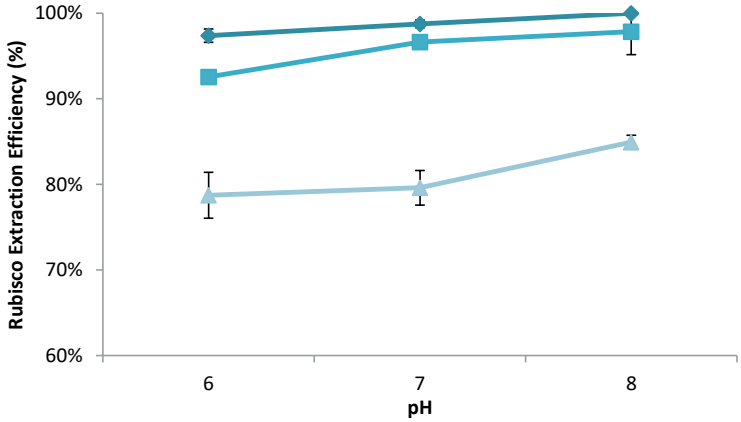


Figure 2.4 Effect of pH on Rubisco extraction efficiency in:◆ ,Iolilyte 221PG-Citrate;■ , PEG400-Citrate;▲, PEG400-Ch DHp

Stability studies

Native-PAGE was used to evaluate the conformation of Rubisco after separation in ATPS. The gel in Figure 2.5a) shows the top phase of the three systems at optimal conditions, same phase-forming components composition and pH 8. Figure 2.5b) shows Rubisco interaction with PEG400-Ch DHp at different pH conditions. Although Iolilyte 221PG-Citrate shows the best extraction efficiency compared with the other two systems, the band of Rubisco was very soft in the gel (well 2). Opposite to this, in PEG-based systems (PEG400-Citrate and PEG400-Ch DHp) Rubisco bands are more intense (well 1, 3). The native form of Rubisco is influenced by the pH of the ATPS (well 4, 5 and 6). This can be observed with the difference in the Rubisco band visibility when lowering the pH from 8 to 6. The visibility in the gel can also be affected by the low concentration of protein in the top phase when decreasing the pH of the system.

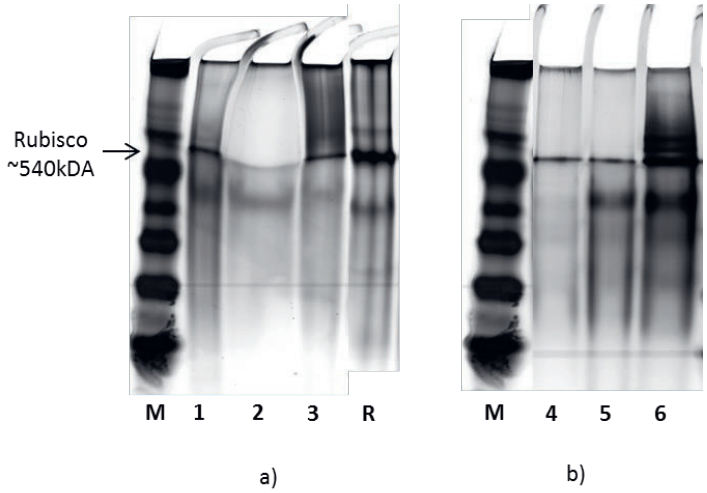


Figure 2.5. Native-PAGE of top phase after Rubisco separation in ATPSs. a)Three ATPS using the same conditions. Marker (M), PEG400-citrate pH 8 (1); Iolilyte 221PG-citrate pH 8 (2); PEG400-Ch DHph pH 8 (3); Rubisco (R). b) PEG 400-Ch DHP ATPS at three pH conditions. Marker (M); PEG400-Ch DHph pH 6 (4); PEG400-Ch DHph pH 7 (5); PEG400-Ch DHph pH 8 (6)

Choline dihydrogen phosphate (Ch DHp) has been identified as one of the most promising media for the stabilization of proteins and other biomolecules (58, 60, 78, 79). This protein stabilization can be related according the Hofmeister series, which describes the ability of anions to stabilize /destabilize the native state of proteins (80, 81). It is already known that Rubisco is more vulnerable to form aggregates in presence of ionic liquids than other proteins due to its subunits and size/complexity (54). Samples of the protein were prepared with increasing amount of ionic liquid (10-40w/w%) to evaluate the interaction of Rubisco with the two ionic liquids used: Iolilyte 221PG and Ch DHp. Size exclusion chromatography shows a decreasing of Rubisco signal when increasing the amount of Ch DHp (Figure 2.6a). However, comparing the two ionic liquids, Native-PAGE shows no big difference in the bands when increasing Ch DHp concentration. On the other hand, Iolilyte 221PG shows a clear decrease in the Rubisco band and increase in aggregates (well 8) (Figure 2.6b). This suggests that Iolilyte 221PG leads to denaturation of the protein at lower concentration (30% of IL) than Ch DHp. We conclude that PEG-based ATPSs (PEG400-citrate and PEG400-Ch DHp) keep the native form of Rubisco and do not destabilize Rubisco by formation of aggregates compared to Iolilyte 22PG-Citrate, which promotes formation of aggregates (54). The fact that Rubisco prefers to separate in the top phase in the PEG-based ATPSs is beneficial for the stability of the protein due to PEG which is the main component of that phase and stabilizes the protein.

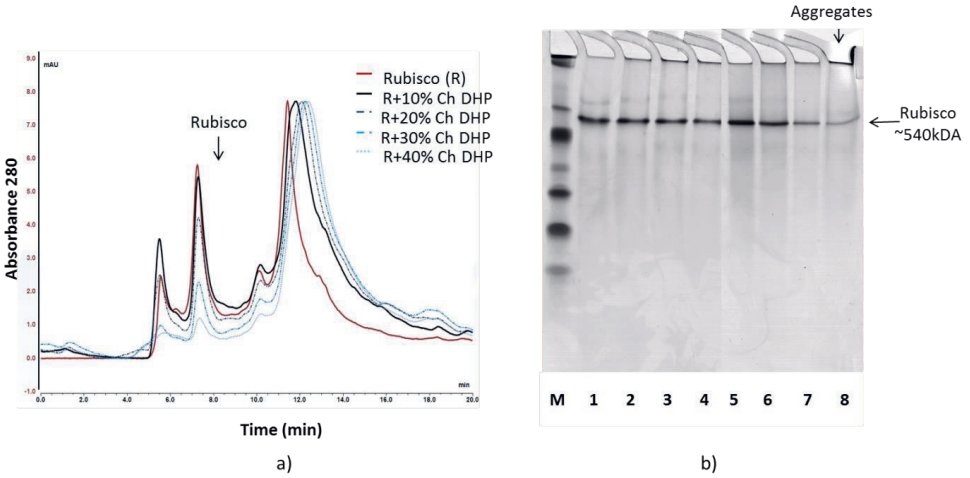


Figure 2.6 a) SEC-HPLC of Rubisco at increasing concentrations of Ch DHph. b) Native page: effect of increasing amount of IL on Rubisco native form: Marker (M); Rubisco +10%Ch DHp (1); Rubisco +20% Ch DHp (2); Rubisco +30% Ch DHp (3); Rubisco +40% Ch DHp (4) ; Rubisco +10%Iolilyte 221PG (5); 20%Iolilyte 221PG (6); 30%Iolilyte 221PG (7); 40%Iolilyte 221PG (8).

Conclusions

In this research, two biocompatible ionic liquid-based ATPSs were investigated and compared with a traditional PEG-salt ATPS. We selected biocompatible phase-forming components and ionic liquids suitable for protein separation. The potential of the ATPSs was demonstrated based on the characterization of the systems. Phase equilibrium data of the three systems were here reported for the first time and the parameters evaluated were useful to understand the phenomena behind each ATPS. Iolilyte 221PG-Citrate had the highest phase-forming ability and was the most efficient ATPS in Rubisco separation. This performance is related with the higher salting out effect of potassium citrate over Iolilyte 221PG and the higher hydrophobicity of the ionic liquid. In addition, electrostatic interactions between the IL and Rubisco positively influence the extraction efficiency. However, Iolilyte 221PG-citrate ATPS was not able to keep the protein in its native form at high concentration due to formation of aggregates.

Keeping the functionality of the proteins is very important in the development of an effective separation method for proteins. PEG400- citrate and PEG400-Ch DHp are good alternatives to replace the commonly used inorganic salts, which can cause environmental problems. These alternatives can replace also highly toxic ionic liquids. Additionally, these systems are able to keep the native conformation of the protein after separation. Rubisco prefers to separate into the polymer-rich (PEG400) phase, which is a nice environment for Rubisco. Cholinium-based ATPS could be very promising in the separation of microalgae components as well. This work reported a good start in the development of an effective and environmentally friendly separation process for microalgae components. In future studies the behavior of other microalgae components in ATPS will be investigated. Furthermore, techniques for recovery and reuse of the ILs need to be explored to design an economically feasible process.

Acknowledgements
The authors would like to thank Bert Vanwinsen student from Wageningen University for his contribution collecting some of the data of this work. This work is performed within the TKI AlgaePARC Biorefinery program with financial support from the Netherlands' Ministry of Economic Affairs in the framework of the TKI BioBased Economy under contract nr. TKIBE01009.

Supplementary material

Screening of Ionic liquids:

Table S2.1 Screening of ionic liquids based on formation of ATPS with PEG 400 and potassium citrate.

Ionic Liquid Names	**Formation of Aqueous two phase system**	
Ammonium-based ionic liquids	**Potassium citrate-Ionic liquid**	**PEG 400- Ionic liquid**
Ethylammonium nitrate [$EtNH_3$][NO_3]	NO	NO
Methyltrioctylammonium bis(trifluoromethylsulfonyl)imide [N1888][Tf_2N]	NO	NO
IoLiLyte 221PG (AMMOENG 110 ™)	YES	NO
Choline dihydrogen phosphate[Ch][DHp]	NO	YES
Choline bis(trifluoromethylsulfonyl)imide [Ch][Tf_2N]	NO	NO
Choline acetate [Ch][OAc]	NO	NO
Imidazolium-based ionic liquids		
1-Butyl-3-methylimidazolium bis(trifluoromethylsulfonyl)imide [Bmim][Tf_2N]	NO	NO
1-Butyl-3-methylimidazolium chloride [Bmim][Cl]	YES	NO
1-Butyl-3-methylimidazolium dicyanamide [Bmim][DCA]	YES	NO
1-Butyl-3-methylimidazolium tetrafluoroborate [Bmim][BF4]	YES	NO
1-Butyl-3-methylimidazolium dibutylphosphate [BMIM][DBP]	YES	NO
1-Butyl-3-methylimidazolium acetate Bmim[AcO]	NO	NO
1-Ethyl-3-methylimidazolium dibutylphosphate [EMIM][DBP]	YES	NO
Phosphonium-based ionic liquids		
Trihexyltetradecylphosphonium chloride [P14666][Cl]	NO	NO
Trihexyltetradecylphosphonium dicyanamide [P14666][DCA]	NO	NO
Trihexyltetradecylphosphonium bis(2,4,4-Trimethylpentyl)phosphinate [P14666][TMPP]	NO	NO
Triisobutylmethylphosphonium tosylate [Pi(444)1][Tos]	YES	NO
Triisobutylmethylphosphonium metyl sulfate [Pi(444)1][$MeSO_4$]	YES	NO
Tributylmethylphosphonium bis (trifluoromethylsulfonyl) imide [P4441][Tf_2N]	NO	NO

Screening of Ionic liquids based on the stability of the protein:

Experiments were performed using 2mg/ml of Rubisco stock solution. The protein was then mixed with each Ionic liquid at concentrations from 10-40%w/w. The analysis were performed using SEC-HPLC, Native-PAGE and enzymatic assay.

Table S2.2 Screening of ionic liquids based on protein stability after incubation with ionic liquids

Ionic Liquid Names	**Rubisco stability**	
Ammonium-based ionic liquids	**10-20%(w/w) IL**	**30-40%(w/w)IL**
IoLiLyte 221PG (AMMOENG 110 ™)	stable	~stable
Choline dihydrogen phosphate [Ch][DHp]	stable	stable
Imidazolium-based ionic liquids		
1-Butyl-3-methylimidazolium chloride [Bmim][Cl]	stable	no stable
1-Butyl-3-methylimidazolium dicyanamide [Bmim][DCA]	no stable	no stable
1-Butyl-3-methylimidazolium tetrafluoroborate [Bmim][BF4]	no stable	no stable
1-Butyl-3-methylimidazolium dibutylphosphate [BMIM][DBP]	stable	no stable
1-Ethyl-3-methylimidazolium dibutylphosphate [EMIM][DBP]	stable	no stable
Phosphonium-based ionic liquids		
Triisobutylmethylphosphonium tosylate [Pi(444)1][Tos]	no stable	no stable
Triisobutylmethylphosphonium metyl sulfate [Pi(444)1][$MeSO_4$]	no stable	no stable

Characterization of ATPS:

Table S2.3 Experimental binodal curve weight fraction data for PEG 400-Citrate ATPS at 25 ºC.

PEG 400 (wt%)	Citrate (wt%)	PEG 400 (wt%)	Citrate (wt%)	PEG 400 (wt%)	Citrate (wt%)
57.412	8.920	35.928	17.541	24.825	24.642
53.852	10.521	34.873	18.137	23.895	25.305
50.101	11.573	33.775	18.723	22.336	26.475
48.417	12.490	32.790	19.330	21.110	27.447
46.245	12.992	31.516	20.004	19.850	29.619
44.073	13.743	30.639	20.576	14.812	34.013
42.822	14.327	29.738	21.168	11.553	38.011
41.181	15.014	28.902	21.739	8.111	42.452
39.508	15.817	27.608	22.665	3.721	49.322
38.298	16.356	26.503	23.431		
37.080	16.913	25.592	24.025		

Table S2.4 Experimental binodal curve weight fraction data for Iolilyte 221PG-Citrate ATPS at 25 ºC.

Iolilyte 221PG (wt%)	Citrate (wt%)	Iolilyte 221PG (wt%)	Citrate (wt%)
54.927	4.812	8.958	32.65
47.694	6.463	9.096	33.298
43.602	8.758	6.777	34.309
38.346	11.432	6.864	35.308
34.072	13.314	5.622	36.076
28.567	17.569	4.169	40.506
21.701	21.722	3.593	42.364
18.136	25.024	2.573	44.988
15.779	25.881		
12.003	29.544		

Table S2.5 Experimental binodal curve weight fraction data for PEG 400-Ch DHP ATPS at 25 ºC.

PEG 400 (wt%)	[Ch][DHp] (wt%)	PEG 400 (wt%)	[Ch][DHp] (wt%)	PEG 400 (wt%)	[Ch][DHp] (wt%)
61.877	8.123	33.253	25.423	15.344	40.964
51.490	11.281	27.072	30.928	14.711	41.575
49.464	13.380	24.037	33.138	13.671	43.409
48.554	13.228	22.699	34.300	13.666	42.723
45.428	15.441	20.741	36.046	8.225	49.325
43.746	16.739	19.589	37.024	3.318	66.682
42.135	18.001	18.717	37.808		
40.133	19.594	17.968	38.446		
38.283	21.167	17.361	39.039		
37.692	22.324	16.793	39.588		
36.762	22.418	16.006	40.330		

To fit the experimental data, different empirical equations were explored (Equation. S2.1-S2.6). The best fit was then plotted.

$$Y = A + BX^{0.5} + CX \quad \text{(S2.1)}$$

$$Y = AX^3 + BX^2 + CX + D \quad \text{(S2.2)}$$

$$Y = A * \exp[BX^{0.5} + CX^3] \quad \text{(S2.3)}$$

$$Y = A * \exp\left[-\frac{X}{B}\right] + C * \exp\left[-\frac{X}{D}\right] + E \quad \text{(S2.4)}$$

$$Y = -\ln\left(V_{evX} * \frac{X}{M_X}\right) * \frac{M_Y}{V_{evX}} \quad \text{(S2.5)}$$

$$Y = \exp\left[A + BX^{0.5} + CX + DX^2\right] \quad \text{(S2.6)}$$

The tie-lines (TLs) of each phase diagram were determined according to the gravimetric method proposed by (*Merchuk et al*. 1998). Eventually, the lever-arm rule was used to calculate each tie line (Equations S2.7-S2.9). These equations correlate the phase composition

(X_T , Y_T , X_B , Y_B) with the overall system composition (X_M , Y_M). Where the subscripts T is top, B is bottom and M is mixture. α is named weight fraction.

$$Y_T = \left(\frac{Y_M}{\alpha}\right) - \left(\frac{1-\alpha}{\alpha}\right) Y_B \quad \text{(S2.7)}$$

$$X_T = \left(\frac{X_M}{\alpha}\right) - \left(\frac{1-\alpha}{\alpha}\right) X_B \quad \text{(S2.8)}$$

$$\alpha = \frac{weight\ of\ the\ top\ phase}{weight\ of\ the\ mixture} \quad \text{(S2.9)}$$

To solve the previous set, the empirical equations (S2.10-S2.11) were used. The equation form depends on the fitting of the binodal curve of each system.

$$Y_T = f(X_T) \quad \text{(S2.10)}$$

$$Y_B = f(X_B) \quad \text{(S2.11)}$$

To calculate each tie line length, the equation (S2.12) was used:

$$TLL = \sqrt{(X_T - X_B)^2 + (Y_T - Y_B)^2} \quad \text{(S2.12)}$$

Table S2.6 Correlation parameters adjusted to the experimental binodal data at 25 ºC with corresponding errors of the fitting (σ) and the correlation coefficients R^2

System	A	B	C	R^2	σ
PEG 400-Citrate	183.2922	-0.3789	$7.8148*10^4$	0.9984	0.48335287
Iolilyte 221PG-Citrate	98.481	-0.2716	$2.3642*10^{-5}$	0.9986	0.71510372
PEG 400-Ch DHP	$1.1145*10^2$	-0.2165	$8.3424*10^{-6}$	0.9995	0.81136675

Tie-lines data

Table S2.7 Tie line data: detailed weight fraction data and respective correlations, tie line length (TLL) and slope of the line

PEG 400-Citrate						
Tie line	**Y_T(wt %)**	**Y_B(wt %)**	**X_T(wt %)**	**X_B(wt %)**	**Tie Line Length (TLL)**	**Slope**
1	36.534	7.679	17.229	43.91	39.3	-1.08
2	40.105	5.67	15.474	47.831	47.251	-1.06
3	49.106	2.815	11.844	55.674	63.749	-1.06
4	54.285	1.321	10.173	62.753	74.632	-1.01
Iolilyte 221PG-Citrate						
Tie line	**Y_T(wt %)**	**Y_B(wt %)**	**X_T(wt %)**	**X_B(wt %)**	**Tie Line Length (TLL)**	**Slope**
1	33.613	5.054	14.095	38.45	37.534	-1.17
2	41.918	2.319	9.827	45.404	53.233	-1.11
3	53.518	1.83	4.604	48.326	67.7	-1.18
4	59.9	1.76	2.54	55.12	78.39	-1.11
PEG 400-Ch DHP						
Tie line	**Y_T(wt %)**	**Y_B(wt %)**	**X_T(wt %)**	**X_B(wt %)**	**Tie Line Length (TLL)**	**Slope**
1	50.065	9.799	12.632	47.301	53.134	-1.16
2	62.477	5.659	7.806	53.509	72.918	-1.24
3	67.69	2.393	6.594	61.527	85.33	-1.19
4	72.986	1.459	5.642	65.517	90.096	-1.22

Table S2.8 Composition of mixture prepared for protein partitioning and the corresponding α

PEG 400-Citrate			
Tie line	**X_M (wt %)**	**Y_M(wt %)**	**α**
1	31.292	21.325	0.473
2	32.626	21.851	0.470
3	35.723	23.887	0.455
4	39.055	25.192	0.451
Iolilyte 221PG-Citrate			
1	26.777	18.742	0.479
2	28.871	20.721	0.465
3	28.432	25.348	0.455
4	31.257	28.611	0.445
PEG 400-ChDHP			
1	30.413	29.412	0.487
2	31.275	33.301	0.487
3	35.016	33.905	0.483
4	36.759	35.813	0.480

Chapter 3

Fractionation of proteins and carbohydrates from crude microalgae extracts using an Ionic liquid based-Aqueous Two Phase System

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Abstract

Mild, simple and efficient recovery methods are required to obtain high-value microalgae proteins. As a promising extraction method, an Aqueous two phase system (ATPS) was used to partition proteins from crude microalgae extracts obtained from two green microalgae of industrial interest: *Neochloris oleoabundans* and *Tetraselmis suecica*. Furthermore, the Non-Random Two Liquids model (NRTL) was applied to describe both the phase diagram and the partition coefficient of total protein. It was observed that total protein preferentially concentrates in the top phase. Additionally, no significant effect on partition or extraction efficiency was noted at different tie lines. Experimental data indicate that proteins and sugars are selectively fractionated in top and bottom phases respectively. The model provided a good representation of the experimental data for the liquid-liquid equilibrium. Moreover, the model also led to a good representation of the partitioning data for two reference proteins, Rubisco and Bovine Serum Albumin (BSA), as well as for total protein from crude microalgae extracts.

Keywords. Crude Protein, partitioning, ionic liquid, phase diagram, tie lines.

Introduction

Due to their rich composition, microalgae are a potential source of biomolecules for food, feed, chemical and pharmaceutical products, of which proteins are of paramount industrial relevance. Microalgae can accumulate up to 60% protein under different cultivation conditions (35). Because of their sustainability, techno-functionality and broad range of applications, algae proteins have been in the spotlight of numerous studies (82). However, microalgae proteins are often present intracellularly or forming complexes with pigments and polysaccharides and thus, their recovery and purification still represents a challenge (83). Several processes have been developed for the extraction and fractionation of proteins from microalgae. pH-shifting, filtration and adsorption are commonly reported (84). However, such processes, are often characterized by low yields, poor selectivity and harsh conditions. Further research on alternative separation methods is therefore required.

Aqueous two-phase system (ATPS) is a liquid-liquid extraction method that has been presented as a mild, easily scalable, efficient and cost competitive technology for the recovery of a broad range of biomolecules (85). Although large scale applications are reported (13), its widespread implementation has been constrained by the poor understanding of the partitioning mechanism and by the selection of the phase forming components, in terms of sustainability, recyclability and costs. Ionic liquids (ILs) have gained significant attention in the last decades as phase forming components in ATPS due to their chemical versatility and physicochemical properties. They are non-flammable and non-volatile. Moreover, their physicochemical properties (e.g. polarity, viscosity, miscibility) can be tuned by manipulating their cations and anions, allowing the tailor-made design of extraction processes (86). Ionic liquid-based ATPS have been studied by several authors for the extraction of proteins (87). High extraction efficiencies and partition coefficients 3-4 times higher can be achieved in comparison with traditional polymer-salt systems (54).

Partitioning of proteins in IL-based ATPS is a complex phenomenon. It depends on several factors including type and concentration of phase-forming components, pH, temperature, ionic strength and chemical nature of the target molecule(s) (86). In the case of proteins, hydrophobicity, isoelectric point, molecular weight and conformation play a critical role (43). Significant progress has been made in the theoretical understanding of the underlying mechanisms of protein partitioning as well as phase equilibrium in ATPS. For the latter, several thermodynamic models have been successfully implemented for systems containing polymers (88), salts (89) and ILs (90). Thermodynamic models have also been used to describe and predict the partition coefficients of model proteins in ATPS; satisfactory estimations are reported for polymer/salt systems using an extension of the Pitzer's model (89) and multicomponent Wilson model (91). For polymer-polymer systems, modifications of the Pitzer's model (92), Flory Huggins theory (93) and UNIQUAC model (94), have been successfully implemented. A correct understanding and prediction of equilibrium and partitioning can lead to further developments in design, scale up and process optimization.

Despite the large number of publications in the field of ILs and protein extraction (44, 87), the application of ATPS for the extraction of microalgae proteins and in particular for crude

microalgae extracts is limited. The published research have centred mostly on extracting *C-phycocyanin* from *Spirulina* strains (95), *B*-phycoerythrin from *Porphyridium cruentum (96)* and proteins from *Chlorella pyrenoidosa* (97) and *Chlorella sorokiniana* (98). Combination of several disintegration-extraction methods have also been described. Lee and co-workers (99) extracted proteins from *Chlorella vulgaris* using ultrasound and IL-based buffers, proving that the IL aids in the disintegration process. This was also demonstrated by Orr et al.(100) for the extraction of lipids from wet microalgae.

In the present investigation, we study the equilibrium of an IL-based ATPS and the partitioning of crude protein extracts obtained from two green microalgae: *Neochloris oleoabundans* and *Tetraselmis suecica*. *N. oleoabundans* have been extensively investigated and it is considered a promising industrial strain due to its versatility, high growth rate, and biomass composition (101). *T. suecica* has been traditionally used in aquaculture (102) and recently it has been highlighted due to the techno-functional properties of its proteins (103). In addition, the Non Random Two Liquids (NRTL) model is used to describe equilibrium and partition coefficients. The NRTL model was selected because of its flexibility to describe systems of different chemical nature, including electrolyte solutions and IL (104), and because of its simplicity compared with models like UNIQUAC or UNIFAC (105). To our knowledge, this is the first attempt to describe the partitioning of crude microalgae proteins in ATPS containing ILs using thermodynamic models.

Experimental section

Materials.

The Ionic liquid Iolilyte 221PG (> 95 %) was purchased from Iolitec®. Citric acid monohydrate (> 99.0 %) was purchased from Merck Millipore®. Bovine serum albumin (BSA, > 96 %, 66.4 kDa), potassium citrate tribasic monohydrate (> 99.0 %) and D-Ribulose 1,5- diphosphate carboxylase (Rubisco, ~ 540 kDa), a partially purified protein from spinach, were purchased from Sigma-Aldrich®. Potassium citrate buffer stock solution was prepared by weighing and mixing 60 % (w/w) citric acid monohydrate with 60 % (w/w) potassium citrate tribasic until pH 7 was reached.

Microalgae cultivation and harvesting. Two microalgal strains were used for this study: *Neochloris oleoabundans and Tetraselmis suecica*. *N. oleoabundans* (UTEX 1185, University of Texas Culture Collection of Algae) was cultivated in fresh water using a fully automated 1400 L vertically stacked tubular photobioreactor supplied with Bold's basal medium (106). *T. suecica* (UTEX LB2286, University of Texas Culture Collection of Algae, USA) was cultivated in 25 L flat panel photobioreactors in sea water supplied with Walne medium. Cultivation details are given elsewhere (106). Both photo-bioreactor systems were located in AlgaePARC (Wageningen, The Netherlands). After harvesting, biomass was stored at 4 ^{o}C until further use.

Fractionation Process. The harvested microalgae were suspended in MilliQ® water to obtain a biomass concentration of ~ 90 g L^{-1}. The microalgae suspension was disrupted in a horizontal stirred bead mill (Dyno-Mill Research Lab, Willy A. Bachofen AF Maschinenfabrik, Switzerland) using 0.5 mm beads as described by Postma et al. (106). The milled suspension

was then centrifuged at 14000 rpm and 20 ºC for 30 min in a Sorval® LYNX 6000® centrifuge (ThermoFisher Scientific®). The supernatant was recovered and subjected to a two steps filtration process. First, ultrafiltration was conducted on a laboratory scale tangential flow filtration (TFF) system (Millipore®, Billerica, MA) fitted with a membrane cassette with a filtration area of 50 cm^2 and a cut-off of 1000 kDa (Pellicon® XL Ultrafiltration Biomax®). The process was run at constant transmembrane pressure until a 5x concentration factor was achieved. The resulting permeate was then filtered three times over a 3 kDa Ultracel® Amicon® Ultra centrifugal filter (Millipore®, Tullagreen, IRL). Each run was performed for 20 min at 4000 xg and 20 ºC; MilliQ® water was used as feed for the second and third run. The resulting retentate, regarded as crude protein (CP), was stored at -20 ºC until further use.

Characterization of the crude protein extract. The crude protein (CP) extract was characterized based on proteins, carbohydrates, lipids and ash content. Soluble proteins were quantified using the DC Protein assay (Bio-Rad), which is based on the Lowry assay (107). Bovine serum albumin (Sigma–Aldrich) was used as protein standard and absorbance was measured at 750 nm using a microplate reader (Infinite M200,Tecan, Switzerland).
Total carbohydrates content was determined by the method of Dubois (108), which is based on a colorimetric reaction in phenol-sulfuric acid which is measured at 483 nm. Glucose (Sigma–Aldrich) was used as standard. Total lipids were analysed following the method of Folch (109). Lipids were extracted three times with chloroform/methanol/Phosphate buffer saline (1:2:0.8 v/v). After extraction, the excess of solvent was removed in a vacuum concentrator (RVC 2-25 CD+, Christ GmbH) and total lipid content was determined gravimetrically. Ash content was measured gravimetrically after burning in a furnace at 575 ºC. Free glucose was determined as described below.

Electrophoresis. To investigate the native conformation of the microalgae proteins before and after partitioning in the ATPS, the samples were analysed by native gel electrophoresis. The samples were diluted with native sample buffer at a ratio 1:2 and applied on a 4-20 % Criterion TGX (Tris-Glycine eXtended) precast gel. The gel was run in 10x Tris glycine native buffer at 125 V for 75 min. The native gel was stained using the Pierce Silver Stain Kit from Thermo Scientific®. The precast gels and buffers were procured from Bio-Rad®.

Aqueous two phase systems, tie lines and protein partitioning. The aqueous two phase system Iolilyte 221PG (1) - citrate (2) - water (3), further regarded as Iol-Cit, was selected to study the partitioning of proteins. This system was chosen from several ionic-liquid based ATPS (data not shown) due to its strong ability to form two phases and to partition proteins, biocompatibility and mild interaction with proteins; furthermore, the corresponding phase diagram and 4 tie lines are readily available as described by Suarez Ruiz et al. (110). To study the partitioning behaviour of the model proteins BSA and Rubisco, four tie lines were selected. For the CP, two tie lines more were constructed to assess in total six tie lines . Along each tie line, a sole point was selected where the volume of top and bottom phases are equal. The final protein concentration in the system was ~ 0.3 mg g^{-1}. The mixture was stirred in a rotary mixer for 10 min. Subsequently, bottom and top phases were separated by centrifugation at 2500 rpm for 5 min and transferred to separate flasks. Experiments were conducted at room temperature and pH 7.

Protein determination. Reference proteins BSA and Rubisco were quantified by measuring the absorbance at 280 nm using a Tecan infinite M200® plate reader. The CP from both strains were quantified with bicinchoninic acid using the Pierce BCA protein assay kit from Thermo Scientific®. For both methods, BSA was used as protein standard. Sample blanks (ATPS without protein) were prepared to correct for the influence of solvent composition. Because of the strong interference of the phase forming components in the top phase and the protein determination methods, the concentration of the protein in the top phase was calculated as function of the total protein fed in each tie line and the protein concentration in the bottom phase.

Free glucose determination. Soluble glucose in the ATPS was determined by reaction with a solution containing *p*-hydroxybenzoic acid, sodium azide (0.095 % w/v), glucose oxidase plus peroxidase and 4-aminoantipyrine (GOPOD reagent) from Megazyme®. Samples were mixed with the reagent at a ratio 0.1:3 (v/v) and incubated at 50 ºC for 30 min. After cooling down to room temperature, quantification was conducted by measuring absorbance at 510 nm using a Tecan infinite M200® plate reader. GOPOD reagent and glucose were used as blank and standard respectively.

Partition coefficients and extraction efficiencies. The partition coefficient for proteins (k_p) between top (T) and bottom (B) phases was estimated according to Eq. 3.1 where x_p is the mole fraction of protein in each phase. Additionally, the extraction efficiencies for proteins EE_p and for free glucose EE_g were calculated as shown in Eq. 3.2. EE% indicates the extent of extraction of a compound in the top phase compared to the total feed.

$$k_p = \frac{x_{p,T}}{x_{p,B}} \qquad (3.1)$$

$$EE\% = \frac{C_T * V_T}{C_B * V_B + C_T * V_T} * 100 \qquad (3.2)$$

Where *C* stands for concentration and *V* stands for volume in each phase.

Statistics. All experiments were conducted in duplicates. Statistical analysis was performed using R (V 3.4.1). One way ANOVA and Tukey HSD tests were implemented at 95 % confidence level to assess significant differences among treatments.

Thermodynamic framework. In this investigation, the NRTL model was implemented for the description of phase equilibrium and protein partitioning in the ATPS. Although this model was not developed for electrolyte solutions, it has been shown to accurately calculate equilibrium data of systems containing salts and ILs (104). With this model, we assume that the ILs and salts do not dissociate; this means that each pair cation-anion is treated as a single molecule. The NRTL model assumes that the liquid is made up of molecular "cells" in a binary mixture. Each cell comprises a central molecule interacting with its neighbouring molecules (105)[28]. Such interaction is characterized by the parameter g_{ij}, which accounts for the interaction energy between the pair *i-j*. The model details are given in Appendix A.

Liquid-liquid equilibrium was estimated assuming constant pressure and temperature (isothermal flash calculation) as described by Denes et al. (111) In this approach, equality of

activities for component *i* in both phases (Eq. 3.3) and mass conservation equations are coupled with the activity coefficient model.

$$(\gamma_i x_i)_{Top} = (\gamma_i x_i)_{Bottom} \qquad (3.3)$$

Where x_i and γ_i are the mole fraction and activity coefficient of component *i* in each phase.

Experimental equilibrium data collected for the characterization of the system Iol-Cit (110) was used to estimate the corresponding interaction parameters τ_{ij} via model regression. First, the non-randomness parameter α was fixed between 0.1-0.3 (105). The three remaining parameters were correlated following the solution algorithm proposed by Haghtalab and Paraj (112) and described in Appendix B. To summarize, two objective functions involving molar compositions and activities are minimized in order to find the best set of integration parameters τ_k (IP) that correlate best the experimental data.

To describe the partitioning of crude proteins in the system Iol-Cit, the following assumptions are made:

i. The proteins in the CP behave like a single molecule. This implies that the presence of other components (carbohydrates, lipids, ash) do not affect the protein's partitioning behaviour; LLE is also unaltered.
ii. The CP does not affect the system equilibrium. In other words, the interaction parameters for components 1, 2 and 3 remain unaltered by the presence of small amounts of CP. In the present study, a maximum CP concentration of ~ 0.3 mg g^{-1} was used. This assumption has been already implemented by Hartounian et al. (94) and Perez et al.(89), and allow us to treat LLE and protein partitioning separately.
iii. The protein partition coefficient (k_p) is calculated as follows:

$$k_p = \frac{x_p^T}{x_p^B} = \frac{\gamma_p^B}{\gamma_p^T} \qquad (3.4)$$

Where x_p and γ_p are the molar fraction and activity coefficient of the pure (reference protein) or crude protein (CP) in each phase.
iv. γ_p can be estimated using the NRTL model.
v. Although the CP is a mixture of proteins of different molecular weight and chemical nature, no distinction is made on the type of protein that is present in each phase. In this regard, x^T_p and x^B_p refer to total protein found in top and bottom phase respectively. To estimate the corresponding molar fractions, the molecular weight of Rubisco was selected to represent all proteins present in the crude extract.
vi. Furthermore, we assume that the protein remains in solution, without aggregation or precipitation. We also ignore protein-protein interactions.
vii. The partitioning process takes place at constant pressure and temperature.

It was assumed that the presence of protein is not altering the established equilibrium of the system Iol-Cit. If we consider a fourth component (CP or pure protein), the interaction parameters $\tau_{p\text{-}Iolilyte}$, $\tau_{p\text{-}citrate}$ and $\tau_{p\text{-}water}$ can be calculated by experimentally measuring x^T_p and

$x^{B}{}_{p}$, and by an optimization procedure adopted by Perez et al.(89). In this procedure (Appendix B), the interaction parameters are obtained by minimization of the following objective function:

Results and discussion

Since retaining the native conformation and the stability of valuable proteins is crucial for a variety of industrial applications, we selected the ionic liquid Iolilyte 221PG based on a previous screening (110). The screening was conducted over 19 ILs of different chemical nature and included aspects like ability to form two phases and the IL effect on protein stability. Iolilyte 221PG did not significantly affect the native conformation of Rubisco (present in microalgae) and other reference proteins (BSA and IgG1) up to a 50% concentration of IL (43, 54). Citrate was selected as phase forming component due to its strong salting out character, and because it has a more environmentally friendly character as opposed to inorganic phosphate salts (113). The system Iolilyte 221PG-Potassium citrate showed outstanding extraction efficiencies in the partitioning of Rubisco compared to the commonly used polymer/salt systems.

The phase diagram for the system Iol-Cit is presented in Figure 1. Equilibrium compositions mark the transition from single to two phases, and are linked from top to bottom with tie lines. The points linked by a tie-line represent the composition of the existing phases, which are identical along a tie line. We have studied the partitioning of CP in an area covering six tie lines, with compositions of IL ranging from 2 to 60% (w/w) and salt concentrations from 1 to 55% (w/w). Furthermore, the NRTL model was implemented in order to describe the experimental data. The experimental equilibrium compositions, extended from four tie lines (110) to six tie lines and the corresponding model estimations are shown in Figure 1. The resulting interaction parameters are listed in Table 1. A good representation of the experimental data is achieved in both cases, demonstrating the flexibility of the NRTL model and its applicability to systems containing IL and salts, as indicated in other studies (104).

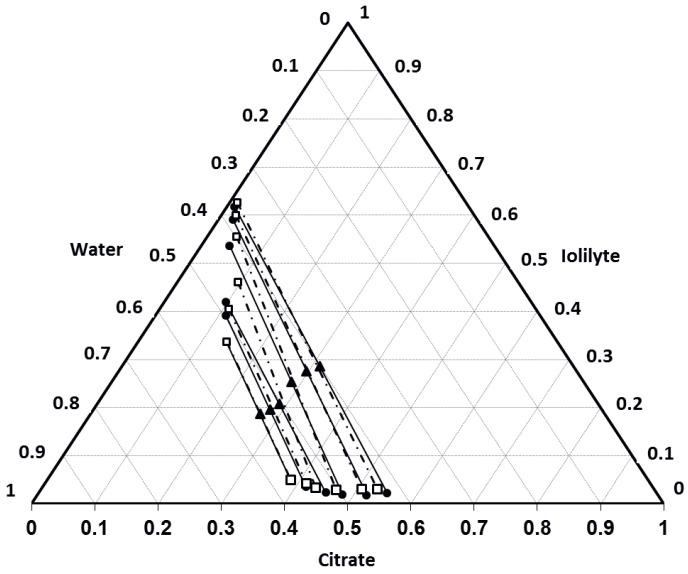

Figure 3.1. Phase diagram (% w/w) for the system Iolilyte (1) - Citrate (2) - Water (2). Experimental data (filled symbols and solid line for tie lines, ▲ for feed compositions) and model estimation (open symbols and dashed line for tie lines).

Protein partitioning. The equilibrium data and interaction parameters for the system Iol-Cit (Figure 3.1 and Table 3.1) were used to evaluate the partitioning of proteins. Two reference proteins, BSA (~ 67 kDa) and Rubisco (~ 540 kDa), were assessed in the system with four tie lines and microalgae proteins (CP) were assessed with six tie lines. For each case, the Tie Line Length (TLL) was calculated according to Eq. 3.7. TLL is proportional to the concentration of the phase forming components and thus, it reflects the effect of the system components on the fractionation process. Higher values of TLL correspond to tie lines farther away from the origin (Figure 1).

$$TLL = \left(\frac{\Delta x_1^2}{\Delta x_2^2} \right)^{1/2} \quad (3.5)$$

Where subscripts 1 and 2 stand for Iolilyte and citrate respectively, and Δ refers to the difference between top and bottom composition for each tie line.

The experimental partition coefficients presented in Figure 2A indicate that the proteins are preferentially concentrated in the IL-rich phase. A notably high standard deviation occurs for TLL 53 %, which can be due to the inherent stability of Rubisco in Iolilyte 221PG. At a TLL of 53 %, the concentration of IL in the top phase is ~ 42 % (w/w). This appears to be a turning point for the molecular stability of Rubisco, leading to strong responses in solubility and therefore uncertainties in the analytical determination. In fact, Desai et al. (54)[9] found that the molecular conformation of Rubisco in the same IL changes already from 30 % IL but becomes significant at 50 % IL. This was also observed in our earlier publication. Partition

coefficient results are in agreement with studies on protein partitioning in ATPS containing the same ionic liquid (43, 54, 110). Dreyer et al. 54 postulated that molecular weight and net protein charge are the most important factors explaining the enrichment of proteins in the IL-rich phase. When comparing the partition coefficients of BSA and Rubisco, no significant differences were found ($p > 0.05$). Considering the remarkable difference in size of these reference proteins, molecular weight does not seem to play a relevant role in our case. On the other hand, the system pH (~6.5) is higher than the isoelectric point (pI) of Rubisco (pI ~ 5.5) (114) and BSA (pI ~ 4.7) (54), which means that both proteins are negatively charged in the ATPS. Electrostatic interactions between the negatively charged protein and the ionic liquid cation influence positively the concentration of the protein in the ionic liquid-rich phase. We also propose that the citrate anion plays an important role in the partitioning of the proteins to the top phase. Citrate is a highly hydrated anion with a strong salting out capability (115) and therefore proteins in the CP migrate to the IL rich phase in which the charge density is lower. Thus, it appears clear that the protein net charge in the ATPS is an important driving force for the preferential concentration of proteins in the IL-rich phase (116).

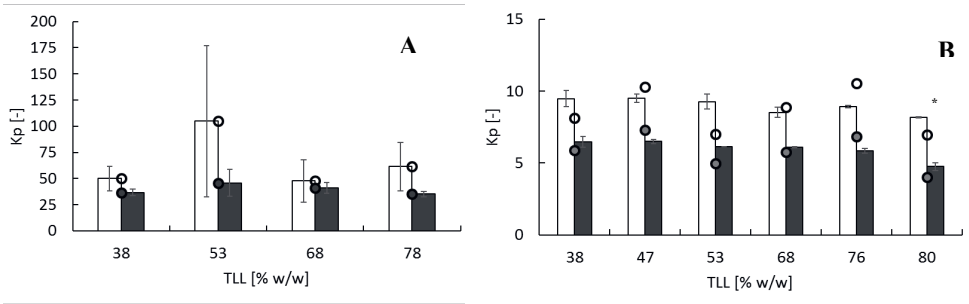

Figure 3.2. Partition coefficients as function of tie line length in the system Iolilyte (1) - citrate (2) - water (3). Experimental data is shown in bars and model estimations with markers. (A) Rubisco (open marker and bars) and BSA (filled bars and marker). (B) Crude protein from *N. oleoabundans* (open marker and bars) and *T. suecica* (filled marker and bars). Error bars indicate standard deviation. * Significant difference ($p < 0.05$).

Figure 3.2B displays the partition coefficient of total protein from the CP derived from *N. oleoabundans* and *T. suecica.* Although a slight decreasing trend is observed, statistically only the k_p for the tie line length (TLL) 80 % is significantly lower ($p < 0.05$). A decrease of k_p with TLL have been previously reported by other authors (117, 118). The TLL changes the free volume available, therefore, the migration of the protein to the opposite phase seems to be consequence of the high concentration of the ionic liquid in the top phase which reduces the free volume available for the protein. This was the case for the CP from both microalga strains. The statically lower value of k_p at TLL 80 % can be due to protein loss as result of the high concentration of IL at TLL 80 %, which reaches nearly 62 % (w/w). At such concentrations, some proteins from the CP are excluded from the top phase into the bottom phase or interphase. Although we expected protein loss already from TLL 68% (where the concentration of IL is higher than 50 % (54), it appears that above 60 % IL the effect becomes significant. The corresponding k_p for TLL from 38 to 76 % confirm the observations for the reference proteins

(Rubisco and BSA) presented in Figure 3.2A, in which the partition coefficients remained statistically stable through the different tie lines. In this regard, the proteins present in the CP are of different molecular size (119) and their chemical nature is unknown, which makes it challenging to predict accurately the mechanism of partitioning. Furthermore, when increasing the tie line length, the concentration of phase forming components also increases, making the upper phase richer in IL and the lower phase richer in salt. Hence, composition difference between phases becomes larger. In the system studied, however, the conductivity of the top phase varied between 81.3-89.2 mS cm^{-1} while that of the bottom phase reached 5.1-8.7 mS cm^{-1} depending on the tie line. This narrow range gives additional insights in how to explain the constant trend observed for k_p at different TLL.

The effect of TLL on protein extraction appears to be minor. Similar observations have been made for proteins from microalgae. Zhao et al. (120) noted a slight effect on protein yield when the TLL varied from 20 to 33 %, while Patil and Raghavarao (117) observed an increase in protein yield from 90 to 97 % when the TLL changed from 13 to 33 %. In both cases, c-phycocyanin was extracted from *Spirulina platensis* in a system containing Polyethylene glycol and phosphate. Suarez Ruiz et al. (110) found a small increase in *EE* for Rubisco (91 to 98 %) in the system Iol-Cit for TLL in the range 37 to 80 %.

After microalgae cultivation and harvesting, a fractionation process involving bead milling, centrifugation, filtration and diafiltration was implemented to produce a crude protein extract from two microalgae strains. The composition of the resulting CP extracts is presented in Figure. 3.3A. The protein content reached 43.5 ± 2.7 and 48.1 ± 1.8 % (dw of the CP) for *N. oleoabundans* and *T. suecica* respectively. These values are in good agreement with protein extracts obtained from several microalgae strains using various separation processes (121). As expected, a higher value of ash was observed for the marine strain *T. suecica* (18.5 ± 0.5 % dw) compared to *N. oleoabundans*, which was grown in fresh water. A maximum lipid content of ~ 2 % dw was found for both strains; these are probably prosthetic groups (e.g., porphyrins) and lipoproteins (122).

The carbohydrates content in the CP is notably high, in particular for *N. oleoabundans* (~ 40 % dw) This could be due to an elevated carbohydrates content in the initial biomass. Since both microalgal strains used in this investigation can accumulate starch during cultivation, we anticipate that glucose is the most abundant sugar. In fact, Brown (123) measured the sugar composition of 16 species of microalgae including the classes *Chlorophyceae* and *Prasinophyceae*, finding that glucose accounts for 55.2 to 85.3 % of the total carbohydrates content. The fraction of free glucose to total carbohydrates in the CP is 21.49 ± 0.55 % and 36.47 ± 1.28 % for *N. oleoabundans* and *T. suecica* respectively.

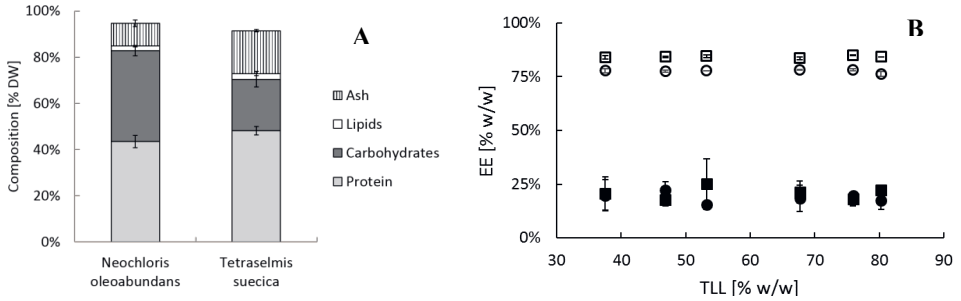

Figure. 3.3. (A) Overall composition (% dw) of crude protein extract from *T. suecica* and *N. oleoabundans*. Error bars indicate standard deviation. Experiments run in duplicate. (B) Extraction efficiencies for proteins (open symbols) and free glucose (filled symbols) for *T. suecica* (circles) and *N. oleoabundans* (squares).

The corresponding extraction efficiencies (*EE*) for total protein and free glucose, according to Eq. 3.2, are presented in Figure. 3B for different tie lines. We have determined free glucose as an indirect measure of carbohydrates in the ATPS, as the common methods for the quantification of total sugars show strong interference with Iolilyte 221PG. It is clear that proteins are preferentially accumulated in the top phase while sugars are mostly concentrated in the bottom phase. The high affinity of sugars for water and their lack of charge seems to be responsible for their separation into the most hydrated phase (bottom phase). This confirms that the system Iol-Cit effectively fractionate proteins from sugars in a single step. Only few studies have demonstrated the separation of proteins from saccharides in ATPS containing IL (87); the reported extraction efficiencies (> 82 % for proteins and > 100 % for saccharides) are in good agreement with our findings. Furthermore, to our knowledge, the present research is the first evidence of extraction of proteins and carbohydrates from microalgae crude extracts in an IL based ATPS. Moreover, there is no significant difference ($p > 0.05$) in the values of *EE* for *N. oleoabundans* and *T. suecica*. This is unexpected, but indicates that the chemical nature of the proteins and sugars from both microalgal strains are comparable.

Protein conformation. Previous research conducted on several ionic liquids-salt pairs (data not shown) revealed that Iol-Cit forms two phases at relatively low concentrations of IL. This is important considering protein stability; Desai et al. (54) studied the stability and activity of Rubisco in Ioliliye 221 PG and found that above 30 % IL there is aggregation and significant loss of enzyme activity but no signs of protein fragmentation. In our experiments, the highest concentration of IL was 60 % (w/w), and occurs in the top phase of the sixth tie line. Visually, we did not identify aggregation or the formation of precipitates. In order to evaluate protein conformation, native gel electrophoresis was performed for each tie line in the top phase and the resulting protein bands are presented in Figure. 4. The expected protein band for Rubisco (~540 kDa) is observed for all tie lines, with a gradual decrease in band intensity at higher values of tie lines for *N. oleoabundans*. There is a clear loss of distinctive protein bands in the range < 20 kDa for both microalgal strains (dotted squares). We hypothesize that these proteins could have suffered denaturation due to the presence of IL and thus they migrated to the bottom

phase. To understand the partitioning of all proteins present in microalgae, their chemical nature should be investigated.

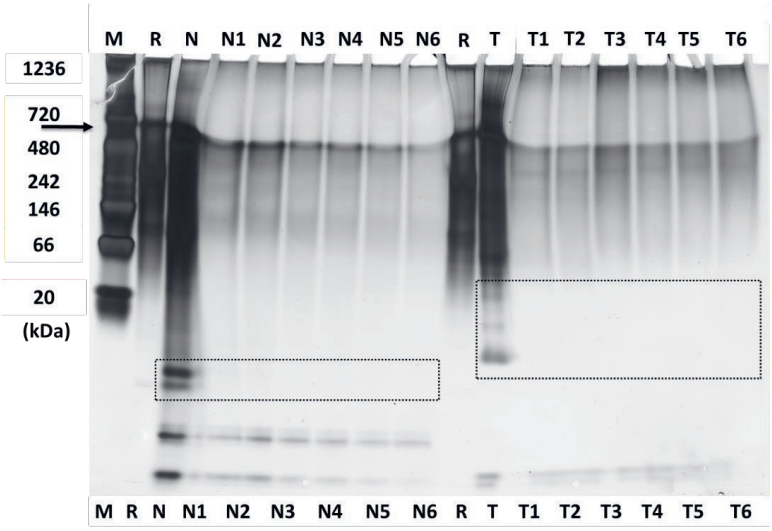

Figure 3.4. Native gel electrophoresis for crude protein (CP) from *N. oleoabundans* (N) and *T. suecica* (T). M: protein marker. R: Rubisco (arrow at ~540 kDa). Numbers 1-6 refer to tie lines. Wells N and T refer to CP in water. Dotted squares indicate region in which protein bans are lost.

Protein partitioning estimations. The corresponding estimations of protein partitioning with the NRTL model are given in Figure 3.2A for the reference proteins and in Figure 2B for the microalgae CP. The respective interaction parameters are listed in Table 3.1. Excellent description of the experimental data is obtained with the system containing four tie lines. This is due to an almost perfect fit to the equilibrium data of four tie lines, for which the optimization procedure provided a more accurate set of interaction parameters to estimate the partition coefficients. The calculated values of k_p for the CP, nonetheless, show the expected trend at different TLL. It can be seen that the largest deviation from the experimental data occurs for TLL 53 %, for which the equilibrium prediction also presented high deviations.

For the implementation of the NRTL model, it was assumed that the IL and salt do not dissociate. Besides, it is also considered that the protein does not possess a net charge. However, for all proteins evaluated, this assumption is not correct, as their pIs are far from the working pH for all tie lines (89). The fact that the model's output provides a good representation of the experimental data means that the adjustable parameters in the NRTL model covers the uncertainty regarding the partitioning mechanism.

We implemented the NRTL model to describe equilibrium and protein partitioning in a biphasic system. Experimental phase equilibrium data is used to estimate the interaction parameters, and thus, the results presented in this research are only valid for the system Iol-Cit. Furthermore, partitioning data is used to calculate the interaction parameters for total protein in the system.

The resulting model, is therefore applicable in further studies and design of extraction systems containing Iol-Cit and crude proteins from green microalgae.

IP	6 TL	4 TL	IP	BSA	Rubisco	CP *N. oleoabundans*	CP *T. suecica*
α	0.3	0.3	α	0.15	0.15	0.15	0.15
Δg_{12}	102156.3	-36699.2	Δg_{14}	-40428.6	-31452.3	414.1	-11067.5
Δg_{13}	-221631.5	-9904.1	Δg_{24}	-43176.1	-23007.2	-680.3	-10935.9
Δg_{21}	-232114.1	-36118.3	Δg_{34}	70799.3	38794.7	71785.3	42233.4
Δg_{23}	-202039.8	-11047.9	Δg_{41}	-23487.7	-26218.9	83437.0	62510.4
Δg_{31}	-110600.3	24359.0	Δg_{42}	-15807.2	-22231.3	13001.1	7529.9
Δg_{32}	160368.3	34645.4	Δg_{43}	-37491.9	-41216.8	1786957.8	265463.7
rmsd[+]	0.21	0.073	***ssq***[**]	2.06E-4	3.74E-4	3.44	1.99

[+] Root mean square deviation
* Sum of squares

Table 3.1. Binary interactions parameters (IP) [j mol^{-1}] according to the NRTL model for the system Iolilyte (1) - Citrate (2) – water (3) and protein (4), using 6 and 4 tie lines (TL), reference proteins and Crude Protein (CP).

1.1. Outlook. In this investigation we implemented the NRTL model to describe equilibrium and protein partitioning in a biphasic system. Experimental phase equilibrium data is used to estimate the interaction parameters, and thus, the results presented further are only valid for the system Iol-Cit. Furthermore, partitioning data is used to calculate the interaction parameters for total protein in the system. The resulting model, a function of composition and interaction parameters, is therefore applicable in further studies and design of extraction systems containing Il-Cit and crude proteins from green microalgae.

We have demonstrated the partitioning of crude protein from algae in an IL based aqueous two phase system. We have also provided evidence of the simultaneous extraction of proteins from carbohydrates. However, a potential application of IL in algae biorefinery remains challenging. In particular, the following aspects require further development:

- Chemical nature of the CP: A more accurate knowledge on the chemical nature of the proteins in the CP would lead to a better understanding of the partitioning behaviour.
- Binary interaction parameters: In this research we have used an algorithm which depends on equilibrium compositions in order to estimate the corresponding interaction parameters. Although the model output shows a good representation of the experimental data, the calculated Δg's may not correspond to experimental interaction parameters. In this regard, phase analysis is recommended. Furthermore, the values presented in Table 3.1**.** for the partitioning of CP reflect not only the influence of proteins and phase forming components (Iolilyte and salt) but also carbohydrates, lipids and ash present in the crude fraction.
- Recovery of IL: Due to their inherent costs and limited knowledge regarding toxicity and environmental concerns, further research is needed in order to develop effective strategies for recycling the IL after protein extraction.
- Application of the protein extracts: The functionality and potential use of the proteins obtained after the purification process remain unknown.

Conclusions

In this research we demonstrated the partitioning of total proteins from crude algae extracts in an aqueous two phase system containing an ionic liquid and an organic salt. It was determined that sugars are preferentially accumulated in the opposite phase as proteins, demonstrating a simultaneous extraction. The extraction efficiencies and K_p of proteins did not vary significantly as function of the tie line length. The same behaviour was observed for the partition coefficient of two reference proteins namely BSA and Rubisco. It was proposed that the partitioning is determined mostly by the net protein charge rather than by the molecular weight of the proteins. Good representation of the experimental equilibrium and partitioning data was achieved with the Non Random Two Liquids model, confirming clearly its flexibility and applicability in algae biorefinery.

Nomenclature

Letter	**Definition**	**Units**
a	Activity	*[-]*
CP	Crude Protein	[-]
EE	Extraction Efficiency	%
g	Interaction energy	[J mol^{-1}]
k	Partition coefficient protein	[-]
Q	Penalty term	[-]
R	Universal gas constant	[J mol^{-1} K^{-1}]
T	System temperature	[K]
x	Mole fraction	[mol mol^{-1}]
Greek characters		
α	Non-randomness (NRTL model)	[-]
τ	Adjustable interaction parameter	[-]
γ	Activity coefficient	[-]
Subscripts		
i, *k, l*	Component, parameters and tie lines respectively	[-]
ij	Refers to binary interaction between components *i* and *j*	
g	Free glucose	[-]

T, *B*	Top and bottom phase respectively	[-]
exp, calc	Experimental and calculated value respectively	[-]
p	Protein	[-]

Acknowledgements

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Appendices

Appendix A: Thermodynamic framework

NRTL model. The general expression for the molar excess Gibbs energy (g^E) according to the NRTL model, for *n* components, is given by:

$$\frac{g^E}{RT} = \sum_{i=1}^{n} x_i \frac{\sum_{j=1}^{n} \tau_{ji} G_{ji} x_j}{\sum_{k=1}^{n} G_{ki} x_k} \quad \text{(A1)}$$

Where:

$$G_{ij} = \exp(-\alpha_{ij} \tau_{ij}) \quad \text{(A2)}$$

$$\tau_{ij} = \frac{g_{ij} - g_{jj}}{RT} = \frac{\Delta g_{ij}}{RT} \quad \text{(A3)}$$

Here, *R* is the universal gas constant (J mol^{-1} K^{-1}) and *T* the system temperature (K). The parameter g_{ij} characterizes the interaction energy of the pair *i*-*j*, while the parameter α_{ij} accounts for the non-randomness in the mixture; x_i is the corresponding molar fraction of component *i*. For both parameters it holds that $g_{ij}=g_{ji}$ and $\alpha_{ij}= \alpha_{ji}$. Although α_{ij} is adjustable, a fixed value of 0.2-0.3 is frequently used in most studies (105). The binary interaction parameters τ_{ij} are usually obtained from experimental and mutual solubility data. The corresponding general expression for activity coefficient γ of component *i* is given by:

$$\ln \gamma_i = \frac{\sum_{j=1}^{n} x_j G_{ji} \tau_{ji}}{\sum_{k=1}^{n} x_k G_{ki}} + \sum_{j=1}^{n} \frac{x_j G_{ij}}{\sum_{i=1}^{n} x_k G_{kj}} \sum_{j=1}^{n} \left(\tau_{ij} - \frac{\sum_{m=1}^{n} x_m G_{mj} \tau_{mj}}{\sum_{k=1}^{n} x_k G_{kj}} \right) \quad \text{(A4)}$$

Liquid-liquid equilibrium. The equilibrium condition implies equality in the chemical potentials of both phases and minimal Gibbs free energy (111). For a two-phase system and *n* components:

$$a_i^T = a_i^B \quad \text{(A5)}$$

$$a_i = \gamma_i x_i \quad \text{(A6)}$$

Here, a_i and γ_i are the activity and activity coefficient of component i. T and B stand for top and bottom phases. Furthermore, the material balances can be written as:

$$\sum_{i=1}^{n} x_i^{T,B} = 1 \qquad \text{(A7)}$$

If the molar compositions of both phases are known, Eq. 1-4 are reduced to a system of non-linear algebraic equations in which only the interaction parameters τ_{ij} and α_{ij} are still unknown. The values of the parameters τ_{ij} are therefore calculated by assuming a constant value of α_{ij} and by an iterative procedure described in appendix B and C.

Appendix B: Computational algorithms

The solution method presented in this research is adopted from Haghtalab and Paraj (112) and involves the estimation of the interaction parameters starting from known experimental compositions (Figure B1). Briefly, experimental compositions in both phases are used to initialize the interaction parameters. Next, the activity of all species is calculated using the activity coefficient model (NRTL). In the following step, by minimization of the objective function 1 (OF1). This function conducts a fit based on the equality of activities, and thus, after convergence, a new set of calculated compositions is obtained.

$$OF_1 = \sum_{l}^{6}\sum_{i}^{3}\left[\left(x_{li}\gamma_{li}\right)^{T} - \left(x_{li}\gamma_{li}\right)^{B}\right]^2 + Q\sum_{k}^{6}\tau_k^2 \qquad \text{(B1)}$$

Subscripts l, i and k refer to tie lines (1,2..N), components (Iolilyte 221PG (1), citrate (2), water (3) and adjustable parameters (1, 2..6), respectively. T and B indicate top and bottom phase. τ_k are the adjustable interaction parameters. Q is a penalty term to reduce the risks of multiple solutions associated with parameters of high value (112). In this case Q was set to 10^{-6}.

Finally, to estimate the binary interaction parameters that lead to the best correlation between the experimental and calculated molar compositions, the objective function 2 (OF2) is implemented.

$$OF_2 = \sqrt{\frac{1}{6N}\sum_{l}^{N}\sum_{i}^{3}\sum_{k}^{2}(x_{lik}^{cal} - x_{lik}^{\exp})^2} \qquad \text{(B2)}$$

As can be seen, OF2 is the root mean square deviation for the compositions. For both objective functions, the criterion for minimization (ε) was set to 10^{-6}.

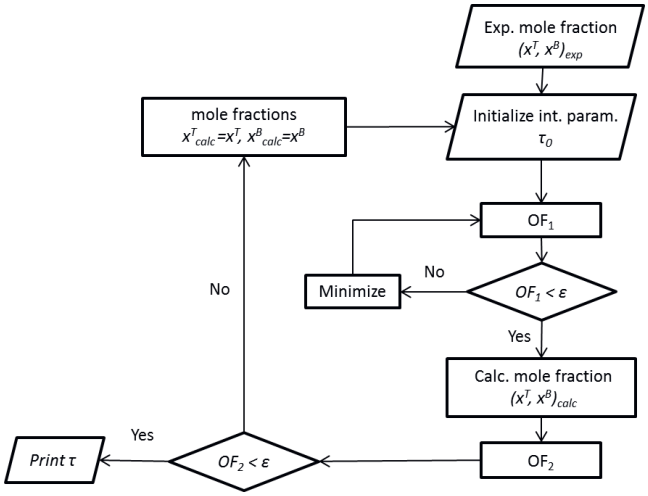

Figure B1. Solution algorithm proposed by Haghtalab et al. (112) for estimating liquid-liquid interaction parameters.

The set of calculated interaction parameters is used to estimate the binary interaction parameters for the system containing protein. The solution algorithm is presented in Figure B2. In short, we assumed that the presence of proteins and other biomolecules do not alter the equilibrium compositions or binary interaction parameters of the phase forming components. Then, we used experimentally obtained compositions to initialize the interaction parameters. This allowed us to have a first estimate of the activity coefficients for protein in each phase. Then, we have used objective function 3 (OF3) to estimate the interaction parameters that lead to a better agreement between the experimental and calculated protein composition, expressed in terms of the partition coefficient k_p.

$$OF3 = \left(\sum_{l=1}^{6} \frac{(k_{p,\exp} - k_{p,calc})^2}{6} \right)^{1/2} \quad \text{(B3)}$$

Where the subscript p indicates protein, l refers to tie lines and $k_{p,exp} - k_{p,calc}$ is the deviation between experimental and calculated partition coefficients. OF3, which also corresponds to a root mean square deviation, was adapted from the work of Perez et al. (89). The criterion for minimization (ε) was set to 10^{-6}.

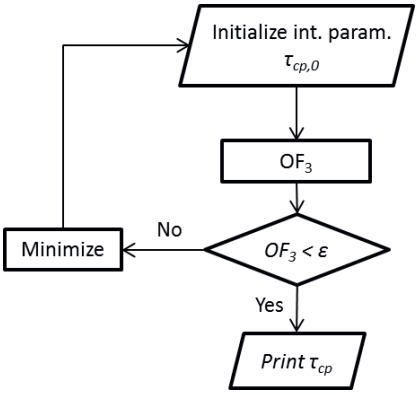

Figure B2. Computational algorithm for estimating interaction parameters of crude proteins (CP).

Chapter 4

Selective and mild fractionation of microalgal proteins and pigments using aqueous two phase systems.

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Abstract

BACKGROUND: Microalgal biomass is generally used to produce a single product instead of valorizing all the cellular components. The biomass production and downstream processes are too expensive if only one product is valorized. A new approach was proposed for the simultaneous and selective partitioning of pigments and proteins from disrupted *Neochloris oleoabundans* cultivated under saline and fresh water conditions.

RESULTS: An aqueous two phase system composed of polyethylene glycol and cholinium dihydrogen phosphate selectively separated microalgal pigments from microalgal proteins. 97.3 ± 1.0% of lutein and 51.6 ± 2.3 % of chlorophyll were recovered in the polymer-rich phase. Simultaneously, up to 92.2 ± 2.0% of proteins were recovered in a third phase (interface) in between the aqueous phases (interface). The recovered proteins, including Rubisco with a molecular weight of ~560 kDa, seem to be intact and pigments did not suffer degradation, demonstrating the mildness of this system to fractionate microalgal biomolecules.

CONCLUSION: The ability of aqueous two phase system (ATPS) to simultaneously and efficiently fractionate different biomolecules in a mild manner from disrupted microalgae is demonstrated. This is an important step towards the development of a multiproduct microalgae biorefinery.

Keywords: Microalgae biorefinery, proteins, pigments, cholinium-based ionic liquids, aqueous two phase systems

Introduction

Microalgae are a promising feedstock for many industries including biopharmaceuticals, biomaterials, bioenergy, nutraceuticals, agriculture, animal health and cosmetics and personal care.(124) Feasibility studies demonstrate that a biorefinery focus on one single product is not cost-efficient (6) and that several microalgal components should be extracted to supply different markets and thus increase the overall value of microalgal biomass (10).

Depending on the strain, microalgae can contain chlorophylls (green), carotenoids (red, orange and yellow) and phycobiliproteins (red and blue) (6). *Neochloris oleoabundans* accumulates mainly lutein, cantaxanthin, zeaxanthin, and astaxanthin monoesters and diesters, which are promising ingredients for pharmaceutical and nutraceutical applications (125), (126). Moreover, microalgal proteins are recognized for their high quality, showing outstanding nutritional, functional and techno-functional properties that are in some cases superior to conventional protein concentrates (127, 128).

Although microalgal cells contain several valuable biomolecules, the currently applied separation processes valorize only one specific product (e.g. astaxanthin, phycobiliproteins) (129, 130). The development of efficient, mild and scalable methods/processes, capable of fractionating different microalgal biomolecules, is of particular importance (131). For the fractionation of hydrophobic molecules such as lipids and pigments from disrupted microalgae, organic solvents are often used, while water soluble components such as proteins and carbohydrates are discarded or undervalued (132). Proteins are fragile molecules that tend to denature during extraction using organic solvents or other harsh conditions (54). For a complete valorisation of microalgal biomass, it is favorable to use mild separation methods.

Aqueous two phase systems (ATPS) have been adopted as a new technology in a microalgae biorefinery framework. ATPS is composed of two immiscible aqueous phases formed generally by two polymers, a polymer and a salt or two salts. Since an ATPS contains mainly water, the phases formed can provide a mild and suitable environment for biomolecules (133). Furthermore, the process of ATPS extraction is easy to scale up, in the process non-flammable/volatile and low toxic components are used, and multiple products can be separated simultaneously (134).

The potential for the refinery of biomolecules by using ionic liquids (IL) based ATPSs has grown exponentially (135). IL-based ATPSs provide advantages over conventional ATPSs because: They are highly tuneable through variations in pH, molecular structure, composition and temperature. The wide variety of potential ions spans the entire hydrophobicity–hydrophilicity range (136). Thus, IL-based ATPSs potentially have a higher selectivity, flexibility and can provide more efficient separations (54). However, reports on integrated separation processes of biomolecules from biomass using ATPS and IL based-ATPS are limited (137-140). The application of IL based-ATPS is generally studied using pure biomolecules (e.g. proteins), facilitating the understanding of the partitioning behavior of certain molecule . However, these compounds are normally present in complex biological matrices such as microalgae biomass, which contain a large number of other components (e.g. carbohydrates,

pigments, lipids). Therefore, to provide a realistic scenario, investigations using IL-ATPS need to focus on the fractionation of these compounds from complex matrices (86).

Studies of ATPSs for fractionation of biomolecules from microalgae extracts are limited. These studies mainly focus on the recovery of specific molecules such as the fluorescent pigment-protein complex *C-phycocyanin* from *Spirulina* strains, (95) *B*-phycoerythrin from *Porphyridium cruentum* (96, 141) and proteins from *Chlorella sorokiniana* (98). The simultaneous separation of different molecules from microalgae has not been addressed (142). To our knowledge, the current study is the first study using Ionic liquid based-aqueous two phase systems for the simultaneous fractionation of proteins and pigments from disrupted microalgae.

In this research, *Neochloris oleoabundans* is used to study the fractionation of pigments and proteins by using one conventional ATPS (polymer/salt) and two IL-based ATPSs. Mild and biocompatible phase forming components were selected based on previous investigations (110). ATPS was used for the fractionation of pigments and proteins from disrupted *N. oleoabundans* grown under both fresh and saline water conditions.

Materials and methods

Chemicals.

Potassium citrate tribasic monohydrate, polyethylene glycol (PEG) 400, hydrochloric acid, acetic acid and Bovine serum albumin protein (BSA), >98% were purchased from Sigma-Aldrich. Citric acid was obtained from Merck. The ionic liquids: IoliLyte 221 PG, >95% and cholinium dihydrogen phosphate (Ch DHp), >98% were obtained from Iolitec. Acetonitrile and Methanol (HPLC grade) were provided by Biosolve and ethyl acetate by Fisher Scientific.

Microalgae cultivation, harvesting and cell disruption.

N. oleoabundans (UTEX 1185, University of Texas Culture collection of Algae, USA) was cultivated in a fully automated 1300L vertical stacked tubular photo bioreactor (PBR) located at AlgaePARC, The Netherlands. It was cultivated under saline and fresh water conditions, both in Bold's Basal medium (143) at a pH value of 8.0 and the temperature was controlled at 30 °C. To cultivate microalgae under saline conditions artificial sea water was used: NaCl: 24.5g/L; $MgCl_2$: 9.8 g/L; CaCl2: 0.53g/L; K_2SO_4: 0.85g/L; $NaSO_4$: 3.2g/L; $NAHCO_3$: 0.8g/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). The biomass paste was suspended in MilliQ® water to obtain a solution containing 6% of solids and was disrupted by using a horizontal stirred bead mill (Dyno-Mill ECM-AP) using zirconia beads with bead size of 0.5 mm. Bead milled microalgae was centrifuged (20 min, 20000×g) to separate most of the cell debris from the supernatant. The resulting extract (supernatant) was stored at -20ºC until further use.

Fractionation of pigments and proteins from microalgae using ATPS.

Previously, three promising ATPSs were selected and characterized for the separation of microalgae components (110). The selection of the ionic liquids was based on their interaction with the protein Rubisco (protein present in microalgae with a molecular mass of ~560 kDa

consisting of non-covalently bound 8 large subunits (~56 kDa) and 8 small subunits (~14 kDa). Potassium citrate and PEG 400 were selected due to their biocompatibility and ability to form ATPS with these ionic liquids. The composition of the mixtures prepared for the partitioning of microalgal pigments and proteins is shown in the Table S4.1 of the supporting information. The ATPS mixtures were prepared gravimetrically (10^{-4} g) by using mixture points along four tie-lines (TLs) with a volume ratio (Vr) of one between the top and bottom phase as described by Suarez Ruiz et al.(110). One gram of microalgal extract was added and MilliQ® water was used to complete 10 g in each system. Mixtures were protected from light during the fractionation process. These were stirred for 1 hour in a rotatory shaker (50 rpm) and left to equilibrate at room temperature. To facilitate the separation of the aqueous phases from the interface all systems were centrifuged for 5 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 MilliQ® water to facilitate the quantification of the biomolecules. Possible interferences of phase forming components on the analytical method were taken into account, and control samples were prepared using water instead of microalgae extract. At least two individual samples for each condition were prepared and the biomolecules were quantify. The results were reported as the average of two independent experiments with the respective standard deviation.

Pigment analysis.

Pigments in each phase (top, bottom and interface) were analyzed by measuring the absorption spectrum between 200 and 750 nm. Total chloroplast carotenoids and chlorophylls were determined by measuring their absorbance at 470 nm using a spectrophotometer (DR6000, Hach Lang, USA). Relative partition coefficients (K_p) for total pigments were calculated by Eq. 4.1. Pigments from the initial microalgal extract and interfaces after ATPS were completely extracted with methanol as a control for further calculation.

$$Kp_{Pigments} = \frac{A_{470nm,top}}{A_{470nm,bottom}} \qquad (4.1)$$

Reversed-phase high-performance liquid chromatography.

RP-HPLC was used to identify and quantify the pigments after separation in ATPS. HPLC analysis was performed in a Shimadzu system coupled with a photo-diode array detector (SPD-M20A) and an Acclaim™ C30 LC reversed-phase column from Thermo Scientific™ was used. Three mobile phases were used: A) Acetonitrile, B) Methanol/Ethyl acetate 1:1 (v/v), C) 200mM Acetic acid in water with the gradient shown in Table 4.1. The flow rate was set at 1.5 mL/min and the column temperature at 30°C.

Table 4.1. HPLC gradient method

Time (min)	%A	%B	%C
0	85.0	14.5	0.5
2	85.0	14.5	0.5
15	65.0	34.5	0.5
25	65.0	34.5	0.5
30	85.0	14.5	0.5

Three pigments were identified and quantified (lutein, chlorophyll a, chlorophyll b) and extraction efficiencies (%w/w) were calculated with Eq. 4.2, where $m_{Lutein,initial}$ is the initial mass of lutein in the microalgae.

$$EE_{Lutein}\% = \frac{c_{Lutein,top} * V_{top}}{m_{Lutein,initial}} \quad (4.2)$$

Protein analysis.

Proteins in each phase were separated from the phase forming components and quantified by Size Exclusion Chromatography (SEC) on an Äkta pure FPLC system equipped with a Hi Trap 5mL desalting column with Sephadex G-25 resin (both GE Healthcare), inline detectors and a fraction collector. Samples were injected using a 100 µL injection loop system and were eluted with 0.05M sodium phosphate, 0.15M sodium chloride pH 7 buffer at a flow rate of 1mL/min at room temperature. Peak detection was conducted by a UV/Vis absorbance detector at a wavelength of 280nm and to control the purification process conductivity and pH monitors were used. For protein quantification, FPLC chromatograms at 280nm were integrated using the GE Unicorn software. Samples were collected after purification by FPLC and protein content was determined by Bradford's method(144) using the Pierce™ Coomassie Plus (Bradford) Assay Kit. Absorbance at 595 nm was measured using a Tecan infinite M200® plate reader. Calibration curves were prepared for both quantification methods with Bovine (BSA) pure protein in MilliQ® water.

Partition coefficient (K_p) was calculated from the ratio of protein concentrations between top and bottom phase (Eq. 4.4). Protein distribution between the three phases was described by the protein extraction efficiency in each phase ($\boldsymbol{EE_{Protein}}\%$) which expresses the ratio of protein amount between the top, interface or bottom phase and the total amount (Eq. 4.5). $\boldsymbol{m_{protein,initial}}$ is the initial mass of protein in the microalgae extract added.

$$K_{p\ protein} = \frac{cprotein_{,top}}{c_{protein,bottom}} \quad (4.4)$$

$$EE_{Protein}\% = \frac{C_{protein,top} * V_{top}}{m_{protein,initial}} \quad (4.5)$$

The selectivity of pigments considering the presence of proteins in the top phase was calculated with Eq. 4.6

$$S_{pigment/protein} = \frac{K_{p\ pigments}}{K_{p\ proteins}} \quad (4.6)$$

Statistical Analysis

All experiments were conducted in duplicates and results were reported as the average of two independent experiments 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 the different treatments.

Electrophoresis.

To investigate the conformation of the proteins before and after the partitioning step, the samples were analysed by native gel electrophoresis (Native-PAGE). The samples were diluted with native sample buffer in the ratio 1:2 and applied on a 4-20% Criterion TGX (Tris-Glycine eXtended) precast gel. The gel was run in a 10 X Tris glycine native buffer at 125 V for 75 min. The native gel was stained with the Pierce Silver Stain Kit from Thermo Scientific. The precast gels and buffers were procured from Bio-Rad.

Results and discussion

Three ATPSs: polyethylene glycol (PEG) 400-potassium citrate, Iolilyte 221PG-potassium citrate and PEG400-Cholinium dihydrogen phosphate (Ch DHp) were selected to evaluate the partitioning of pigments and proteins from *N. oleoabundans* extract. These systems were previously selected based on their interaction with the protein Ribulose-1,5-biphosphate carboxylase/oxygenase (Rubisco) as explained by Suarez Ruiz et. al (110). Microalgae cultivated under saline and fresh water conditions were used to investigate the feasibility of this technique in a multiproduct biorefinery approach. A brief schematic representation of the process presented in this paper is shown in Figure 4.1a.

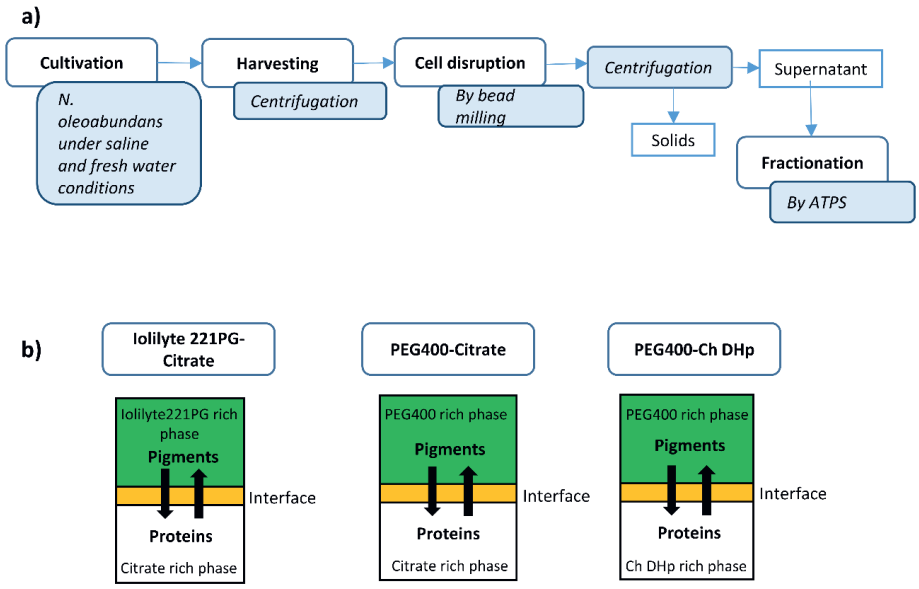


Figure 4.1. (a) Brief diagram of the process followed in the separation of proteins and pigments from microalgae; (b) Description of the main phase forming components in each aqueous two phase system.

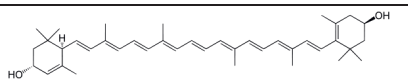
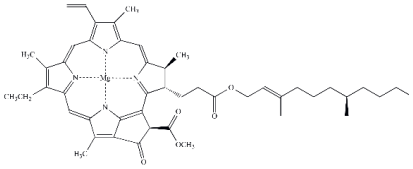
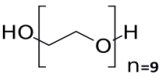
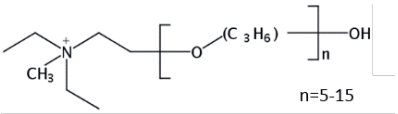
Pigments partitioning in ATPS.

As described in Figure 4.1(b) Iolilyte 221PG-citrate consists of a top phase rich in ionic liquid (Iolilyte 221PG) and a bottom phase rich in citrate. PEG400-citrate and PEG400-Ch DHp ATPSs both consist of a top phase rich in polymer (PEG400) and a bottom phase rich in potassium citrate and Ch DHp respectively. *N. oleoabundans* pigments (carotenoids and chlorophylls) are hydrophobic molecules and therefore tend to partition to the least hydrated phase (top phase) as shown by the partition coefficient values (K_p>1) in Figure 4.2a). The highest partition coefficients were obtained with Iolilyte221PG–citrate ATPS (K_p=62 ± 11), followed by PEG 400-citrate (K_p=36 ± 4) and PEG 400-Ch DHp (K_p=7 ± 0.7). Partition coefficient values and extraction efficiencies presented in Figure 4.2 were obtained using mixture points along the highest tie line lengths (TLLs). Additionally, in the supporting information, Table S4.1 provides the concentration of phase forming components for each TLL and Table S4.2 provides the effect of the tie line length (TLL) on the partition coefficient (Kp) of total pigments.

The effect of the phase composition was investigated based on the tie line length (TLL), which represents the composition and thermodynamic difference of the two phases. The partition coefficient of pigments tend to increase with the TLL and this effect seems to be consistent in the three systems (Table S4.2). Increasing the components concentration in the three systems will enhance the salting out effect, resulting in a less hydrated top phase and an increase of the pigments concentrated in this phase.

Few authors have studied the partitioning of pigments and carotenoids in aqueous two phase systems and they all show that the recovery of these highly hydrophobic molecules is higher when the hydrophobic nature of the top phase is increased (138, 145, 146). Montalvo-Hernandez et al.(147) studied the partitioning of crocins (carotenoids) in four types of ATPSs: polymer-polymer, polymer-salt, alcohol-salt and ionic liquid-salt. In the polymer-salt system, increasing molecular weight and tie line length leads to higher recovery of the carotenoids due to the higher hydrophobicity of the upper phase. Similarly, for ionic liquid-salt, hydrophobic interactions related with the alkyl chain length of the cation seem to be the main driving force for the partitioning of this carotenoid. PEG molecular weight is directly related to PEG hydrophobicity. PEG and Iolilyte 221PG were previously compared and the higher ability of Iolylite 221PG to form ATPS is related with its higher hydrophobicity nature as consequence of its alkyl chain length and molecular weight.(110) Comparing the molecular weight of the polymer and the ionic liquid, Iolilyte 221PG (n=5-15) is a more hydrophobic molecule in comparison with PEG 400 (n=9)(Table 4.2). This could explain the higher recovery of total pigments in Iolilyte221PG-citrate.

Table 4.2. Molecular structure of two important pigments in N. oleoabundans and the main components of the top phases.

Molecule	Chemical structure
Lutein	
Chlorophyll a	
Polyethylene glycol 400 (PEG 400)	
Iolilyte 221PG	

The hydration of the bottom phase main component is also important in the separation of hydrophobic molecules (146). In the case of PEG400-Ch DHp, the high affinity of the IL for water may lead to a higher partitioning of pigments to the top phase (58, 146). However, interactions between the polymer and the ionic liquid play an important role in the partitioning of biomolecules, making the partitioning of the molecules more difficult to predict (148).

Nevertheless, the extraction of hydrophobic molecules in polymer-ionic liquid ATPS could be enhanced by the correct selection of the ionic liquid employed based on these observations.

The salinity of the medium did not affect the preference of the pigments to migrate towards the most hydrophobic phase ($K_p > 1$). Microalgae cultivated under saline and fresh water conditions were used to evaluate the feasibility of using ATPS to fractionate biomolecules from different sources (e.g. microalgae cultivated under different conditions). No significant differences were found ($p > 0.05$) when comparing the partition coefficients of total pigments from microalgae cultivated under saline and fresh water conditions. This is in agreement with the K_p results obtained increasing the concentration of NaCl% in the ATPS mixtures. Figure S4.1 in the supporting information presents the effect of salt concentration (NaCl (%w/w) on the partitioning of pigments. Although a slight increase trend is observed, only the K_p obtained with 3% NaCl in PEG 400-Ch DHp was significantly higher ($p < 0.05$). The medium to cultivate microalgae under saline conditions was prepared using 2.5 (%w/w) of NaCl. Thus, it does not seem that the salinity in the medium of cultivation affect the partitioning of pigments. This is an advantage for the future application of the process, since no washing steps are needed before the fractionation process.

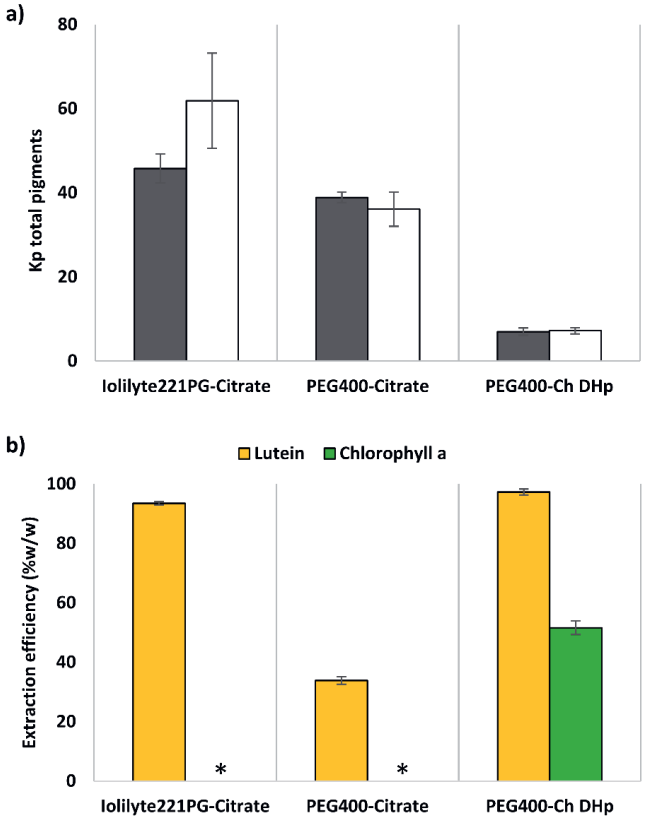


Figure 4.2. Pigments separation from *N. oleoabundans* extract in ATPS: (a) Partition coefficient (K_p) for total pigments. Saline (filled bars) and fresh water (open bars) cultivation condition of *N. oleoabundans*. (b) Extraction efficiencies (%w/w) in the top phase for lutein and chlorophyll a from microalgae cultivated under saline water. The results represent the average of two independent experiments and error bars indicate standard deviations. Asterisks (*) denote chlorophyll a not detected by the RP-HPLC method.

Relative partition coefficients were calculated based on the absorbance of total pigments using UV-Vis spectrophotometry at 470nm. UV-Spectroscopy is widely used to analyse the absorption spectrum of pigments in different solvents (149). Although, it provided information about the overall pigment spectrum, the quantification of specific pigments (e.g. chlorophyll a) was affected by their degradation products. The absorption spectrum of the degradation products of pigments overlapped with the target pigments (e.g. chlorophyll a) (150, 151). Therefore, the quantification of specific chlorophylls and carotenoids (Lutein, chlorophyll a and chlorophyll b) was done by HPLC. This method allowed us to identify the individual pigments of interest in the microalgae and to calculate the recovery of these pigments in the top phase. Chromatograms of the pigments present in microalgae under saline conditions and recovered in the top phase using ATPS are shown in the Figure S4.2 of the supporting

information. This figure shows that lutein had the highest peak followed by chlorophyll b and chlorophyll a (quantified at 660nm). Lutein was efficiently recovered by Iolilyte 221PG-citrate (93.1 ± 0.6 %) and PEG400-Ch DHp (97.3 ± 1.0 %), while PEG400-Citrate only recovered 34.8 ± 1.26 %. Chlorophyll a was recovered by PEG400-Ch DHp (50.6 ± 2.3 %), but it was not detected in the other two systems at 660nm (Figure 4.2b). Chlorophyll b was exceptionally not found in any of the top phases. The lack of chlorophyll a and chlorophyll b in some ATPSs could be due to low extraction efficiencies or a change in the molecule structure by oxidation. Chlorophyll stability is affected by temperature, light irradiance, acids, bases, and oxygen, causing the loss of its magnesium ion and/or phytol group (152) and a change in color to olive-brown (153). Pigments were not detected in the interfaces and bottom phases by RP-HPLC, due to the lack or too low amount of pigments present in those phases.

The nature, stability and amount of the pigments extracted by ATPS depends on the phase forming components. It was observed that the green color of the top phases was different in each case, being more brownish for Iolilyte-221PG and PEG400-Citrate than for PEG-Ch DHp. The brown color might indicate modification of chlorophyll caused by oxidation, which is in agreement with former studies (100), where it was established that the quality of chlorophyll could be affected by some ILs like propylammonium nitrate and on the contrary enhanced by others like dimethylethylammonium methanoate.

Protein partitioning in ATPS.

Proteins were analyzed in the phases after fractionation with ATPS. Figure 4.3. shows the partition coefficient of proteins in the three systems using saline and fresh water cultivated *N. oleoabundans*. K_p values show large differences between PEG400/Citrate, Iolilyte 221PG/Citrate, which proteins migrate preferentially to the top phase (high K_p values) and PEG400/Ch DHp which K_p values are below 1, showing a preference of the proteins to the opposite phase.

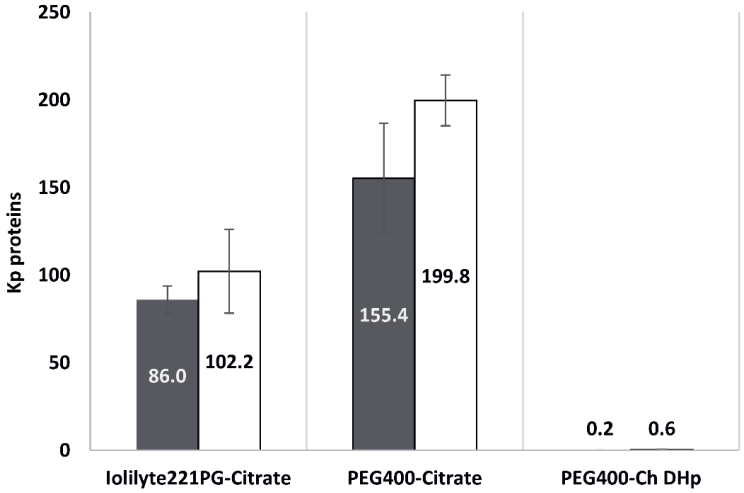


Figure 4.3. Protein partition in ATPS: Partition coefficient (K_p) values for proteins using three different ATPSs. Saline (filled bars) and fresh water (open bars) cultivation condition of *N. oleoabundans*. The results represent the average of two independent experiments and error bars indicate standard deviations.

The distribution of the proteins among the three phases is shown in Figure 4.4a using *N. oleoabundans* cultivated under saline conditions and Figure 4.4b using *N. oleoabundans* cultivated in fresh water. Extraction efficiency of proteins from microalgae cultivated under saline conditions (Figure 4.4a) into the top phase was 94.3 ± 2.8 % using Iolilyte 221PG-Citrate and 61.3 ± 0.9 % using PEG400-Citrate. These extraction efficiencies (%w/w) are 8 to 13 times higher than that found using PEG400-Ch DHp (6.7 ± 0.5 %). In previous studies using Rubisco as model protein, results suggested that the high recovery of protein into the Ionic liquid-rich phase (Iolilyte 221PG) compared to polymer-based ATPSs is a result of hydrophobic and electrostatic interaction between the phase forming components and the protein (110). This conclusion is in line with our current results. But despite the high protein recoveries obtained with Iolilyte 221PG-citrate, the pigments are also recovered in the top phase. As the pigments are also recovered in the top phase, this does not result in the multiproduct biorefinery we are aiming for. Furthermore, a third phase was formed increasing the complexity of the phenomena behind the partitioning of the molecules.

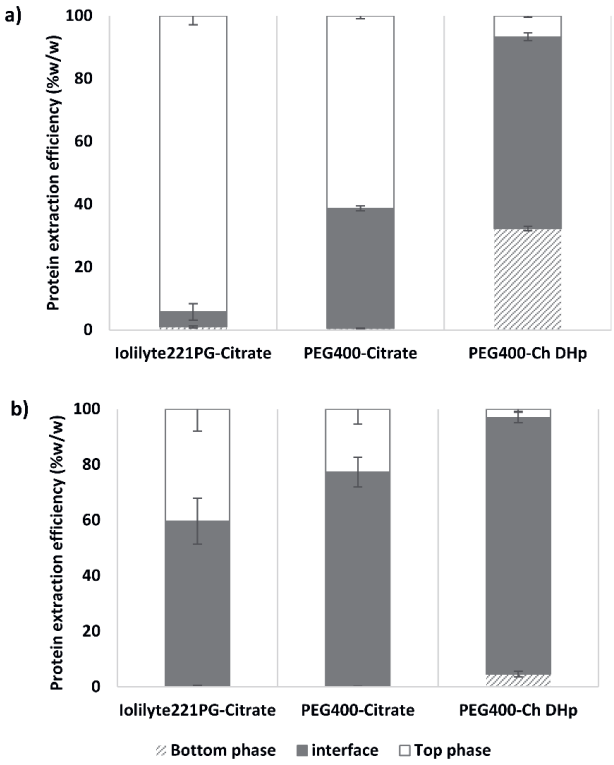


Figure 4.4. Distribution of microalgae proteins among the three phases in the three ATPSs. a) Saline and b) fresh water cultivation condition of *N. oleoabundans*. The results represent the average of two independent experiments and error bars indicate standard deviations.

Aqueous two phase systems can generate interfacial partitioning of different molecules, including proteins, and it has gained attention in the last years for large scale-processes (154). In the traditional ATPS (polymer-salt), protein partitioning is governed by hydrophobic interactions and salting out effect. Similarly, the interfacial concentration of proteins in polymer-salt ATPS was described by Kim et al.(155) based on a protein solubility model, in which protein precipitation was a result of increasing the salt concentration in the bottom phase. Thus, salting out was considered to be the most important driving force together with polymer excluded volume to concentrate proteins in the interface. In conventional ATPS, polymer steric exclusion effects and hydrophobic interactions between the polymer and proteins were often mentioned to predict the maximum concentration that can be added to separate proteins into the top phase (39, 156). Protein precipitation is caused by phase saturation, determined mainly by hydrophobic interactions and by salting out effect. However, the solubility of proteins seem to be different in each ATPS (157), due to the different partitioning driving forces involved.

Ionic liquid-based three phase partitioning (ILTPP) has been investigated because it combines the advantages of IL-based ATPS and TPP for the concentration and recovery of different

molecules including proteins (158-160). ILTPP is capable to induce the formation of a dense and stable protein layer in the middle with IL-based ATPS phase forming components. Alvarez-Guerra et al.(161) suggested that the salt concentration has the greatest influence on the amount of lactoferrin recovered at the interface. However, pH, temperature and protein concentration also influence the partitioning (162).

The three systems studied in the current research were previously used for the partitioning of different proteins, including Rubisco (54, 58, 110). No precipitation of these proteins was reported, demonstrating that the studied systems do not always form three phases. Protein partitioning was previously studied using a total concentration of 0.3 mg/mL of purified Rubisco. In the current study similar microalgal protein amounts were used; 0.4 mg/mL for fresh water cultivated *N. oleoabundans* and 0.2 mg/mL for *N. oleoabundans* cultivated under saline conditions. However, the disrupted microalgae added to the systems contained other biomolecules, such as pigments, lipids, carbohydrates and other proteins apart from Rubisco. The overall composition of microalgae is influenced by cultivation conditions (5). Microalgae cultivated in fresh water has higher amount of proteins and pigments than microalgae cultivated under saline conditions. When using *N. oleoabundans* cultivated in fresh water more proteins were concentrated in the interface (Figure 4.4b) than when using *N. oleoabundans* cultivated under saline conditions. This increase reflects a lower solubilizing capacity of the ATPSs on microalgae molecules (e.g. proteins) in these conditions, which may be caused by protein content but also by other molecules present in the microalgae. PEG400-Ch DHp recovered 92.2 ± 1.9 % of proteins in the interface, followed by PEG400-Citrate (77.2 ± 5.3 %) and Iolilyte 221PG-Citrate system (59.2 ± 8.2 %). We hypothesized that the interfacial precipitation can be caused by the amount of feedstock (including microalgae proteins) added and by the presence of other biomolecules from the microalgae that benefit the emulsification and precipitation of the proteins.

Other authors have reported that ATPS may form an interface when high protein concentrations are added to the system as well as by increasing the tie line length (TLL) (163-165). In table 3S of the supporting information, the distribution of proteins among the three phases and the influence of the tie line length (TLL) were reported. By increasing the TLL (phase forming components concentration), more proteins were recovered in the interface instead of the top phase. Temperature, polymer molecular weight, TLL and protein loading affect the solubilizing capacity of ATPS (157). In a descriptive model of interfacial partitioning in ATPS (polymer-salt), Luechau et al.(154) suggested that the interfacial concentration of molecules depends on phase system, feedstock composition and bioparticles at the interface. Particle size and interfacial tension of the system were used to describe the adsorption of particles in the interface.

In the PEG400-Ch DHp system, proteins were concentrated mainly in the interface and in the bottom phase when using both cultivation conditions. Previous studies have reported the preference of pure proteins to the bottom phase when using ATPS combinations with a more hydrophobic polymer (PPG400) and cholinium-based ionic liquids (55, 58). Li et al.(55) studied protein partitioning using systems composed of PPG 400 and various cholinium based-ILs. They reported a decrease in the protein extraction efficiency in the IL-rich phase due to an

increase in protein size. The proteins studied were Lysozyme, Papain, Trypsin and BSA. They argued that protein partitioning to the IL-rich phase (bottom phase) requires energy to break the interactions between phase components. Thus, smaller proteins require less energy than larger proteins to migrate into the bottom phase. Previous results reported by us agree with this theory (110). Rubisco with a higher molecular weight (~560 kDa) than the proteins reported by Li et al.(55) was partitioned preferentially to the PEG-rich phase (top phase) when using PEG400-Ch DHp. This indicates the importance of protein size in this particular ATPS formed by polymer and cholinium based-ionic liquids. Other studies explained the effect of protein structure (e.g. surface amino acid residues) on the partitioning of these molecules using ATPS formed by polymer and ILs (58, 166). Concluding that protein partitioning depends on specific interactions (e.g. electrostatic) between the protein and the ionic liquid.

Microalgae contain a high amount of proteins with different molecular weights and structures, making the prediction of protein partitioning in PEG400-Ch Dhp even more difficult. Molecular interactions between the proteins and the ionic liquid (Ch DHp) seems to be a strong driving force for the partitioning of proteins to the bottom phase. However, protein size, feedstock load and the presence of other molecules could trigger the interfacial partitioning of the proteins.

To study the conformation of proteins before and after the separation process, Native-PAGE electrophoresis was performed to the initial microalgae added and to the proteins recovered in the interface. Figure 4.5a shows the proteins in the extract of microalgae cultivated under saline and fresh water conditions. Rubisco (~560 kDa) was identified as the most abundant protein known in *N. oleoabundans* and was therefore used as biomarker (167). Other proteins present in the extract have not been characterized, but other bands were detected. In Figure 5b Rubisco band appears for the three systems, indicating that this protein remains intact also after interfacial partitioning with ATPS. However, the band of Rubisco in Iolilyte 221PG-Citrate system is faint, indicating the influence that the Iolilyte221PG has on the protein. The effect of Iolilyte221PG on Rubisco was previously studied, showing that increasing the amount of this ionic liquid the protein losses its native conformation and forms aggregates (54, 110). PEG-Ch DHp and PEG-citrate seem to retain the native conformation of not only Rubisco but also of other proteins from *N. oleoabundans* (Figure 4.5). These systems are therefore considered milder for the separation of proteins. However, further characterization and techno-functional evaluation of the proteins recovered is needed for their use in different industrial applications.

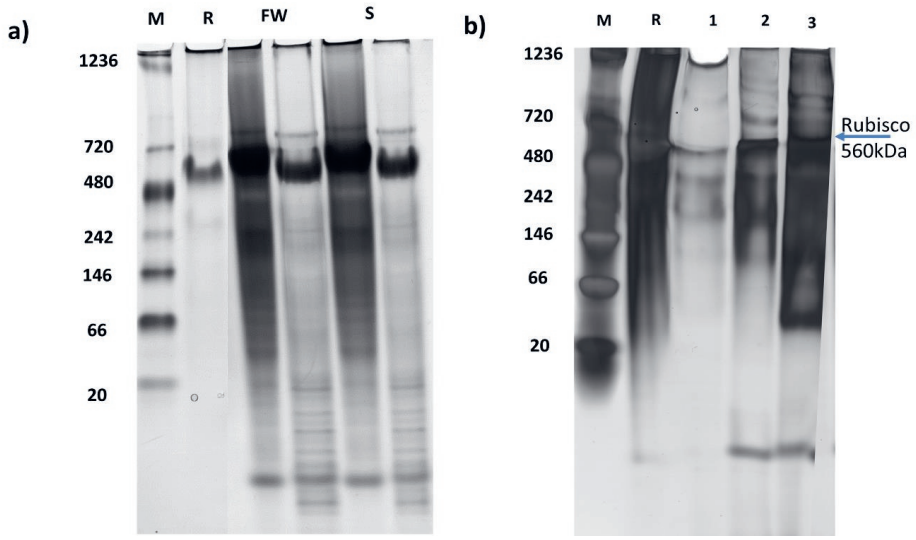


Figure 4.5. Protein conformation by Native-PAGE. a) M: marker; R: Standard Rubisco; Two concentrations of FW: fresh water *N. oleoabundans* and S: Saline *N. oleoabundans* b) M: marker; R: Standard Rubisco; 1: Interface PEG-Citrate; 2: Interface Iolilyte-Citrate; 3: Interface PEG-ChDHp. The interfaces were recovered from *N. oleoabundans* cultivated in fresh water.

Selectivity.

Table 4.3. shows the selectivity results of ATPS for the fractionation of pigments and proteins from microalgae extracts. Polymer-ionic liquid ATPS shows higher selectivity between proteins and pigments due to the preference of the pigments for the top phase and of the proteins for the bottom phase. These results suggest that polymer-IL systems do not exclude molecules by salting out, but hydrogen bonding and molecular interactions between the protein and ATPS components allow the molecules to partition, allowing a high selectivity between protein and pigments. As discussed before, this depends on protein concentration as high protein concentrations will cause significant size exclusion effects (interfacial partitioning).

Table 4.3. Selectivity results of the separation pigments/proteins from *N. oleoabundans* extract.

	Selectivity $_{pigment/protein}$	
ATPS	Saline	Fresh water
PEG 400-Citrate	0.26	0.18
Iolilyte 221PG-Citrate	0.54	0.64
PEG 400-Ch DHp	14.18	11.61

PEG400-Ch DHp partition behavior is clearly beneficial for the partitioning of the molecules in different phases (Figure 4.6). Besides that, proteins recovered in the interface seem to conserve their native conformation based on electrophoresis experiments and pigments did not suffer oxidation in the top phase. Recovery of the proteins in the bottom phase and recycle of the cholinium dihydrogen phosphate for reuse is possible by ultrafiltration as described by Ramalho et al.(166) A large number of biocompatible ionic liquids as Ch-DHp are being synthetized (168), which opens up research opportunities for polymer-ionic liquid ATPSs. This type of ATPS and their recyclability will be studied in the future to separate microalgae biomolecules in an integrated biorefinery approach. The partitioning behavior of other molecules of industrial interest such as lipids and carbohydrates from microalgae should be investigated to design a correct biorefinery approach.

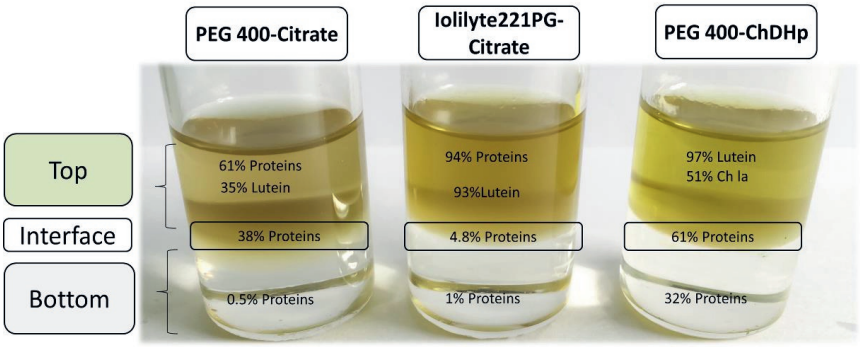


Figure 4.6. Summary of highest extraction efficiencies (%w/w) obtained in the separation of pigments and proteins from *N. oleoabundans* extract cultivated under saline conditions.

Conclusions

As a first step towards the development of a multiproduct microalgae biorefinery, three kind of ATPSs were investigated to separate proteins and pigments from microalgae extract. The traditional polymer-salt (PEG400-citrate), and two ionic liquid-based ATPSs: ionic liquid-citrate (Iolilyte221PG-citrate) and polymer-ionic liquid (PEG400-Ch DHp). Although Iolilyte221PG-citrate showed outstanding partition coefficient for pigments, a high amount of proteins also moved to the top phase. This behavior resulted in low selectivity between pigments and proteins. The quality of pigments and proteins separated by Iolilyte 221PG-citrate were considered low as pigments suffered degradation(oxidation) and the proteins did not retain their native form.

PEG400-Ch DHp system was the most selective to separate pigments (top) and proteins (interface and bottom). 97.3 ± 1.0 % of the lutein content in *N. oleoabundans* extract was separated into the top phase and very low amount of proteins moved to the top phase. Proteins moved preferentially to the bottom phase and interface. High protein concentration load resulted in interfacial precipitation, up to 92.2 ± 2.0% of proteins precipitated in the interface. This interfacial partitioning is considered an advantage in the separation of complex matrices as microalgae, since it combines separation and concentration of proteins. Thus, phase forming

components recyclability by back extraction is simpler. Besides that, proteins recovered in the interface seems to conserve their native conformation based on electrophoresis experiments and pigments did not suffer oxidation in the top phase. We demonstrated the potential of ATPS to separate different biomolecules simultaneously, giving value to different microalgae components for a sustainable multiproduct biorefinery. Furthermore, microalgae cultivated in saline and fresh water condition can be separated by this technique, which is an advantage in view of a sustainable process.

Acknowledgements

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Supporting information

Table S4.1 Composition of mixtures prepared for the partitioning of microalgae pigments and proteins.

PEG 400-Citrate		
Tie line	**X_M (wt %)**	**Y_M(wt %)**
1	31.292	21.325
2	32.626	21.851
3	35.723	23.887
4	39.055	25.192
Iolilyte 221PG-Citrate		
1	26.777	18.742
2	28.871	20.721
3	28.432	25.348
4	31.257	28.611
PEG 400-ChDHP		
1	30.413	29.412
2	31.275	33.301
3	35.016	33.905
4	36.759	35.813

Table S4.2 Effect of the tie line length on the partitioning of pigments by the ATPSs. The results represent the average of two independent experiments with the respective standard deviations

		Fresh water	Saline
ATPS	**TLL(w/w%)**	**K_p**	**K_p**
PEG 400-Citrate	39.3	10.7±0.6	21.6±1.2
	47.3	14.7±0.3	20.8±4.8
	63.8	22.2±2.1	37.8±2.5
	74.6	36.1±4.1	38.9±1.3
Iolilyte 221PG-Citrate	37.5	15.7±6.1	15.8±0.2
	53.2	21.9±0.9	14.8±0.3
	67.7	31.3±3.3	31.8±1.7
	80.3	61.9±11.3	45.8±3.5
PEG 400-Ch DHp	53.1	1.1±0.02	2.2±0.0
	72.9	2.2±0.3	4.8±0.3
	85.3	3.7±0.5	2.8±1.0
	93.3	7.2±0.7	7.0±0.8

Table S4.3 Effect of the tie line length (TLL) on the distribution of proteins among the three phases. The results represent the average of two independent experiments with the respective standard deviations

		Extraction efficiencies (%w/w)					
		Fresh Water			Salt		
ATPS	TLL	Bottom	Top	Interface	Bottom	Top	Interface
PEG 400-Citrate	39.3	0.6 ± 0.1	36.70± 7.2	62.72± 7.3	1.44± 0.2	70.06± 6.4	28.50± 6.7
	47.3	0.3± 0.01	28.36± 0.8	71.31± 0.8	0.92± 0.3	66.48± 5.3	32.59± 5.7
	63.8	0.2± 0.02	24.47± 3.2	75.38± 3.2	0.55± 0.1	61.37± 2.6	38.08± 2.5
	74.6	0.1± 0.04	22.68± 5.4	77.20± 5.3	0.52± 0.1	61.32± 0.9	38.16± 0.8
Iolilyte 221PG-Citrate	37.5	1.6± 0.4	52.53± 2.7	45.89± 3.1	1.40± 0.4	90.73± 3.4	7.87± 3.8
	53.2	1.3± 0.4	51.64± 10.2	47.02± 10.7	1.08± 0.2	91.26± 3.7	7.67± 3.9
	67.7	0.9± 0.1	42.41± 5.7	56.68± 5.8	0.81± 0.4	86.93± 4.0	12.26± 4.4
	80.3	0.4± 0.2	40.37± 8.0	59.22± 8.2	0.96± 0.3	94.25± 2.8	4.79± 2.6
PEG 400-Ch DHp	53.1	11.5± 2.3	5.78± 2.0	82.68± 4.4	43.07± 5.3	12.52± 1.8	44.41± 14.0
	72.9	7.5± 0.7	4.36± 0.9	88.14± 0.3	34.13± 0.5	10.62± 0.1	55.25± 0.4
	85.3	5.3± 0.5	4.23± 1.0	90.52 ± 1.5	24.87± 1.7	10.81± 0.8	64.32± 2.4
	93.3	4.7± 1.0	3.08± 0.9	92.23± 1.9	32.21± 0.7	6.73± 0.5	61.06± 1.2

Figures

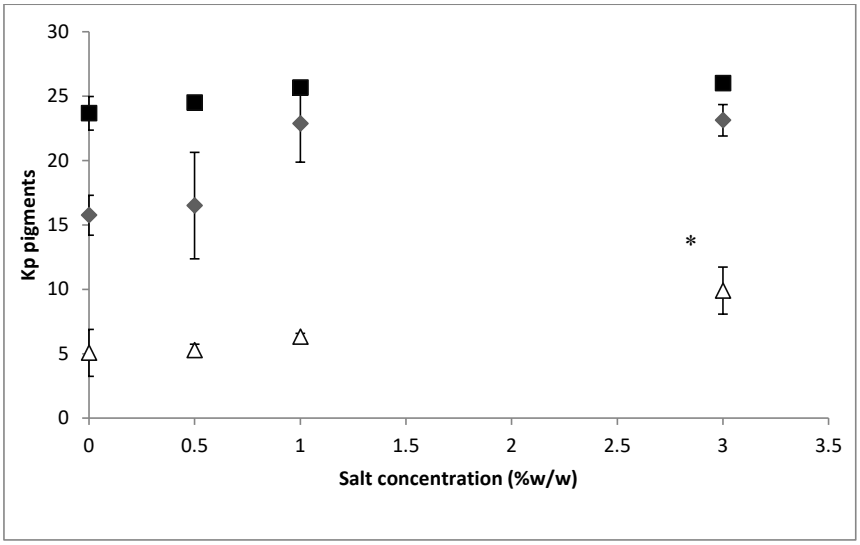

Figure S4.1 Effect of NaCl (%w/w) increase in the partitioning of pigments. ■) Iolilyte 221PG-citrate; ◆)PEG400-Citrate and Δ) PEG400-Ch DHp. Error bars indicate standard deviations. *Significant difference ($p < 0.05$).

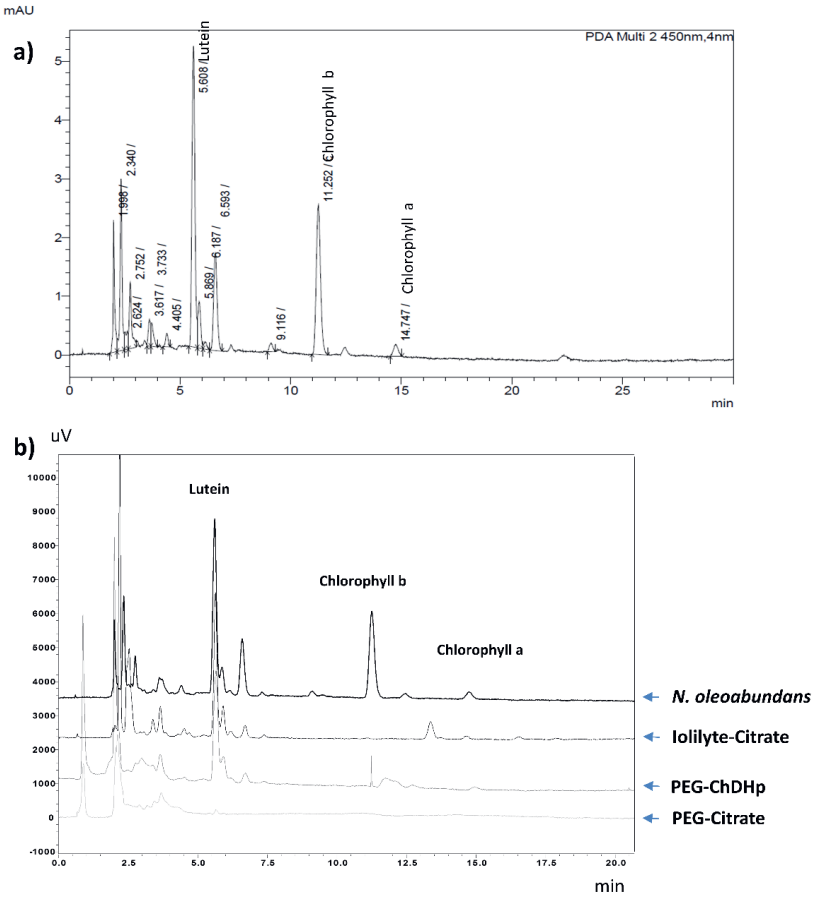

Figure S4.2 Effect of ATPS components on pigments. a) RP-HPLC chromatogram of pigments present in saline microalgae; b) RP-HPLC chromatograms of pigments recovered in the top phase by ATPSs (450 nm).

Chapter 5

Selective fractionation of free glucose and starch from microalgae using aqueous two-phase systems

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Abstract

Microalgae are a promising source of lipids, pigments, proteins and carbohydrates, which are valuable compounds for many industries. However, optimal fractionation and valorization of all produced compounds is necessary to improve the economic viability of microalgae production. This paper aims to understand the fractionation of microalgae carbohydrates (free glucose and starch) in aqueous two-phase systems. Three aqueous two-phase systems were investigated to efficiently and mildly separate carbohydrates from disrupted *Neochloris oleoabundans*. This strain contains 16 w/w% of proteins, 48 w/w% total fatty acids and 27 w/w% carbohydrates when cultivated under saline water and nitrogen depletion conditions. The protein content decreases and the amount of fatty acids and carbohydrates increases notably under stress conditions and glucose becomes the main carbohydrate in this microalgae. Glucose is present in the disrupted microalgae as part of polymeric carbohydrates (starch) or in monomeric form (free glucose). With the aqueous two-phase system PEG400-ChDHp microalgal free glucose is fractionated up to a recovery of 99% to the most hydrated bottom phase in a single step. Simultaneously, a recovery of 70% is reached for microalgal starch in the interface after two additional liquid-liquid extractions with PEG400-ChDHp. The final fractions obtained were free of pigments.

Keywords: Microalgae, carbohydrates, glucose, ionic liquids, aqueous two-phase systems, extraction

Introduction

Carbohydrates derived from renewable biomass are a promising and sustainable alternative for the petroleum-based products that are used in diverse applications (e.g. renewable energy, commodity chemicals, bioplastics, food and feed additives). For these purposes, microalgae have received increased attention[1-4]. These microorganisms can accumulate up to 60% carbohydrates, depending on the species, growth and environmental conditions[5]. Compared to plant-based feedstock, microalgae are efficient photosynthetic organisms, have high growth rates and they do not need arable land and fresh water, since they can grow in brackish water, seawater and even wastewater[6]. Currently, microalgal carbohydrates are investigated mainly for the production of biofuels (169, 170). However, carbohydrates are also a promising ingredient for the food and animal feed industries[9] and for the production of new materials such as biopolymers[10] or used as fermentation feedstock to produce hydrocarbons, lower alcohols, diols and carboxylic acids[11].

Eukaryotic microalgae and cyanobacteria accumulate carbohydrates in the plastids and cytosol as storage components (e.g. starch and free glucose) or as part of the cell wall in the form of (hemi)cellulose and polysaccharides (171), also some species can produce extracellular polysaccharides (172). The type of carbohydrates present in microalgae are species-dependent. Red algae synthesize floridean starch, while green algae synthesize amylopectin-like polysaccharides (starch). The composition of the cell wall depends on the microalgae strain as they are complex and poorly understood. Moreover, growth conditions might affect the carbohydrate composition as well. Lignin is not present in microalgae, and some species lack hemicellulose, cellulose or a cell wall[8]. These facts can be considered advantages for biorefinery, since harsh pretreatments that are normally used for lignocellulosic feedstock are not needed.

Among the carbohydrates present in green microalgae, starch is an important polysaccharide essential in human nutrition and food industry (173) and even in non-food applications. Its composition and structure varies between the sources, influencing its physico-chemical properties and functionalities (174). It is widely used in food industry as thickener, emulsifier, gelling agent and stabilizer. Some non-food industry applications are: paper, adhesives, rubber production, formulation of pharmaceutic and cosmetic products, microcapsules for small molecules and for the production of high quality biodegradable plastic films (174-176). Limited research has been done on the extraction and use of starch from microalgae for food applications and other industries, which opens up many research opportunities in strain development, biorefinery, starch structure and functionality (177).

Common methods to extract polysaccharides from microalgae cells and convert them into easily fermentable sugars involve: hydrolysis using harsh acidic (H_2SO_4 and H_3PO_4) or alkaline conditions (NaOH and ammonium) or enzymatic methods (178). Additionally, physical methods include: hot-water treatment, microwave-assisted extraction and ultrasonic-assisted extraction (179). Organic solvents are generally used for the separation of hydrophobic and hydrophilic components from the disrupted microalgae cells. These conditions, however, could affect the structure and functionality of other microalgae valuable components such as proteins

and pigments (129, 180). Therefore, it is preferable to find novel and mild alternatives to the conventional extraction technologies.

Aqueous two phase systems (ATPS) have emerged as a more biocompatible and more efficient liquid-liquid extraction method for the fractionation and purification of biomolecules (86, 181). ATPS is formed by two immiscible aqueous phases, providing a mild environment for the biomolecules, because their main component is water. ATPS is a technologically simple process, low cost and easy to scale up (182). The broad collection of phase forming components that exist, make this technology very versatile. Additionally, ionic liquids (ILs) have been studied to enhance the extraction efficiencies of different target molecules. ILs are molten salts with unique characteristics that make them very attractive for separation processes. They possess low vapor pressure, are non-flammable, have good thermal and chemical stabilities and great solvation abilities (183). They are considered a "green" solvent, are tunable and multipurpose materials due to their ionic character, structure and organization (184).

Aqueous two phase systems have been studied mainly for the fractionation of proteins and enzymes (9). The interest in applying this technique to biological mixtures has led to the research of other target molecules like carbohydrates, pigments and small bioactive compounds. ATPSs were employed to purify and concentrate carbohydrates from *Cordyceps sinensis (185), Aloe vera(186), Brassica oleracea L.(187)* and *beetroots(188)*. This is the first study to investigate the separation of microalgal carbohydrates (free sugars such as glucose as well as starch) using aqueous two-phase systems, and its integration in a multi-product biorefinery concept.

Materials and methods

Materials

The ionic liquids used: IoliLyte 221 PG, >95% and choline dihydrogen phosphate (ChDHp), >98% were procured from Iolitec (Ionic Liquid Technologies, Germany). Potassium citrate tribasic monohydrate, polyethylene glycol (PEG) 400, hydrochloric acid, and standard D-(+)-glucose were purchased from Sigma-Aldrich. Citric acid was obtained from Merck and starch from maize was included in the Megazyme® kit (Wicklow, Ireland). The GOPOD kit was also from Megazyme and all other chemicals were from the highest purity.

Microalgae cultivation, harvesting and cell disruption

To study the carbohydrate content in microalgae, four cultivation conditions were used: Fresh water, artificial sea water, nitrogen depletion (N-) and no nitrogen depletion (N+).

Neochloris oleoabundans (UTEX 1185, University of Texas Culture collection of Algae, USA) was cultivated in a fully automated 1300L vertical stacked tubular photo bioreactor (PBR) located at AlgaePARC, The Netherlands. It was cultivated using Bold's Basal medium (143) at a pH value of 8.0 and the temperature was controlled at 30 °C. To cultivate microalgae under saline conditions, artificial sea water was used: NaCl: 24.5g/L; $MgCl_2$: 9.8 g/L; $CaCl_2$: 0.53g/L; K_2SO_4: 0.85g/L; $NaSO_4$: 3.2g/L; $NAHCO_3$: 0.8g/L. The microalgae were harvested (4000rpm)

using a spiral plate centrifuge (Evodos 10, Evodos, The Netherlands) and the biomass obtained was suspended in MilliQ® water to obtain a biomass concentration of ~90g L^{-1}. The algal cells (approx. 28 ml) were disrupted for 10 minutes in a horizontal stirred bead mill (Dyno-Mill Research Lab from Willy A. Bachofen AG Maschinenfabrik, Switzerland) with a milling chamber of approx. 79.6 ml using 0.5 mm ZrO_2 beads (approx. 51.6 ml) as described by Postma et al., (2016)[34]. Bead milled microalgae suspension was stored at -20°C until further use.

Microalgal characterization.

Carbohydrates: The total carbohydrate content was determined with the Dubois method(108) whereby glucose was used as standard and analysis made in triplicate and statistically evaluated by including the standard error. The carbohydrate composition of *N. oleoabundans* was determined by High Performance Anion Exchange Chromatography (HPAEC) as described by Gilbert-López et al.(189). The microalgae were first freeze dried and subsequently acid hydrolyzed before their composition was determined by HPAEC. Deoxy-galactose was used as internal standard and experiments were carried out in single.

Protein: Protein analysis was done according to Gilbert-López et al. (189) using a FlashEA 1112 nitrogen analyzer (Thermo Fisher Scientific, 337 Waltham, MA, USA). D-methionine was used as standard and a N-to-protein conversion factor of 5.5 was used to calculate total protein from total nitrogen. Analyses were made in triplicate and statistically evaluated by including the standard error.

Lipids. Samples were freeze dried before analysis of the total fatty acid concentration. The analysis consisted of a sequence of mechanical cell disruption, solvent-based lipid extraction, transesterification of fatty acids to fatty acid methyl esters (FAMEs), and quantification of FAMEs using gas chromatography (GC-FID) as described by Breuer et al.(190). The triacylglycerides (TAGs) and polar acyl lipids (PLs) were fractionated using solid phase extraction. TAGs were eluted from the column using 10 mL 7:1 (v/v) hexane:diethylether. Subsequently, PLs were eluted using 10 mL 2:2:1 (v/v/v) methanol:acetone:hexane (191). Total fatty acid (TFA) composition and content were calculated by taking the sum of all fatty acids in both fractions (190). Analyses were made in triplicate and statistically evaluated by including the standard error.

Preparation of aqueous two-phase systems.

Based on the total carbohydrate content *N. oleoabundans* cultivated in saline water and nitrogen depleted conditions were used for the fractionation experiments. Three ATPSs were selected for the fractionation of microalgae biomolecules (110). This selection was based on biocompatibility, low toxicity and their interaction with the protein Rubisco (Ribulose-1,5-biphosphate Carboxylase Oxygenase), which is present in microalgae and able to lose its native conformation under non-mild conditions. Mixtures were prepared gravimetrically $\pm 10^{-4}$g with a volume ratio (V_r) between top and bottom of 1. D-glucose and starch from maize were selected as standard molecules to study their partitioning behavior. The total concentration of the standard molecules in the mixture was 0.05g L^{-1}. We prepared the ATPS mixtures with

increasing the phase forming component concentrations along four tie lines previously constructed by Suarez Ruiz et al.(110) (Table 5.1).

Table 5.1. Concentration (w/w%) of phase forming components used for the separation experiments

Tie line	Polymer - Ionic liquid		Polymer – Salt		Ionic liquid - Salt	
	PEG400	ChDHp	PEG400	Citrate	Iolilyte 221PG	Citrate
1	29.4	30.4	21.3	31.3	18.7	26.8
2	33.3	31.2	21.8	32.6	20.7	28.9
3	33.9	35.0	23.9	35.7	25.3	28.4
4	35.8	36.8	25.2	39.0	28.6	31.3

To study the fractionation of microalgae carbohydrates, one gram of bead milled *N. oleoabundans* suspension was added to each system and MilliQ® water was finally added to complete 10 grams in each system. Experiments were made in duplicate and blanks without microalgae or standard molecules were prepared as control for the analysis methods. All mixtures were mixed for 1 hour at room temperature in a rotary shaker at 50 rpm and centrifuged at 2500 rpm (1200 x g) for 10 min at room temperature to ensure phase separation. Afterwards, the phases were separated and the weights and volumes were noted.

To perform the second and third fractionation steps, the interface was gently separated from the aqueous phases and weighted. Subsequently, the same amount of phase forming components as in the first extraction was added to the interface.

Quantification of glucose.

Two methods were used to quantify glucose before and after the fractionation experiments: a YSI 2700® biochemistry analyzer (Yellow Springs Instruments) and the Megazyme® kit (Wicklow, Ireland): Soluble sugars in ATPS were quantified by the reaction with a solution containing *p*-hydroxybenzoic acid, sodium azide (0.095 % w/v), glucose oxidase plus peroxidase and 4-aminoantipyrine (GOPOD reagent) from Megazyme®. Samples were mixed with the reagent at a ratio 0.1:3 (v/v) and incubated at 50 ºC for 30 min. After cooling down to room temperature, quantification was conducted by measuring absorbance at 510 nm using a spectrophotometer (Hach Lange DR6000). GOPOD reagent and glucose were used as blank and standard, respectively. Analyses were made in triplicate and statistically evaluated by including the standard error.

Starch analysis.

Starch content was quantified before and after fractionation experiments by the total starch protocol of Megazyme® kit (Wicklow, Ireland) adapted from Dragone et al.(192). Maize starch was used as a positive control and calibration curves were made from D-glucose. The quantification was performed by absorbance at 510nm using a spectrophotometer (Hach Lange DR6000). Analyses were made in triplicate and statistically evaluated by including the standard error.

To describe the distribution of glucose and starch in the ATPS, partition coefficients (Kp) and extraction efficiencies (EE%) were calculated. For glucose, for example, Eq. 5.1 and Eq. 5.2 were used to calculate Kp and EE%, respectively. $C_{glucose}$is the concentration of glucose in the phase, V the volume and $m_{glucose,initial}$ the initial mass of glucose in the microalgae added to the systems.

$$Kp_{glucose} = \frac{Cglucose_{,top}}{C_{glucose,bottom}} \quad (5.1)$$

$$EE_{glucose}\% = \frac{C_{glucose,top} * V_{top}}{m_{glucose,initial}} \quad (5.2)$$

In the separation of carbohydrates from microalgae using ATPS an interface was formed. This interface was carefully separated from the aqueous phases and its glucose content and starch were analyzed to complete the mass balance Eq. 5.3. $m_{glucose,interface}$ represents the mass of glucose in the interface and $m_{glucose,initial}$ the initial mass of glucose in the microalgae added to the systems.

$$EE_{glucose}\% = \frac{m_{glucose,interface}}{m_{glucose,initial}} \quad (5.3)$$

Results and discussion

Carbohydrate composition of *Neochloris oleoabundans*

Table 5.2. (Bio)chemical composition (w/w%) of *N. oleoabundans.* All measurements were performed in triplicate and standard error included.

Growth condition		Carbohydrates	Protein	Total fatty acids (TFA)
Fresh water	N+	10.7 ±0.7	44.7 ±0.4	12.4±0.0
	N-	17.4 ±0.5	18.7 ±1.4	44.1±0.8
Artificial seawater	N+	13.3 ±0.2	49.6 ±0.6	7.7±0.0
	N-	27.1 ±0.1	16.0 ±0.4	48.0±6.2

Table5.2. presents the biochemical composition (carbohydrates, proteins and total fatty acids) of *N. oleoabundans* cultivated under four different conditions. These conditions were: artificial sea water or fresh water under nitrogen depletion (N-) or nitrogen repletion (N+). It can be observed that the growth conditions notably influence the biochemical composition of the microalgae. Under saline and nitrogen depletion conditions *N. oleoabundans* accumulates more carbohydrates and lipids (TFA), while the protein content decreases. The increase of carbohydrates and lipids in this microalgae has previously been used as a strategy to provide a more economically feasible scenario for microalgal-based biofuels (191, 193). Furthermore, the

use of salt water can reduce production costs and the fresh water footprint of large scale microalgae production (5).

The industrial application of carbohydrates depends on the chemical and physical properties of these biomolecules. Therefore, the carbohydrates accumulated in *N. oleoabundans* cultivated under different conditions were characterized. The total carbohydrate content in *N. oleoabundans* was ~10.7 w/w% dry matter when it is cultivated in fresh water and an excess of nitrogen (N+). When the microalgae grow in artificial saline water and under nitrogen depletion (N-), the content of total carbohydrates increases up to ~27.1 w/w% dry matter.

Table 5.3 Carbohydrate composition in (mol%) of *Neochloris oleoabundans* in different cultivation conditions performed in duplicate.

*total carbohydrate content (w/w% dry matter) carried out in triplicate and standard error included.

Growth condition	**Fresh**		**Artificial saline**	
Carbohydrate	**N+**	**N-**	**N+**	**N-**
Fucose	0.0	0.0	0.0	0.0
Arabinose	3.8	2.9	2.7	0.6
Rhamnose	20.0	10.6	11.8	2.2
Galactosamine	0.0	0.0	0.0	0.0
Galactose	30.0	11.6	23.1	10.3
Glucosamine	7.6	5.2	4.0	0.2
Glucose	22.0	55.3	50.2	76.9
Xylose	4.3	5.1	2.2	0.9
Mannose	8.7	3.3	3.8	3.5
N-Acetyl-galactosamine	0.0	0.0	0.0	0.0
N-Acetyl-glucosamine	0.0	0.0	0.0	0.0
Galacturonic acid	0.0	0.0	0.0	0.0
Glucuronic acid	3.7	6.1	2.1	5.4
Total carbohydrate content*	10.7 ±0.7	17.4±0.5	13.3±0.2	27.1±0.1

The carbohydrate composition (mol%) of *N. oleoabundans* is presented in Table 5.3 which were performed in duplicate with the main focus showing large changes in the composition and not having the intention to know the exact value. Whereas for the total carbohydrate content the exact values are important and performed in triplicate and statistically evaluated. It is clear that depending on the cultivation conditions, not only the total carbohydrate content changes, but also the carbohydrate profile. The amount of galactose, for example, is higher than glucose when *N. oleoabundans* is grown in fresh water under no nitrogen depletion (N+). Instead, when microalgae are grown in artificial saline water, more glucose is accumulated, surpassing galactose as the main carbohydrate in the algal cells. Additionally, when the microalgae grow under nitrogen depletion (N-), the content of glucose increases. Microalgae cultivated in artificial saline water under nitrogen depletion (N-) the content of total carbohydrates is 27.1 w/w%, with glucose as the most abundant monosaccharide after hydrolysis. This glucose is

stored in the microalgae as part of a polysaccharide (starch) or as monomer (free glucose). Microalgae contains under these cultivation conditions 14.3 ± 1.4 w/w% of starch and 9.8 ± 1.3 w/w% of free glucose.

The effect of the cultivation conditions on the carbohydrate profile has been addressed in different studies and this knowledge has been used to accumulate metabolites of interest in microalgae cells (191, 194, 195). Nitrogen depletion is widely used for this purpose, especially to enhance the production of fatty acids. In the absence of a nitrogen source, the flow of fixed carbon is diverted from proteins to energy and carbon storage compounds. Thus, nitrogen depletion leads to the accumulation of lipids and/or carbohydrates (mainly starch). Salt stress also causes the accumulation of carbohydrates in different microalgae strains (194). As NaCl is a commonly encountered inorganic nutrient in microalgae grown in salty water. High salt water stress generates reactive oxygen species that inhibit Rubisco activity and mediates photo-inhibition which concomitantly decreases biomass growth and accumulation of carbohydrates takes place as a response to an immediate NaCl shock. It seems that the sucrose pathway in the metabolism is most affected by this cultivation condition (196).

The high content of total glucose (as monomer as well as part of polysaccharides) in microalgae has also been reported for other microalgae species (197, 198). *N. oleoabundans* with a high content of carbohydrates represents a promising feedstock to produce biochemicals, biofuels such as bioethanol (193) and other valuable products (199).

Carbohydrates separation in aqueous two-phase systems

Aqueous two-phase system (ATPS) was studied as a fractionation method for microalgal carbohydrates. D-Glucose and starch from maize were used as standard molecules and the bead milled suspension of *N. oleoabundans* cultivated in artificial saline water under nitrogen depletion (N-) was used as a complex mixture. This cultivation condition was selected due to the high amount of carbohydrates accumulated by the microalgae. Three ATPSs: polyethylene glycol (PEG)400-potassium citrate, Iolilyte 221PG-potassium citrate and PEG400-cholinium dihydrogen phosphate (ChDHp) were selected based on the screening described by Suarez Ruiz et al.(110). The ionic liquids, polymers and salt used in these experiments were carefully selected for their biocompatibility, low toxicity and ability to form ATPS without affecting the native conformation of the proteins. A schematic summary of the process performed and a description of the systems phases is shown in Figure 5.1.

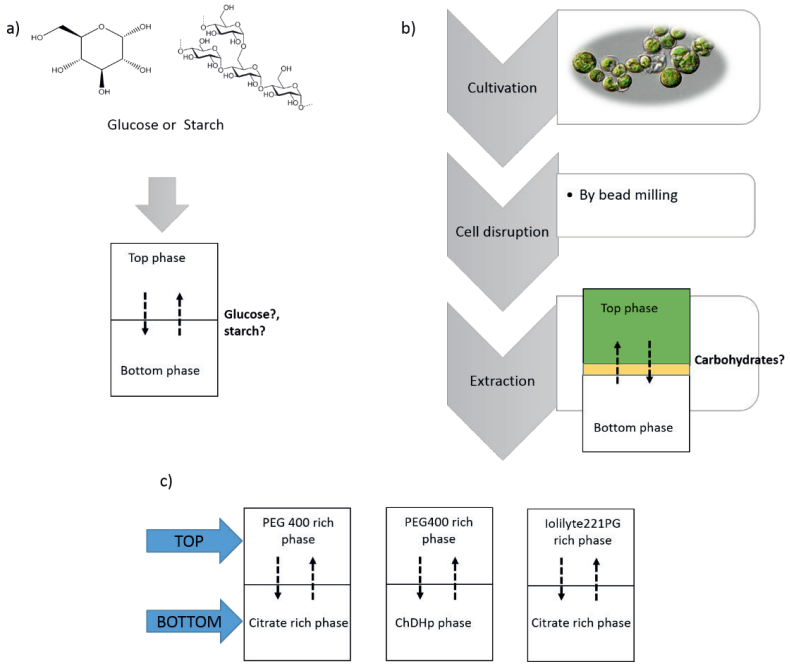

Figure 5.1 Schematic representation of the partitioning experiments. a) Partitioning of standard molecules in ATPS: D-glucose and starch from maize; b) Partitioning of carbohydrates from *Neochloris oleoabundans* (bead milled suspension) cultivated in artificial saline conditions under nitrogen depletion (N-); c) ATPSs used and the description of the two aqueous phases.

Pure D-glucose and glucose from microalgae.

D-Glucose was selected as a standard monosaccharide to study its partitioning behavior in the three ATPSs. The separation of this standard molecule was compared with free glucose from microalgae. Both, the standard monosaccharide (D-glucose) and the free sugar from the microalgae migrate preferentially to the most hydrated phase (bottom phase). This is due to the strong molecular interactions between monomeric sugars and water (200, 201). Recoveries of 82 to 99% of D-glucose were obtained in the bottom phase after a single-step ATPS. The highest extraction efficiencies (%w/w) obtained for D-glucose and microalgae free glucose are shown in Figure 5.2. PEG400-ChDHp concentrated the highest amount of free sugars from microalgae in the bottom phase (99%), followed by PEG400-citrate (93%), while Iolilyte221PG-citrate concentrated 82%. Cholinium-based ionic liquids are highly hydrophilic due to the polar hydroxyl group of one of the cation side chains (87). The hydrophilicity of the IL-rich phase in the PEG400-ChDHp (bottom) phase caused by the amount of water may enhance the separation of carbohydrates in this system.

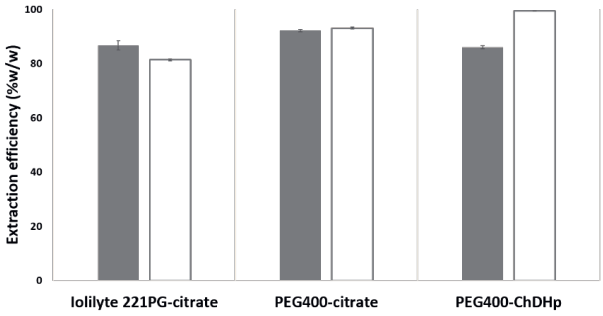

Figure 5.2. Glucose partitioning to the bottom phase: Extraction efficiencies (%w/w) using three different ATPSs; Standard glucose (filled bars) and free glucose from *N. oleoabundans* (bead milled suspension) cultivated in artificial saline conditions under nitrogen depletion (N-) (open bars). Experiments performed in triplicate and error bars indicate standard deviations.

Carbohydrates (also called saccharides) are polar biomolecules that possess many hydroxyl groups. Monosaccharides as well as some polysaccharides have high water solubility as a result of their ability to establish hydrogen bonds with water (202). This high affinity of carbohydrates for water seems to be responsible for their preference to migrate to the most hydrated phase (bottom phase). The polymeric carbohydrate component starch is insoluble in water and although containing many –OH functional groups on the surface, water binds to starch forming a gel and partition towards the interface. This polymer presents low solubility in almost any solvent (203). Starch partitioning in ATPS is discussed in the next section.

The effect of the ATPS phase forming components was studied by increasing the tie line length (TLL), which represents the composition and thermodynamic difference of the two phases. A positive effect was found while increasing the TLL, increasing the concentration of the phase forming components more free sugars are accumulated in the bottom phase (Supplementary data).

Pei et al.(200) describes how the structure of the saccharides seems to influence the partitioning behavior of other molecules (e.g. proteins) present in a solution. A higher amount of hydroxyl groups in the saccharides leads to a higher kosmotropic behavior, enhancing both, hydrogen bonding and hydrophobic interactions. These interactions increase the extraction efficiency of proteins. The hydrogen bond formation between carbohydrates and water reduces the number of free water in the bottom phase, forcing the proteins to migrate to the opposite phase than the carbohydrates. The ability of carbohydrates to form hydrogen bonds is considered an advantage, because carbohydrates migrate to the bottom phase reaching a recovery of 99% while other biomolecules (e.g. proteins) migrate to the opposite phase. Due to this reason ATPS is a very promising technology in the microalgae biorefinery.

Maize starch and starch from microalgae

The partitioning behavior of starch from microalgae was studied in the three ATPSs. This behavior was compared with the partitioning of starch from maize, selected as a standard

polysaccharide. The disrupted microalgal suspension added to the ATPSs forms three phases: top, bottom and a third phase (interface) between the two aqueous phases (top and bottom).

In Figure 5.3a) we show the distribution of microalgae starch in the three phases formed. Up to 86% of the microalgae starch was concentrated in the interface in a single-step Iolilyte 221PG-citrate ATPS. Although the polymer/ionic liquid (PEG 400-ChDHp) ATPS fractionates a high amount of starch in the bottom phase (18%) compared with the other two ATPSs, the partitioning preference of starch is clearly to the interface. Unlike the case of D-glucose and free sugars, starch does not prefer to migrate to the most hydrated phase (bottom phase). Some ionic liquids are able to solubilize up to 20% starch, however, the studies found used high temperature (60-100 °C) (204, 205). It seems that the origin of the starch influences the solubility, due to granule form and size differences.

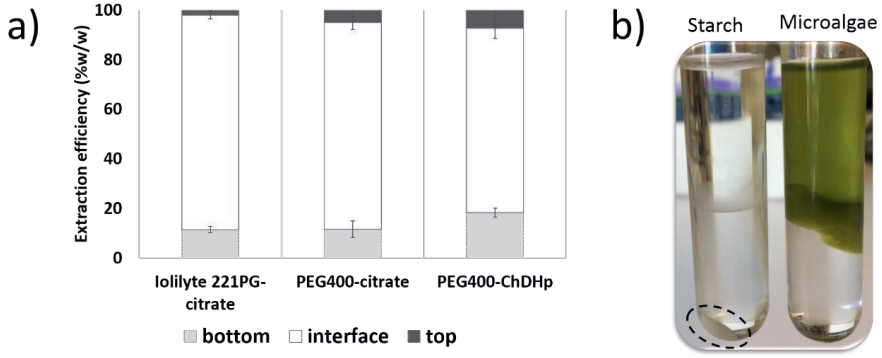

Figure 5.3. Purified starch from maize and microalgae partitioning in three ATPSs. a) Distribution of starch from *N. oleoabundans* (bead milled suspension) cultivated in artificial saline conditions under nitrogen depletion (N-) among the three phases in the ATPSs. Experiments were carried out in triplicate and error bars indicate standard deviations; b) Picture of purified starch from maize and microalgae in PEG400-ChDHp ATPS.

The concentration of starch in the interface seems to be a consequence of its low solubility in the aqueous solutions used. Figure 5.3b) shows the partitioning behavior of purified starch from maize and a bead milled microalgae suspension. The maize starch added to the ATPSs precipitated below the bottom phase and additionally a small layer in the interface was observed. In the aqueous phases (top and bottom) starch was not detected after quantification by the colorimetric method. Starch from maize is not soluble in water at room temperature and it seems that it is neither soluble in the aqueous phases of the ATPSs used. The solubility of starch depends on the amylose and lipid content and granule organization (206), which may explain the partitioning behavior difference between microalgae starch and maize starch. However, limited information is available in literature about the physico-chemical properties of starch granules in microalgae. Other components in the microalgae suspension (e.g. lipids) may influence the partitioning behavior of microalgae starch, compared to purified starch from maize.

Microalgae suspension forms a thick like-emulsion layer at the interface of the ATPSs, where starch is concentrated together with other microalgae biomolecules. The bead milled microalgae suspension used in the fractionation experiments has soluble and non-soluble components such as cell debris. Furthermore, *N. oleoabundans* cultivated under nitrogen depletion (N-) accumulates a high amount of lipids, which may lead to the formation of a stable emulsion after bead milling (207). In fact, several authors have reported emulsion formation in an attempt of extracting lipids from wet biomass (208). Thus, the interface layer formed may be caused by other non-soluble components in the microalgae such as lipids and cell debris.

To further improve the purification of microalgae carbohydrates (starch and free glucose), two extra steps of fractionation with ATPS were performed. Figure 4 shows the distribution of carbohydrates among the three phases in three fractionation steps by PEG400-ChDHp. It was observed that free sugars are separated almost completely in a single-step ATPS (Figure 5.4a). Free glucose migrates to the bottom phase due to their high ability to form hydrogen bonding, and only ~5% remains in the interface after the first fractionation step. On the other hand, a low amount of starch (~8%) migrates to the aqueous phases after three fractionation steps. The microalgae starch remains in the interface (~70%) after three fractionation steps (Figure 5.4b). The purification of starch increases with the number of steps, due to the fractionation of other components of the microalgae (e.g. pigments) to the aqueous phases. After the third ATPS step a whitish emulsion-like solid was observed in the interface (Figure 5.4c). The ATPS phase forming components do not have a big influence in the fractionation and purification of microalgae free sugars and starch (Supplementary data), however, they have a great influence in the separation of other microalgae components such as pigments and proteins (110).

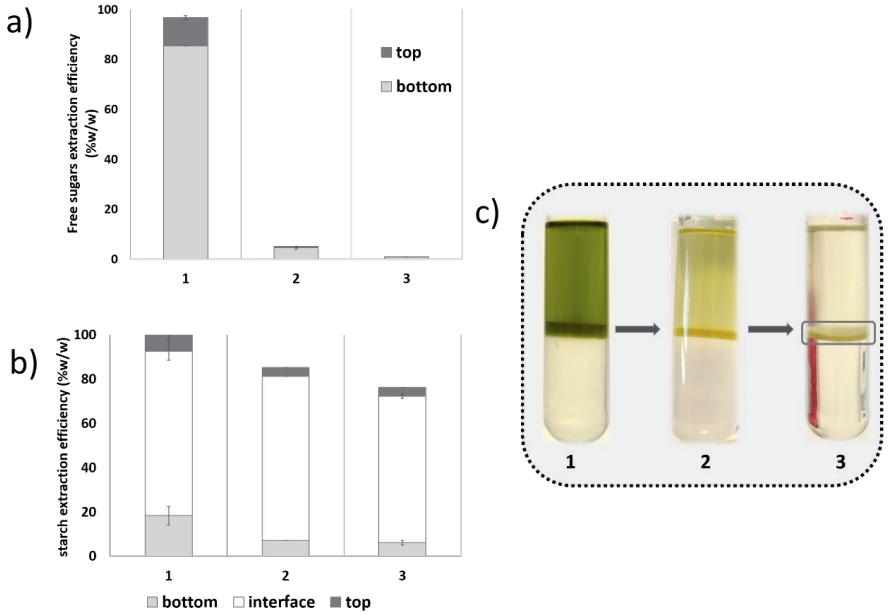

Figure 5.4. Extraction efficiency of starch and glucose from *N. oleoabundans* (bead milled suspension) cultivated in artificial saline conditions under nitrogen depletion (N-) in three fractionation steps by PEG400-ChDHp ATPS: a) distribution of glucose among the two aqueous phases formed in the three fractionation steps; b) distribution of starch among the three phases formed in three fractionation steps. Experiments were carried out in triplicate and error bars indicate standard deviations; c) Picture of carbohydrates purification in three fractionation steps.

Perspectives on ATPS to separate carbohydrates from microalgae

The integration of a microalgae biorefinery to simultaneously fractionate several valuable components could improve the economics of microalgae production. *N. oleoabundans* cultivated under saline conditions and nitrogen depletion (N-) contains free sugars (e.g. free glucose) and polysaccharides such as starch. Microalgae starch, is an important biopolymer that can be used for different industries such as the food industry. The purification of starch from microalgae with a green technology able to concentrate starch and separate other microalgae biomolecules (e.g. free sugars and pigments) at the same time opens great opportunities in the biorefinery field.

Based on the experimental results, Figure 5.5 shows a possible application scenario for the fractionation of microalgal carbohydrates by ATPS. Free glucose (representing free sugars) is separated in the bottom phase with outstanding yields (82-99%) in the first fractionation step. Free sugars can be used as a carbon source in fermentation as a feedstock for alcohols, acids and chemicals. It was previously demonstrated that proteins partition to the bottom phase when using PEG 400-ChDHp ATPS (209). Since the first step separates most of the free glucose from the microalgal suspension to the bottom phase, the proteins can be separated from the free

glucose using ultrafiltration or another ATPS (210), while the salt can be recycled and reused in the following fractionation steps.

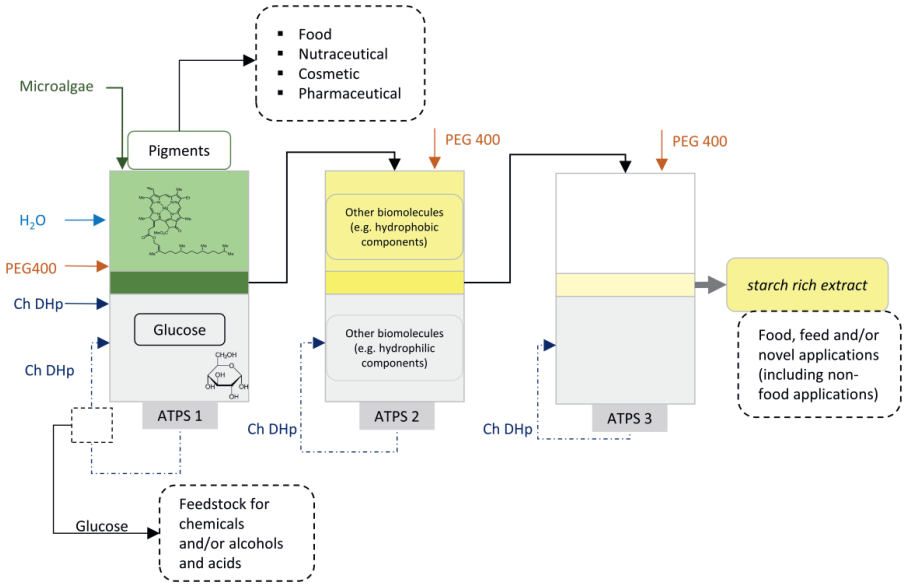

Figure 5.5 Scheme of the possible application scenario for the fractionation of microalgae components in ATPS.

Although pigment quantification data was not presented in this study, these high value biomolecules preferentially migrate to the top phase. After a third ATPS separation step, the green characteristic color disappeared from the interface (Figure 5.4c). *N. oleoabundans* contains chlorophyll a and b and it also accumulates lutein, cantaxanthin, zeaxanthin, and astaxanthin monoesters and diesters, which are promising ingredients with pharmaceutical and nutraceutical applications (125). No recycle step was proposed for the top phase, because its design depends on the biomolecules fractionated in this phase, which might be different for each system. However, other authors have confirmed the recyclability of PEG from aqueous solutions by suitable solvents or by direct distillation (211).

About 70% of the total starch content in *N. oleoabundans* (bead milled suspension) cultivated in artificial saline conditions under nitrogen depletion (N-) was concentrated at the interface free of pigments after three ATPSs steps. Moreover, the recovery of starch in the interface makes the process easier, because extra unit operations and/or solvents are not needed to recover the starch fraction. Microalgae for starch production is considered highly efficient and full of advantages in comparison with traditional crops (corn, rice, potato, oat), because they do not compete for (arable) land space and they do not need fresh water for their cultivation. Starch is an important biopolymer widely used in the food and other industries. Novel applications of

starch are: in free-fat food formulation; novel material in pharmaceutics and cosmetics; encapsulation material for flavor essences; coatings in paper and textile industry and in biodegradable plastic film production (175). The evaluation of the physico-chemical properties of starch from *N. oleoabundans* is necessary to confirm its potential in the different applications.

Carbohydrate partitioning in ATPS is not notably affected by the phase forming components. Since ionic liquid-based ATPS does not alter the yield of extraction of neither free sugar nor starch, the inexpensive PEG400-citrate seems to be a good option to separate carbohydrates from microalgae. However, the partitioning behavior of proteins can be tuned depending on the phase forming components. Previous results indicate that an ionic liquid-salt ATPS concentrates a higher amount of protein in the top phase, while the polymer-based ATPSs concentrate the protein at the interface (209). Thus, depending on the desired final product purity, the ATPS can be selected and designed. The cholinium-based ionic liquids are considered to be biodegradable and in addition these ionic liquids enhance the stability and activity of enzymes and proteins (212). Thus, in the development of a greener separation method this group of ionic liquids are desired.

Conclusions

In this article the separation of microalgae carbohydrates (free glucose and starch) in three biocompatible ATPSs is reported. Bead milled *N. oleoabundans,* cultivated under saline and nitrogen depleted conditions, was used as carbohydrate source. We demonstrated that an ATPS is a promising separation method for carbohydrates and other components from microalgae. Up to 99% of microalgal free glucose was recovered in the bottom phase in a single ATPS with PEG400-ChDHp, which was explained by the ability of these biomolecules to form hydrogen bonds with water. Starch, an abundant polysaccharide present in the microalgae, was simultaneously separated and concentrated at the interface after two additional ATPS steps up to 70% resulting in a pigment-free starch rich extract.

Acknowledgements

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Statement of Informed Consent, Human/Animal Rights, if any, for the work described. No conflicts, informed consent, human or animal rights applicable.
Declaration of authors agreement to authorship and submission of the manuscript for peer review. The authors agree to their contribution, authorship and submission of the manuscript for peer review.

Authors' contributions

Catalina Suarez Ruiz made a proposal for setup, performed all the experiments together with Santiago Zarate Baca including the ATPS studies and the associated analytics. Carbohydrate composition analysis was carried out by Catalina Suarez Ruiz and Lambertus van den Broek and critically analysed. Michel Eppink and Corjan van den Berg were deeply involved in the

setup of the various experiments and performed a critical analysis of the data obtained. Results were also discussed with Rene Wijffels who critically analysed the data. Catalina Suarez Ruiz drafted the manuscript which was critically reviewed by Corjan van den Berg, Lambertus van den Broek, Rene Wijffels and Michel Eppink. Catalina Suarez Ruiz finalized the manuscript and Michel Eppink adapted it so that it could be submitted to Algal Research.

Conflict of interest

The authors declare that they have no competing interests.

Supplementary information

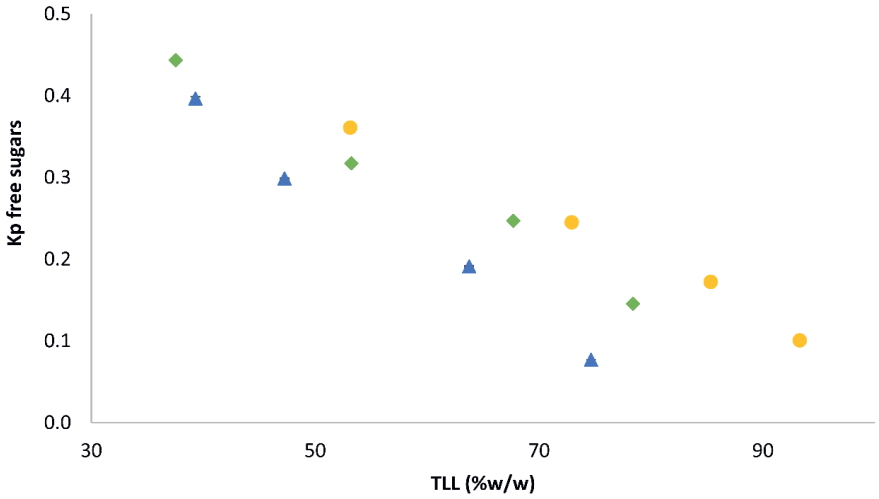


Figure S5.1. Influence of tie line length on Kp of free sugars from microalgae; ● PEG400-ChDHp; ◆ Iolilyte221PG-citrate; ▲ PEG400-citrate. Error bars indicate standard deviations

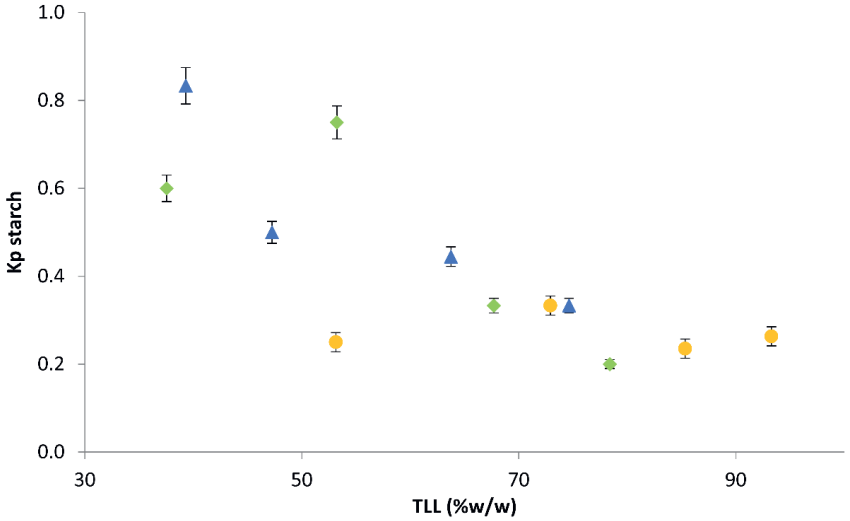


Figure S5.2. Influence of tie line length on Kp of starch from microalgae; ● PEG400-ChDHp; ◆ Iolilyte221PG-citrate; ▲ PEG400-citrate. Error bars indicate standard deviations

Chapter 6

Efficient fractionation of lipids in a multi-product microalgal biorefinery by polymers and IL-based aqueous two-phase systems

Submitted as:

C.A. Suarez Ruiz, Oriol Cabau Peinado, C. van den Berg, R.H. Wijffels, M.H.M. Eppink. Efficient fractionation of lipids in a multi-product biorefinery by polymers and IL-based aqueous two-phase systems.

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 (213) (6).

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 (214, 215). PLs, namely phospholipids are widely used for their emulsifying and structural improvement properties in food matrices (216). Phospholipids are also used in nutritional and pharmaceutical applications (217, 218). 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 (33). Water is the common component in ATPS, which provide a mild condition for the extraction of biomolecules. Mild conditions are crucial when working with functional compounds with applications for the food and pharmaceutical industries (219). Furthermore, ATPS allows the replacement of volatile organic solvents, which are commonly used in liquid-liquid extraction and represent environmental and health hazards (220). The application of ATPS was already proven for many uses, including the separation of proteins, amino acids, antioxidants, pigments, colorants, pharmaceuticals (e.g. antibiotics) from model molecules to real matrices (86).

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 sulphate (221). 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* (222). Phong et al. (98) 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. (95) used an ATPS formed by PEG and potassium phosphate for the extraction of C-phycocyanin from *Spirulina*

maxima. Other studies focussed on the fractionation of polysaccharides (223), lutein (145) and B-phycoerythrin (224). 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 its design and application for the purification of a wide range of biomolecules. Therefore, it is an excellent basis for the development of a multiproduct biorefinery.

Ionic liquids (ILs) are characterized by high design flexibility and tunability, wide range of polarity and capacity to solubilize a wide variety of organic and inorganic compounds (22, 225). 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 (225-227). It is preferred, however, to reuse the IL after regeneration to avoid presence of IL in the waste and to minimize costs for the process.

In Table 6.1 a SWOT (Strength, Weakness, Opportunities, Threats) is presented to compare the more traditional Aqueous two-phase systems (ATPS) such as salt-based or polymer-based ATPS with the Ionic Liquid-based ATPS .

Table 6.1: Comparison of Conventional ATPS and Ionic Liquid based ATPS.

Conventional ATPS	**Ionic Liquid-based ATPS**
Strength	
- Extensive research available - Price (relatively inexpensive) - Biocompatibility (e.g. PEG)	- Flexibility (more possible combinations) - Wider extraction capabilities - Tuneable selectivity - Wide range of applications - Process conditions (e.g. lower viscosity) - Biocompatibility (e.g. Choline) - Recycling feasible
Weakness	
- Flexibility (Less combinations available) - Limited range of polarity of coexisting phases - Process parameters limiting industrial scale (high viscosity) - Still a conservative approach known for years and still not implemented at manufacturing scale	- Price - Insufficient understanding of partition behaviour
Opportunities	
- Primary clarification of cell cultures - Purification of proteins and small molecules (hydrophilic compounds)	- Development of Ionic liquids of low toxicity, low cost and high biodegradability

	- 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)

IL-based ATPS demonstrated higher efficiencies to fractionate microalgal proteins compared with the commonly used ATPS that are formed using only polymers and salts (54). Furthermore, IL-based ATPS have shown versatility to separate different components simultaneously. For example, an IL-ATPS selectively fractionated proteins and sugars in top and bottom phases respectively from different microalgal extracts (210). 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) (209). 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 have been used to extract proteins, pigments and carbohydrates from microalgae (228). However, there are only a few studies where ATPS containing ILs were used for the partitioning of lipids (86). and no studies 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 citrate and Iolilyte 221PG-citrate ATPS were selected based on our previous research (110). The partitioning of commercial canola oil (representing TAGs) and partially purified yeast polar lipids (representing PL) was initially used to understand their behaviour in ATPSs without the influence of other biomolecules present in the microalgal cells. The partitioning behaviour of these model lipids were compared with the partitioning behaviour of lipids from disrupted *N. oleoabundans*. To this end, 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 based on aqueous two-phase solutions.

Materials and Methods

Materials

The ionic liquids: Iolilyte 221PG, ≥ 95% and choline dihydrogen phosphate (Ch DHp, ≥ 98%), were both supplied by Iolitec. 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 an average molecular weight of 400 g mol^{-1}, potassium citrate and citric acid were obtained from Sigma-Aldrich. 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 (229). Canola oil, representing TAGs, was selected in this study because its similar fatty acid profile as *N. oleoabundans* (Total fatty acid profile is provided in Table 1S 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 1300L vertical stacked tubular photo bioreactor (LGem) located at AlgaePARC, The Netherlands. It was cultivated using Bold's Basal medium(143) 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: 24.5g/L; $MgCl_2$: 9.8 g/L; $CaCl_2$: 0.53g/L; K_2SO_4: 0.85g/L; $NaSO_4$: 3.2g/L; $NAHCO_3$: 0.8g/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 MilliQ® water to obtain a biomass concentration of ~90g 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.(230). 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. 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 behaviour 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 0.1g g^{-1} and of polar lipids was 5 mg g^{-1}. For the partitioning of lipids from microalgae, the total concentration of bead milled microalgae suspension in the mixture was 2.5 mg dw.g^{-1}. All mixtures were stirred and incubated for 1h maintaining the desired temperature by a

heating/cooling block thermostat. Then, the mixtures were centrifuged at 2500 rpm (1200 ×g) for 10 min. Afterwards, the phases were carefully separated and weights and volumes were measured. Interfaces were resuspended in MilliQ® water to facilitate the quantification of lipids.

To describe the distribution of lipids in the ATPS, extraction efficiencies (EE%) were calculated with Eq. 6.1. were 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.

$$EE_{lipids}(\%) = \frac{m_{lipids,phase}}{m_{lipids,initial}} x\ 100 \qquad (6.1)$$

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 ATPSs. A two-level full factorial design (FFD) was used for the three systems with three independent variables (factors) each at three levels, three replicates were taken for the centre points. Table 6.1, 6.2 and 6.3 show the coded values for each factor studied in the three ATPSs. Phase forming component concentrations were selected depending on the phase forming range concentrations delimited by phase diagrams previously created (110). Temperatures were selected in a range protecting proteins from denaturation and an optimal pH for the fractionation of proteins in the ATPS was used (110, 231).

Table 6.1 Factors and value levels used in FFD design for the PEG 400-potassium citrate system

Variables	**Factors**	**Low value (-1)**	**Centre 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 6.2: Factors and value levels used in FFD design for the Iolylite 221PG-potassium citrate system

Variables	**Factors**	**Low value (-1)**	**Centre 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 6.3: Factors and value levels used in FFD design for the PEG 400-Ch DHp system

Variables	Factors	Low value (-1)	Centre 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

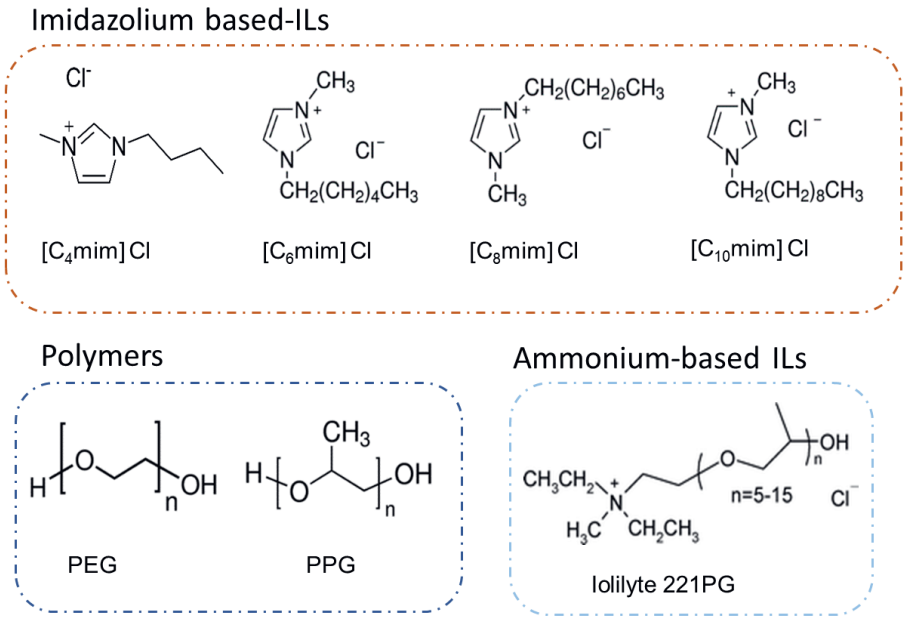
The studied responsive variable was the extraction efficiency of lipids (EE %) in the different phases calculated using Eq. 6.1. The response surface methodology was used to understand the influence of the parameters on the fractionation of lipids. The responses were fitted in a second order polynomial 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 6.1). The IL or polymer concentrations studied were 25 and 50 % w/w. 1g 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 hour. 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.

$$Extraction\ Yield_{lipids} = \frac{m_{lipids,supernatant}}{m_{biomass}} \quad (6.2)$$

Figure 6.1 Structure of the 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 an average molecular weight of 400 g mol-1and Ammonium based ionic liquid: Iolilyte 221PG

Quantification of biomolecules

Quantification of lipids

Total lipids (triacylglycerides (TAG) and polar lipids (PL)) content was determined as described by Remmers et al (232). 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 solvent and helium as carrier gas with a flow rate of 20 mL/min. The column used was a Supelco Nucol^{tm} 25357, 30m x 530µm x 1.0µm. 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. (145).

Results and Discussion

An outline of the results and discussion is given below whereby initially *N. oleabundans* 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 buffer system. Secondly, the solubility of the lipids was determined in the different solutions containing polymers and ionic liquids. Finally, partitioning of the different product (pigments, proteins, soluble sugars and lipids) is investigated with focus on lipids by using a multicomponent approach with PPG 400 and Ch DHp-based ATPS based 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 (125, 233).

The fatty acid profile of *N. oleoabundans* presented in Table 1S is in accordance with the profiles reported in previous studies (191). 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 Fig 6.2 were obtained when using the centre point values (Table 6.1, 6.2 and 6.3) 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 ATPSs, 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. 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.

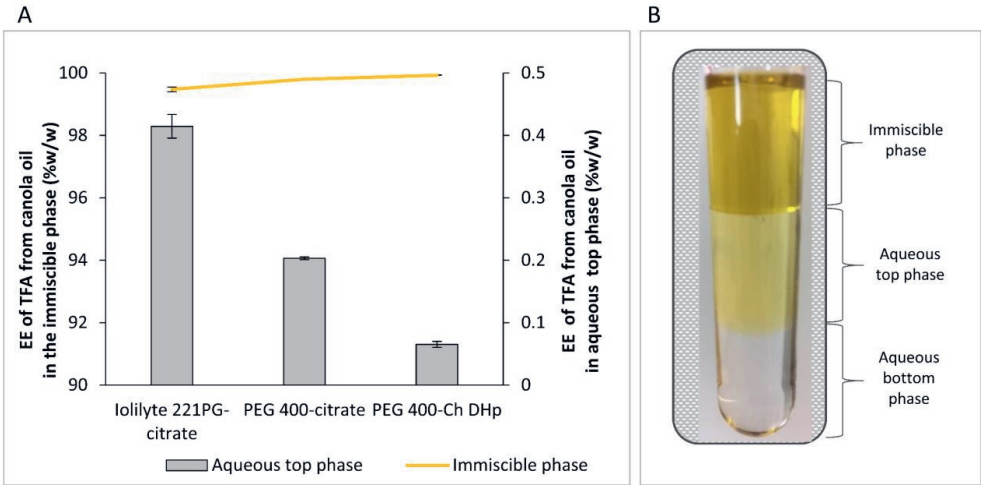


Figure 6.2 A) Extraction efficiency (% w/w) of total fatty acids from canola oil using three different ATPSs at 21.5 °C. The mixture points studied for the systems were: Iolilyte 221PG; citrate (29; 29 % w/w), PEG 400; citrate (28; 32 % w/w), PEG 400; Ch DHp (33; 33% w/w). The results represent the average of three independent experiments and error bars indicate standard deviations. B) Real visual appearance of the partitioning of TAGs from canola oil using PEG 400-Ch DHp ATPS.

Comparing the three ATPSs, 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 ATPSs are presented in figure S6.1 of the supplementary 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 ATPSs.

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 2S of the supporting information. Different from canola oil (representing TAGs); PL added to the ATPSs formed a solid phase between the aqueous phases (interface). The distribution of polar lipids in the three phases: top, bottom and interface is shown in Figure 3A. The results presented in this figure were obtained when using the centre point values (Table 6.2 and 6.3) 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 ATPSs, 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 nature of the Iolilyte 221PG benefits the migration of the phospholipids to the aqueous phases (234) (209).

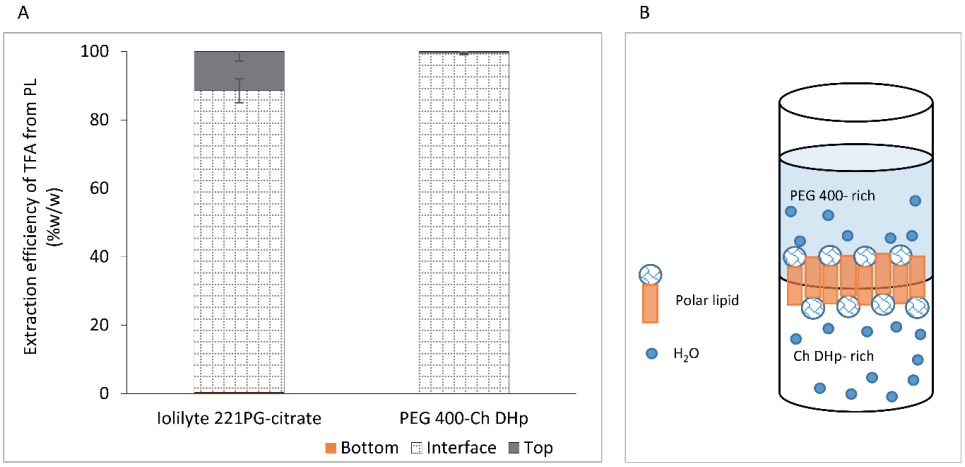


Figure 6.3: A) Distribution of yeast polar lipids in top, bottom and interface at 21.5 °C. The mixture points studied for the systems were: Iolilyte 221PG; citrate (29; 29 % w/w) and PEG 400; Ch DHp (33, 33% w/w). The results represent the average of three independent experiments and error bars indicate standard deviations. B) Visual representation of the partitioning of polar lipids in PEG 400-Ch DHp ATPS.

Phosphate groups are polar and therefore when the phospholipids are suspended in aqueous solutions, they typically arrange themselves in a stable bilayer (Figure 6.3B). 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 towards 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 phosphate buffer was previously used to purify haemoglobin, concentrating cell membrane phospholipids in the interface (235). Aqueous two-phase systems have been widely studied for the purification of proteins from fermentation broths and colloid solutions (236). This liquid-liquid extraction method has the ability of separating cells and cell membrane in the interface from biomolecules such as proteins that are fractionated into the aqueous phases (237). ATPS interfacial tension

allows holding cells and other particles in the interface, achieving efficient cell clarifications and complex separations (238, 239). Liposomes added to ATPSs 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 (61, 240).

We also investigated ATPSs to partition microalgae lipids, 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) phase. Due to the high TAG content of *N. oleoabundans (*80% of the total fatty acids), we expected that 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.

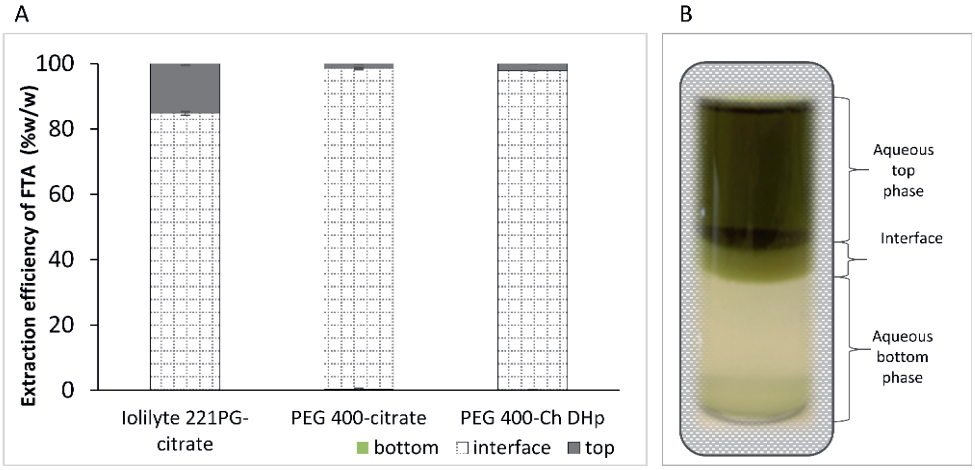


Figure 6.4: A) Extraction efficiency of TFA from disrupted *N. oleoabundans* at room temperature (21.5 °C). The mixture points studied for the systems were: Iolilyte 221PG; citrate (29; 29 % w/w), PEG 400; citrate (28; 32 % w/w), PEG 400; Ch DHp % w/w (33, 33 % w/w). B) Real visual appearance of the partitioning of TAGs from microalgae using PEG 400-Ch DHp ATPS

Figure 6.4A shows the distribution of lipids in the three phases for the three ATPSs studied. These results were obtained using the centre point values (Table 6.1, 6.2 and 6.3) 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 other biomolecules present in the microalgal cells, such as phospholipids and proteins (241). 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 towards the two aqueous phases (forming a bilayer), 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 (242-244). 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) (241). 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 Figure 6.5 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 S6.2 of 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 (71). PEG 400-based ATPSs 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 (69).
A second order polynomial regression model was used to calculate the response contour plot for each response variable. 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 3S 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 ATPSs). The lack of fit test show that errors are not significant for the model ($p>0.05$).

As shown in figure 6.4B, 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 (245).

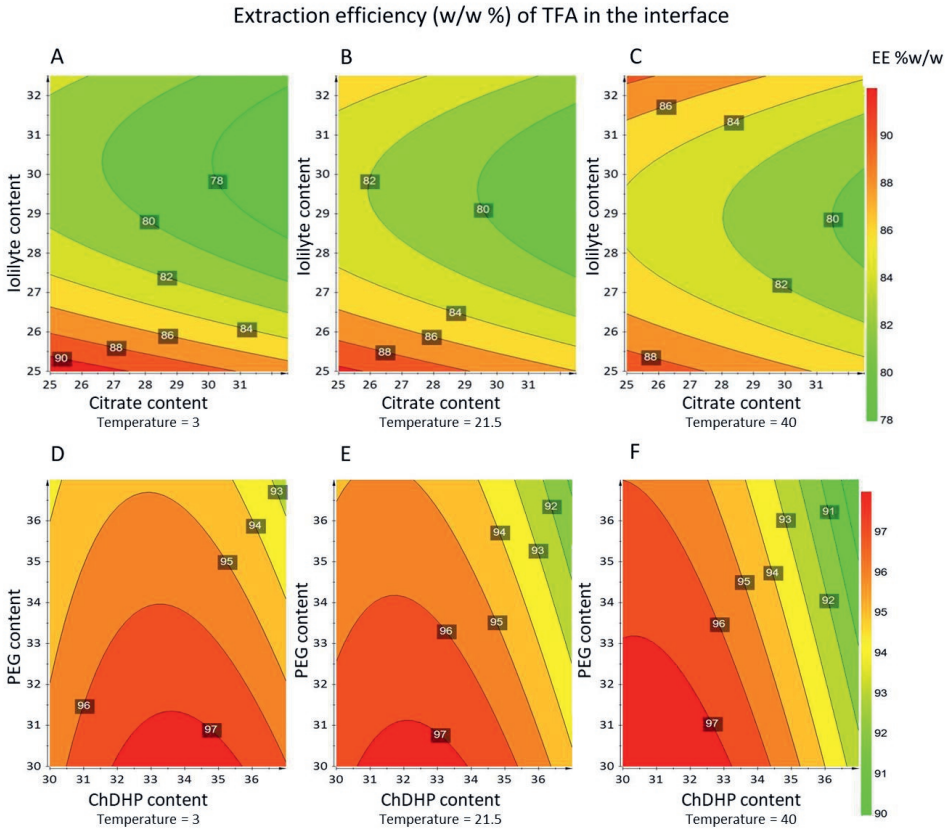


Figure 6.5: Contour plots showing the influence of the concentration of phase forming components and temperature on the extraction efficiency of Total Fatty Acids (TFA) from disrupted *N. oleoabundans* in the interface. A, B and C for Iolilyte 221PG-citrate ATPS. D, E and F for PEG 400-Ch DHp.

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. For a better understanding, disrupted *N. oleoabundans* suspension was mixed with different aqueous solutions composed of ionic liquids or polymers.

Figure 6.6 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 control, the extraction solutions chloroform:methanol (1:1.25) and methanol are shown as well as beadmilling as an extraction method.

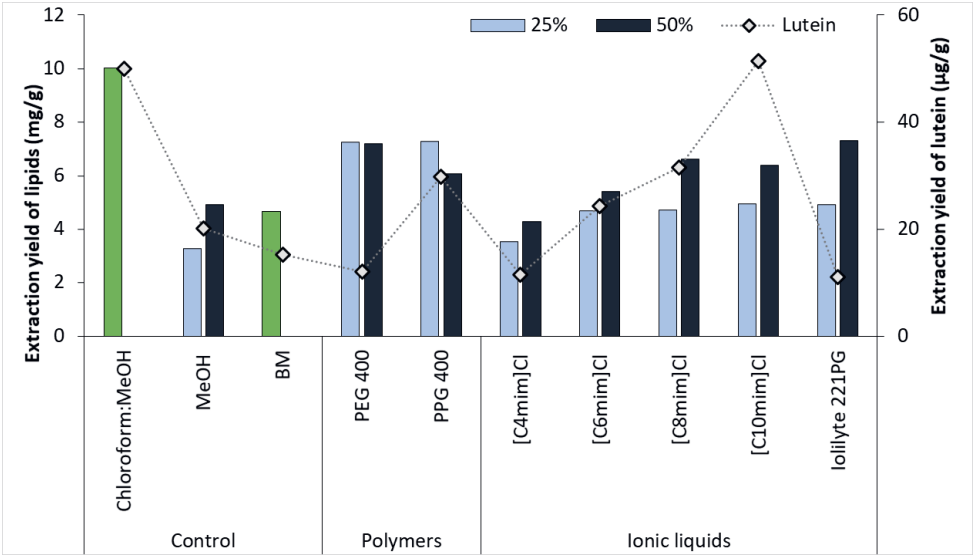


Figure 6.6: Extraction yield of lipids (mg lipids extracted/g microalgae suspension) from disrupted *N. oleoabundans* using aqueous solutions. Chloroform: methanol and methanol were used as control as well as bead milling (BM). The 25% and 50% mentioned in the top part of the figure refer to the IL or polymer concentrations studied (% ww). The extraction yield of lutein using the 50% solutions is also shown.

The extraction yield 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 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 (246, 247). 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 glycol with various molecular weight were previously studied by Manic et al. (248) 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. (249) presented a similar behaviour. The lipids did not dissolve in the ionic liquid, but release 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 (250). 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 (251).

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 posseses both hydrophilic (hydroxyl groups) and hydrophobic (long alkyl side chain) properties (234, 252). This ionic liquid is capable to enhance the solubility of hydrophobic substrates from cell lysate of *E. coli* as described by Dreyer et al. (53). 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 (253) 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 ATPSs.

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 6.6, increasing the alkyl chain length of the ILs cation 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 length of ILs are directly correlated with their hydrophobicity nature, the larger the cation alkyl chain length, the more hydrophobic the ionic liquid is, which explains the extraction trend (254). In addition, long alkyl side chain ILs exhibit surfactant properties in water and have enhanced the extraction of different compounds (255, 256). Cheong et al. (255) found that the alkyl chain length of the cation of ILs have a positive effect on the extraction of polyunsaturated fatty acids from fish oil (n-3 PUFA and n-3 PUFAEE).

The extraction of pigments represented by pigments follows a different trend than lipids (UV-VIS spectra of the extracted pigments in each solvent are shown in Figure S6.3 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 behaviour seems to be highly influenced by the hydrophobicity of the solutions used. Increasing the alkyl chain length of the IL's cation, increases its hydrophobicity and thus more pigments are extracted. Moreover, PPG 400 has an additional methyl group in the ethylene glycol repeating unit than PEG 400 making it more hydrophobic, which explain its better performance in the extraction of pigments.

Fractionation of lipids in a multiproduct biorefinery approach

The results above have shown exclusion of lipids towards 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 (257), that 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 (258).

We propose a fractionation process divided in three steps (Figure 6.7): (1). Recovery of TAGs from microalgae using polymers. 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 (259) (209).

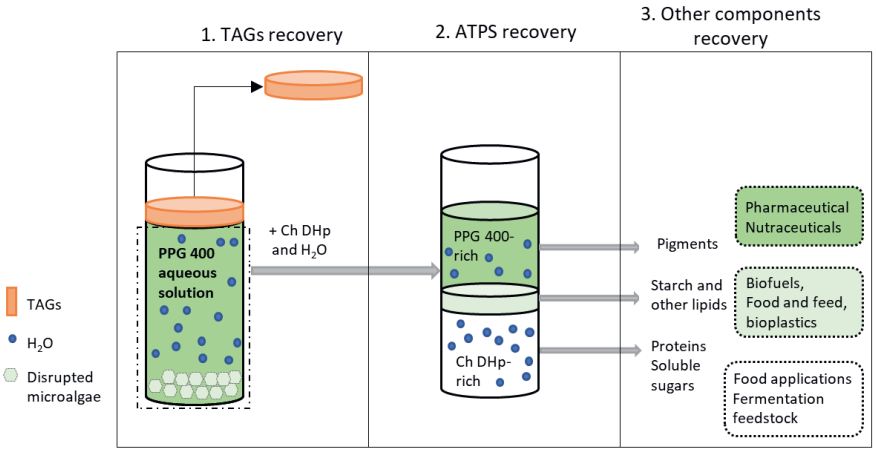


Figure 6.7: Overview of proposed process for the fractionation of microalgal lipids and other cell components

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 (259). 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 recycle of phase forming components, and process kinetics (determining throughput and equipment) need to be assessed first. In this way microalgae multiproduct biorefinery would be feasible.

Conclusions

We studied the fractionation of lipids as part of a biorefinery approach to valorise 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 is not addressed in this manuscript but is part of our future work.

Supporting Information for Publication

The supplementary material, consisting of three figures and four tables, gives information on the lipid composition of *N. oleoabundans* (Table S6.1), canola oil (Table S6.2) and polar lipids in yeast (Table S6.3). Furthermore, Figure S6.1 displays the influence of phase forming components on extraction efficiency of Total Fatty Acid (TFA) from canola oil. Figure S6.2 displays the influence of phase forming components on extraction efficiency of TFA from *N. oleoabundans* and statistically explained in Table S6.4. Finally, Figure S6.3 displays the UV-VIS spectra of extracted pigments using different aqueous solutions.

Acknowledgements

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Supplementary data

Table S6.1: Fatty acid composition of individual lipid classes of *N. oleoabundans*. Each value represents the mean of three determinations ± standard deviation

Fatty acid	Name	PL content (% w/w dry matter)	TAG content (% w/w dry matter)
C14:0	Myristic acid	0.08 ± 0.03	0.19 ± 0.21
C14:1 cis-9	Myristoleic acid	0	0.06 ± 0.05
C16:0	Palmitic acid	2.68 ± 0.11	6.95 ± 0.39
C16:1	Palmitoleic acid	0.22 ± 0.15	0.52 ± 0.01
C16:2	Hexadecadienoic acid	0.29 ± 0.12	0.67 ± 0.04
C16:3	Hexadecatrienoic acid	0	0.11 ± 0.03
C17:0	Margaric acid	0.20 ± 0.07	0.57 ± 0.01
C18:0	Stearic acid	0.29 ± 0.11	1.19 ± 0.08
C18:1	Oleic acid	3.64 ± 0.15	17.54 ± 1.05
C18:2	Linoleic acid	2.01 ± 0.30	4.59 ± 0.30
C18:3	α-linolenic acid	0.44 ± 0.18	1.14 ± 0.06
C20:0	Arachidic acid	0	0.06 ± 0.05
C20:1	Eicosenoic acid	0.15 ± 0.25	0.06 ± 0.02
C20:2-n6	Eicosadienoic acid	0.001 ± 0.00	0
C22:0	Behenic acid	0.08 ± 0.60	0
Total content		**10.07 ± 0.058**	**33.64 ± 0.162**

Table S6.2: Total fatty acid profile of commercial canola oil

Fatty acids	Name	Oil TAG content (w/w %)*	Oil TAG content (w/w %)[a]
C14:0	Myristic acid	0.065 ± 0.000	Not available
C14:1 cis-9	Myristoleic acid	0	Not available
C16:0	Palmitic acid	4.547 ± 0.023	4.2
C16:1	Palmitoleic acid	0.290 ± 0.01	Not available
C16:2	Hexadecadienoic acid	0.066 ± 0.001	Not available
C16:3	Hexadecatrienoic acid	0.129 ± 0.002	Not available
C18:0	Stearic acid	1.421 ± 0.006	2.2
C18:1	Oleic acid	61.36 ± 0.11	67.2
C18:2	Linoleic acid	19.75 ± 0.031	18.9
C18:3	α-linolenic acid	10.43 ± 0.06	7.4
C20:0	Arachidic acid	0.424 ± 0.014	Not available
C20:1	Eicosenoic acid	1.265 ± 0.03	0
C20:2-n6	Eicosadienoic acid	0.06 ± 0.000	Not available
C22:0	Behenic acid	0.194 ± 0.02	Not available

**Each value represents the mean of two determinations ± standard deviation*

[a]*from literature*

Table S6.3: Total fatty acid profile of semi purified polar lipids from yeast

Fatty acids	Name	Commercial PL content (w/w %)*
C14:0	Myristic acid	0.172 ± 0.172
C14:1 cis-9	Myristoleic acid	0
C16:0	Palmitic acid	26.55 ± 0.413
C16:1	Palmitoleic acid	15.37 ± 2.06
C16:2	Hexadecadienoic acid	0
C16:3	Hexadecatrienoic acid	0
C18:0	Stearic acid	24.56 ± 0.747
C18:1	Oleic acid	19.09 ± 0.552
C18:2	Linoleic acid	12.33 ± 1.27
C18:3	α-linolenic acid	1.923 ± 0.007
C20:0	Arachidic acid	0
C20:1	Eicosenoic acid	0
C20:2-n6	Eicosadienoic acid	0
C22:0	Behenic acid	0

**Each value represents the mean of two determinations ± standard deviation*

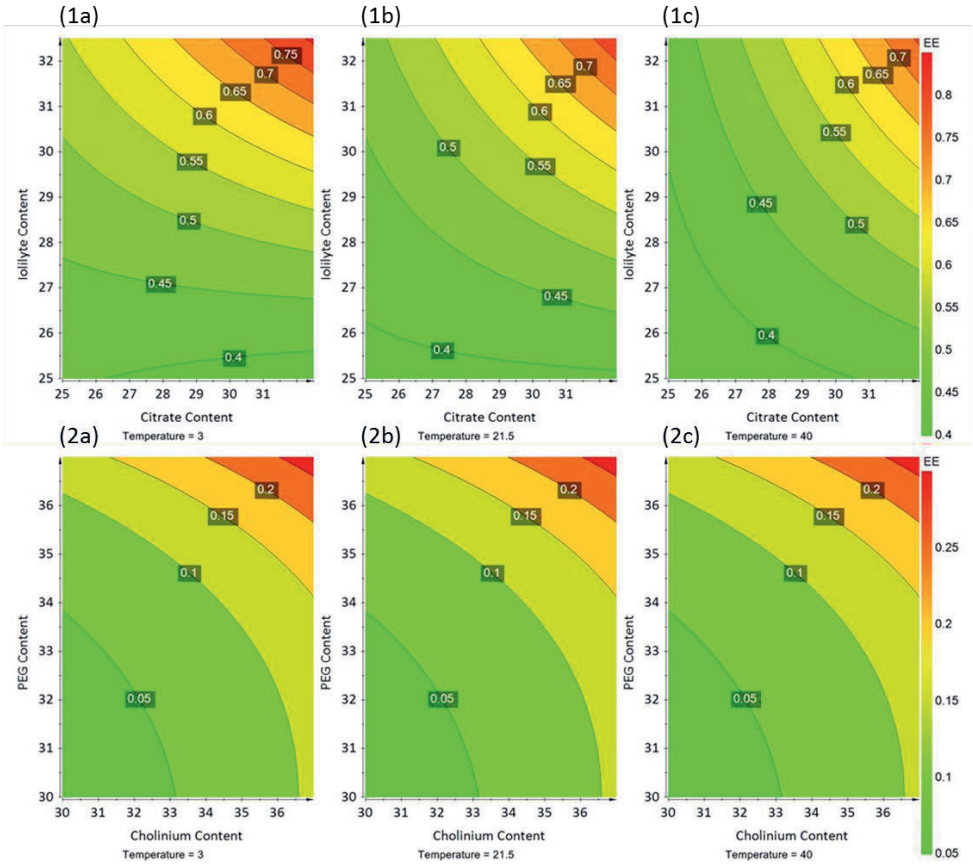

Figure S6.1: Contour plots showing the influence of the concentration of phase forming components and temperature on the extraction efficiency of TFA from canola oil. (1 a, b and c) Iolilyte 221PG-potassium citrate ATPS; (2 a, b and c) PEG 400-Ch DHp ATPS.

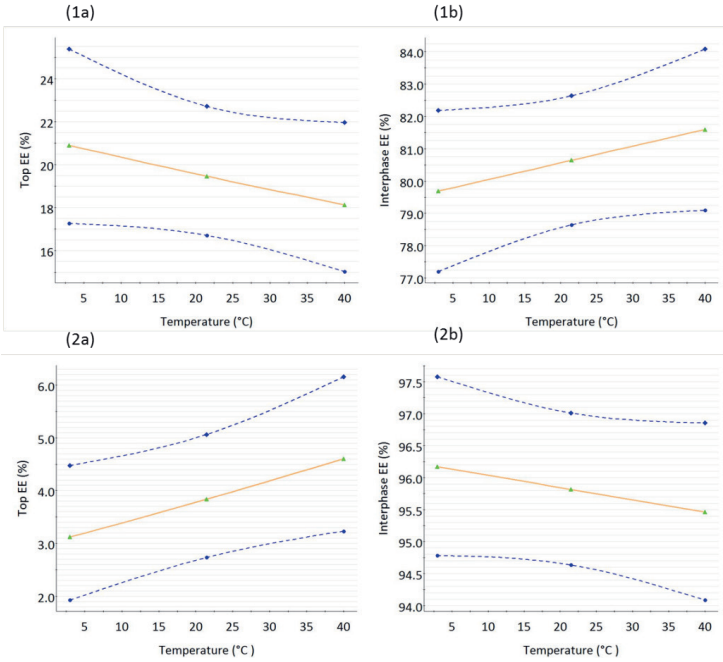

Figure S6.2: Prediction/Response plot of the effect of temperature on (a) extraction efficiency of TFA from *N. oleoabundans* in the top phase and (b) in the interface using (1) Iolilyte 221PG-potassium citrate ATPS and (2) PEG 400-Ch DHp ATPS. Centre line (orange) shows the calculated response. Top and bottom dashed blue lines show the upper and lower confidence interval (0.95), respectively.

Table S6.4 Statistical analysis of variance (ANOVA) for the extraction efficiency of TFA from *N. oleoabundans* to the interface using ATPSs.

System	Regression model	Correlation (R^2)	Variance	Lack of fit
Iolilyte 221PG-citrate	EE %	0.92	0.005	0.347
PEG 400-Ch DHp	EE %	0.92	0.006	0.110
PEG 400-citrate	EE %	0.80	0.007	0.866

Reduced regression equations

Iolilyte 221PG-Potassium citrate ATPS

$$\log EE_{int} = 2.5235 + 0.003342X_3 + 0.015286X_1^2 - 0.00706X_2X_3$$

PEG400-Ch DHp ATPS

$$\log EE_{int} = 2.6809 - 0.00541X_1 - 0.00673X_2 - 0.00622X_2^2 - 0.00541X_2X_3$$

PEG400-potassium citrate ATPS

$$\log EE_{int} = 1.9892 - 0.0018X_2 + 0.00511X_1^2 - 0.00167X_1X_2$$

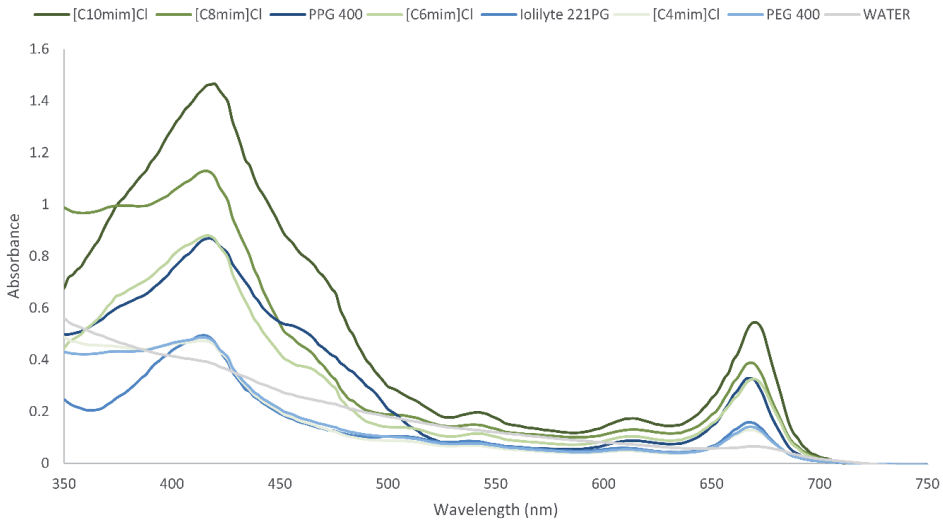

Figure S6.3 UV-VIS spectra of the extracted pigments using different aqueous solutions.

Chapter 7

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.

Synopsis

A selective aqueous two phase system is developed for the separation of multiproduct (proteins, pigments, soluble sugars, starch and lipids) from the microalgae *N. oleoabundans*.

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(132, 260, 261).

Neochloris oleoabundans has been highlighted as a valuable resource for the production of industrially useful materials (2). It has high growth rates and the capacity to accumulate large quantities of lipids after nitrogen depletion (3). 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 (262). Besides triglycerides, *N. oleoabundans* possesses proteins, carbohydrates, and pigments. Carbohydrates from microalgae are excellent substrates for the production of biofuels (e.g. bioethanol), biopolymers (263, 264) and fermentation feedstocks (171). Furthermore, its potential has recently gained attention for their application in other industries, including food, paper production, bioplastics, cosmetics and pharmaceuticals (265-267).

Although not widely investigated, *N. oleoabundans* is a promising source of high-value carotenoids that mainly accumulate lutein (4, 125, 233). These high-value carotenoids can be used as food additives and health supplements due to their antioxidant activity (233). Compared with conventional food proteins of great nutritional quality, proteins from *N. oleoabundans* are also of interest due to their similar amino acid profile (268). 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 (269).

The valorization of all microalgae components can increase the economic feasibility of microalgae production, balancing the high costs of cultivation and downstream processes (270). 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 (271, 272). The interest in developing more efficient and sustainable processes has been extended. Techniques like supercritical CO_2 ($scCO_2$) (273), switchable solvents (272-274), surfactants, and ionic liquids (ILs) were investigated for the extraction of microalgae components (275, 276). 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.

ILs are solvents that have a high solvation power for a wide range of molecules (277, 278). Their tunability is one of their most attractive features, making them adaptable to many technologies, including aqueous two-phase systems (ATPS) (22, 279). The motivation of this study is based on the high solvation ability these “designer solvents” have and their ability to form ATPS (86, 280). 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 non-volatile organic solvents (86, 280). 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 (54, 281) (209). 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 (54, 281) (209).

ATPS can be formed by combining a high variety of aqueous solutions, including polymers, salts, alcohols, and ILs (86, 280). Three kinds of ATPS 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 ATPS, specifically ionic liquid-citrate (Iolilyte221PG-citrate) and polymer-ionic liquid (PEG400-ChDHp), as described by Ruiz et al., (209). The polymer-ionic liquid-based ATPS showed a different partitioning behavior when compared with the other ATPS studied; it fractionates pigments from proteins with a higher selectivity. Furthermore, this ATPS was able to conserve the native form of proteins, namely RuBisCO (110). 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 (247, 282).

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 (22), and their ability to keep the native conformation of RuBisCO (110). 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 (Di stefano et al., 2018) (283).

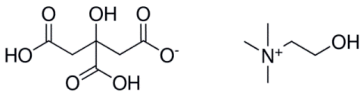
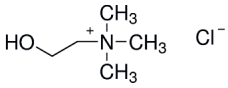
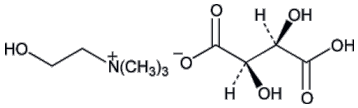
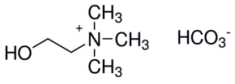
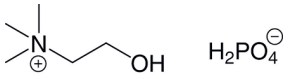
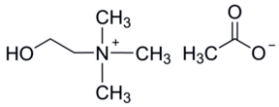
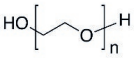
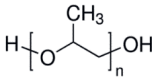
Firstly, different cholinium-based ILs were selected based on literature reviews (58, 79) and evaluated based on their phase-forming behavior and their selectivity to fractionate microalgal pigments and proteins in different phases. Secondly, the best combination was characterized based on the phase 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).

Table 7.1: 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)	

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 sea water on Bold's Basal medium[41]: NaCl: 24.5g/L; $MgCl_2$: 9.8 g/L; $CaCl_2$: 0.53g/L; K_2SO_4: 0.85g/L; $NaSO_4$: 3.2g/L; $NAHCO_3$: 0.8g/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 MilliQ® water to obtain a biomass concentration of ~90g 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.(230). Bead-milled microalgae suspension was stored at -20ºC 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 drop wise 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 drop wise addition of MilliQ® 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.(63). Mixtures in the biphasic system were prepared ($\pm 10^{-4}$ g), mixed and left to equilibrate for at least 12 hours 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) Equation 7.1 was used.

$$TLL = \sqrt{(X_T - X_B)^2 + (Y_T - Y_B)^2} \qquad (7.1)$$

X_T , Y_T , X_B , 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 microalgae.ml^{-1}. The mixtures were subsequently mixed in a rotatory shaker (50 rpm, 1h) 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 MilliQ® 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 (14000 × 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 Equation 7.2, 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.

$$Protein\ recovery\ (\%) = \frac{m_{p,c}}{m_{p,i}} \qquad (7.2)$$

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 Equation 7.3. $m_{IL,p}$ is the mass if the IL in the permeate and $m_{IL,i}$ is the initial mass of the IL added to the filter.

$$IL\ recovery\ (\%) = \frac{m_{IL,p}}{m_{IL,i}} \qquad (7.3)$$

Thermo-separation. PPG 400 is a thermo-sensitive polymer that forms two-phases when heated. This behaviour 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 wt% and 7 wt% of Ch DHp) on the cloud point temperatures (Tc) of PPG 400 was studied. Solutions containing different concentrations of PPG 400 and Ch DHp (2 wt% 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 analysed 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 supplementary information, Table S7.1), 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 nm 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 minutes) to release the pigments from microalgae biomass (284). 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 (285). 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 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)(210). 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 ^{o}C for 30 minutes. 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 Dragone et al.(192). 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.(190). 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^{tm} 25357, 30m x 530µm x 1.0µm. 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 Equation 7.4 and Equation 7.5 respectively. The concentration of certain target biomolecule $C_{biomolecule}$ (e.g. proteins) in the phase (top, bottom or interface) and m the mass of the biomolecule in the phase (top, bottom, interface) or in the initial bead-milled microalgae suspension added to the system.

$$\log(K) = log\left(\frac{C_{biomolecule,top}}{C_{biomolecule,bottom}}\right) \quad (7.4)$$

$$EE_{biomolecule}\% = \frac{m_{biomolecule,phase}}{m_{biomolecule,initial}} \text{x} 100\% \quad (7.5)$$

The selectivity of the systems to fractionate pigments or proteins was calculated with Eq. 7.6

$$S_{pigment/protein} = \frac{K_{pigments}}{K_{proteins}} \quad (7.6)$$

All experiments were conducted in duplicates 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 (210). Glucose was present in the disrupted biomass as part of the starch content and as free glucose. The starch and free glucose content 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 S7.1), which is in agreement with other reports (233) (209). Fatty acid composition (Table S7.2) and pigment chromatogram (Figure S7.1, S7.2) are shown in the supplementary 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 (58, 79) and previous experiments (110, 210) (209) 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 7.1 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 towards the PPG 400- or IL-rich phase not varying, the selectivity varied significantly between the systems.

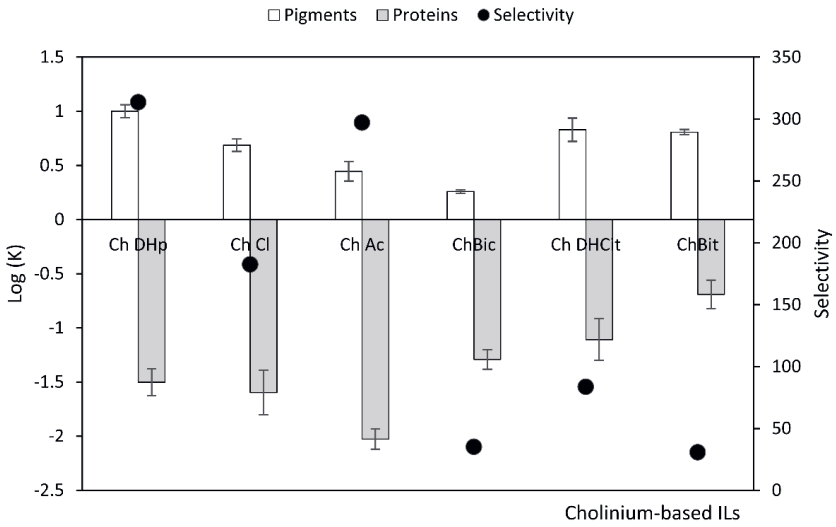


Figure 7.1. Partitioning of pigments and proteins from disrupted *N. oleoabundans* in an ATPS with PPG 400-cholinium based-ILs ATPS. Empty bars and full bars represent pigment and protein partition coefficients in logarithmic scale, respectively. Black dots represent the selectivity values ($S_{pigment/proteins}$) between pigments and proteins. Error bars represent standard deviation.

Being hydrophobic, the pigments were expected to partition towards 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 7.1) 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 (145), 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 (145). 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 (146).

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 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 (69).

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 (166, 212). This property is important since proteins are fragile biomolecules susceptible to denature 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 (58, 166, 286). 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 (287).

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 7.1, Figure S7.3). The interface contains mainly cell debris generated by cell disruption method by bead-milling used in this study (230). 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 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 towards 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 7.1). 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 (58). 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 7.2). 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 supplementary data (Tables S7.3-S7.5). Figure 7.2 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 S7.4) in the bottom phase. This phenomenon, by increasing slightly the PPG 400 concentration in the top phase (Table S7.4) and the concomitant addition of much higher concentration of ChDHp in the bottom phase (Table S7.4) is not understood and needs further investigations.

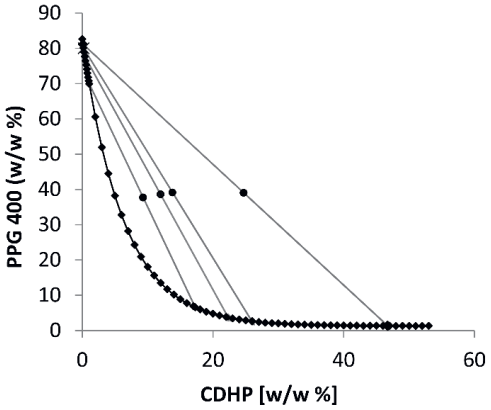


Figure 7.2. Phase diagram of PPG 400-Ch DHp (binodal curve and respective TLs). TLs were calculated considering mixture points at a volume ratio of 1. The mixture points studied for the system (PPG 400; Ch DHp w/w%) were: (38; 9 w/w%), (39;12 w/w%), (40, 14w/w%) and (39, 25w/w%).

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.

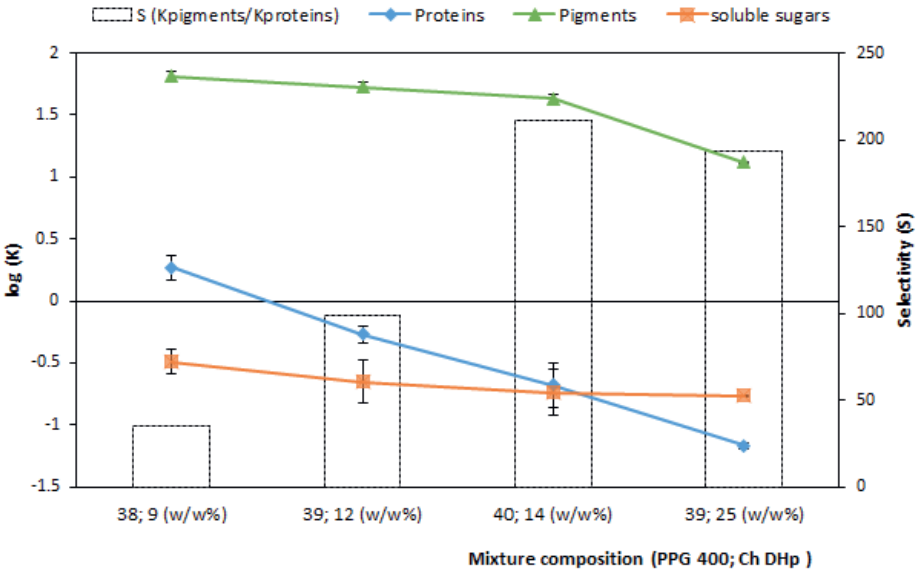


Figure 7.3. Partition coefficients (log (K)) of proteins, pigments and soluble sugars from disrupted *N. oleoabundans*. The mixture points studied for the system (PPG 400;Ch DHp w/w%) were: (38;9 w/w%), (39;12 w/w%), (40;14w/w%) and (39;25w/w%). Bars represent the selectivity between pigments and proteins. Error bars represent standard deviations.

Partition coefficients of pigments, proteins, and free glucose are presented in Figure 7.3. Pigments preferentially migrated to the PPG-rich phase (most hydrophobic phase), while proteins and free glucose tended to migrate towards 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 towards 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 (148). 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 (55). The partitioning of IgG was recently studied by Ramalho et al., using PPG 400 and cholinium-based ILs (166). 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 (288). 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 7.3, 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. (243) 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 (289). 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 (207). An ATPS is not affected by the cell disruption process and is able to selectively fractionate a high amount of biomolecules towards 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 (207). 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.

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 7.3. 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 7.4, 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 7.4.

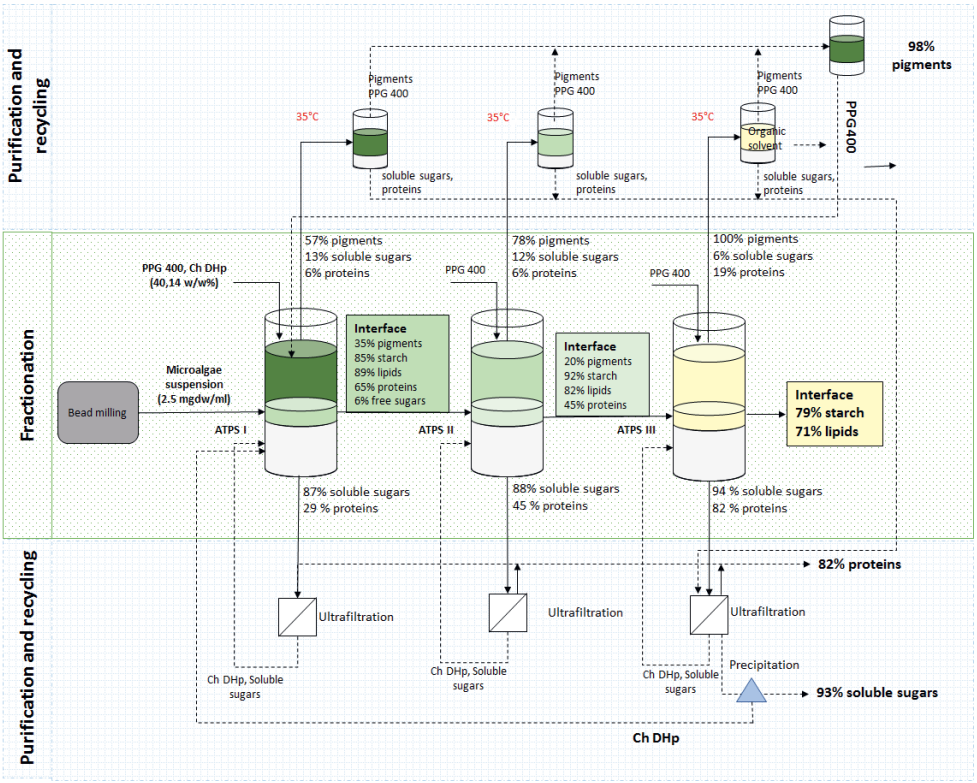


Figure 7.4. Diagram of the multistep approach for the selective fractionation of microalgal pigments, proteins, soluble sugars (free glucose), starch and lipids. The approach studied includes three stages of fractionation, using an ATPS formed by 40% PPG 400 and 14% Ch DHp. Microalgal suspension is introduced 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.

Tables 7.2 and 7.3 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 non-soluble 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 S7.2).

Table 7.2 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.

Stage	**Pigments**				**Proteins**				**Free glucose**			
	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**	-	-

Table 7.3 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.

Stage	**Starch**			**Total fatty acids**		
	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**

Although, proteins preferentially partition to the bottom phase, 65.4 ± 2.3 % remain in the interface in ATPS I (Table 7.2). 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 non-soluble protein fraction is often identified, and 35-50% of the proteins have been reported as water-soluble proteins released by bead milling *N. oleoabundans* (106, 167). 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. 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 (290, 291). 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 (99). 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 (292).

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 7.3). 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 non-polar 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 (148), 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 (293).

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 (206).

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 non-food 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.(294) The Fatty Acids Methyl Esters composition of the lipid fraction recovered in the interface is shown in the supplementary data (Table S7.2).

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 towards 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 thermo-sensitive 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 7.5).

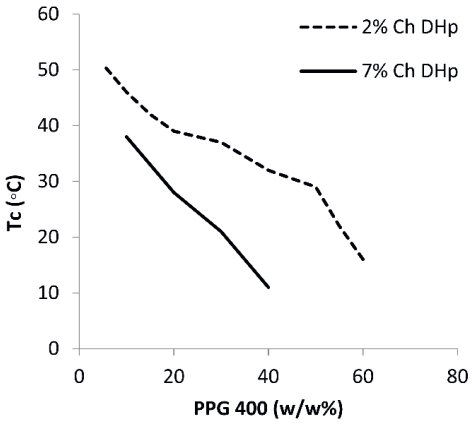


Figure 7.5. Cloud point temperatures of PPG 400 solutions in the presence of Ch DHp (2% and 7%)

In PPG 400-Ch DHp, the top phase is mainly composed of PPG 400 (max. 85%), Ch DHp (max. 7%), and water. Figure 7.5 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 (55). 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 (295). Therefore, mixture points with low cloud point temperatures (<45 °C) were selected for the application of this approach. Table S7.5 in the supplementary 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 towards 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 (296). Furthermore, it is included on the FDA's GRAS (compounds Generally Recognized As Safe) list (58). Therefore, the carotenoids and pigments of interest may be recovered together with the PPG 400 and used subsequently for industrial applications (297). 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 g/mol 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 7.4). 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.

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. 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.

Supporting Information for Publication

The supplementary material, consisting of three figures and five tables, gives information on the pigment (Table S7.1, Figure S7.1) and lipid (Table S7.2) characterizations of the bead milled *N. oleoabundans* biomass. Furthermore, UV-VIS spectra of the three ATPS cycles (Figure S7.2) and a snapshot of the interphase (Figure S7.3) are depicted. Finally, Tables S7.3, S7.4 and S7.5 show the weight fractions of the ternary complex PPG 400 + Ch DHp + H_2O.

Acknowledgements

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Supplementary material

Table S7.1: Reversed-phase high-performance liquid chromatography. RP-HPLC was used to identify the pigments in the disrupted *N. oleoabundans* suspension. HPLC analysis was performed in a Shimadzu system coupled with a photo-diode array detector (SPD-M20A) and an Acclaim™ C30 LC reversed-phase column from Thermo Fisher Scientific™ was used. Three mobile phases were used: A) Acetonitrile, B) Methanol/Ethyl acetate 1:1 (v/v), C) 200mM Acetic acid in water with the gradient shown in Table S7.1. The flow rate was set at 1.5 mL/min and the column temperature at 30°C.

Table S7.1. Gradient used in RP-HPLC

Time (min)	%A	%B	%C
0	85.0	14.5	0.5
2	85.0	14.5	0.5
15	65.0	34.5	0.5
25	65.0	34.5	0.5
30	85.0	14.5	0.5

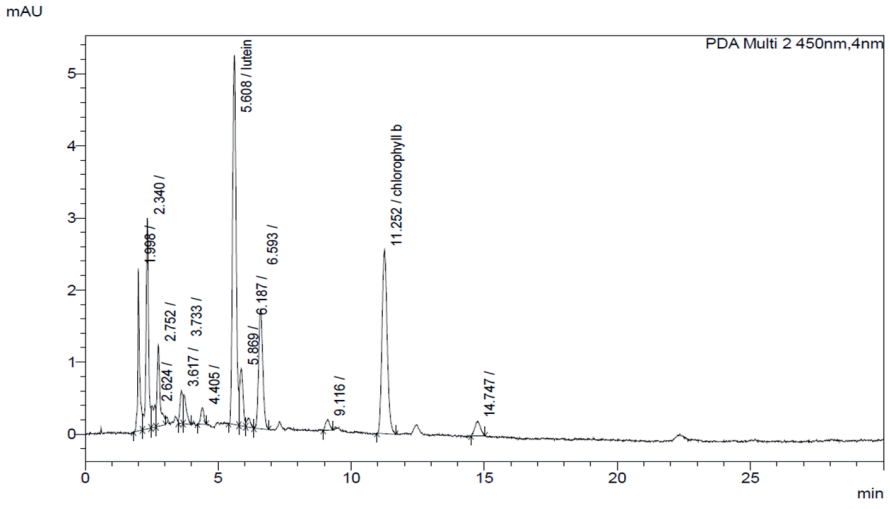

Figure S7.1. RP-HPLC chromatogram at 450 nm of *N. oleoabundans* pigments extracted in methanol with lutein and chlorophyll b as most abundant pigments.

Table S7.*2.* Fatty acid composition of polar lipids (PL) and Triacylglycerides (TAG) of *N. oleoabundans*. Each value represents the mean of three determinations ± standard deviation

Fatty acid	Name	PL content (% w/w dry matter)	TAG content (% w/w dry matter)
C14:0	Myristic acid	0.08 ± 0.03	0.19 ± 0.21
C14:1 cis-9	Myristoleic acid	0	0.06 ± 0.05
C16:0	Palmitic acid	2.68 ± 0.11	6.95 ± 0.39
C16:1	Palmitoleic acid	0.22 ± 0.15	0.52 ± 0.01
C16:2	Hexadecadienoic acid	0.29 ± 0.12	0.67 ± 0.04
C16:3	Hexadecatrienoic acid	0	0.11 ± 0.03
C17:0	Margaric acid	0.20 ± 0.07	0.57 ± 0.01
C18:0	Stearic acid	0.29 ± 0.11	1.19 ± 0.08
C18:1	Oleic acid	3.64 ± 0.15	17.54 ± 1.05
C18:2	Linoleic acid	2.01 ± 0.30	4.59 ± 0.30
C18:3	α-linolenic acid	0.44 ± 0.18	1.14 ± 0.06
C20:0	Arachidic acid	0	0.06 ± 0.05
C20:1	Eicosenoic acid	0.15 ± 0.25	0.06 ± 0.02
C20:2-n6	Eicosadienoic acid	0.001 ± 0.00	0
C22:0	Behenic acid	0.08 ± 0.60	0
Total content		**10.07 ± 0.058**	**33.64 ± 0.162**

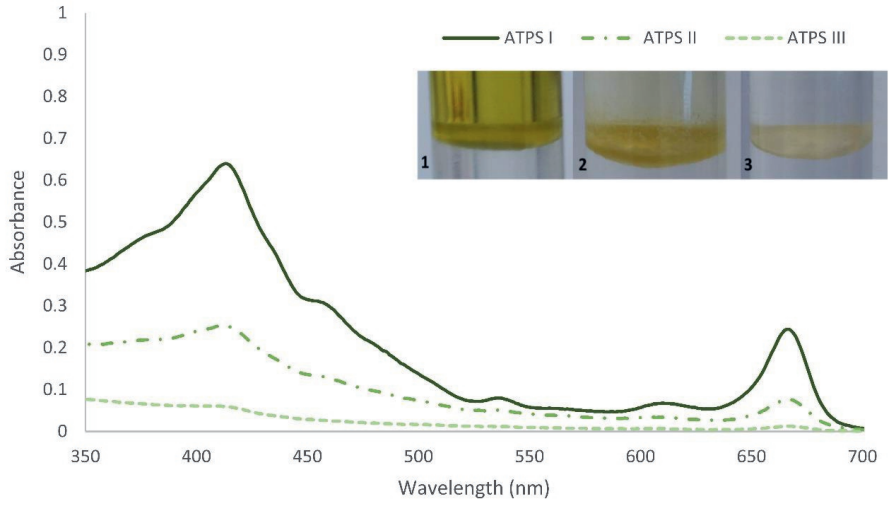

Figure S7.2. UV-VIS spectra of the PPG 400-rich phases when applying the multistep fractionation of disrupted *N. oleoabundans* components. The visual appearance of the interface at each stage is also presented.

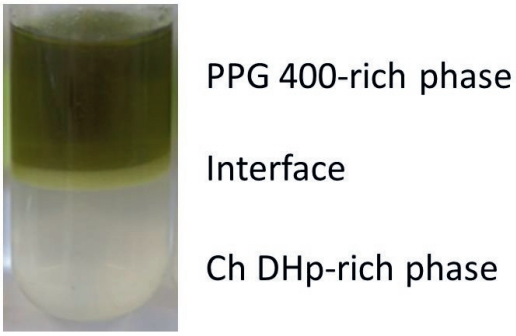

Figure S7.3. Visual appearance of the three phases with the top phase the PPG 400-rich phase containing the green pigments, the bottom phase (Ch DHp-rich phase) containing proteins. The third phase is the interface containing the cell fragments and hydrophobic components such as lipids, proteins and starch.

Table S7.3. Weight fraction data for the ternary system composed of PPG 400 + Ch DHp + H_2O

PPG400 (w/w%)	**Ch DHp (w/w%)**	**PPG400 (w/w%)**	**Ch DHp (w/w%)**
57.01	2.99	2.31	26.53
50.95	3.04	2.41	23.67
48.20	3.44	3.01	22.51
45.90	3.62	3.90	21.30
44.48	3.86	4.25	19.91
42.90	4.35	4.58	19.24
40.67	4.41	5.03	18.79
39.41	4.65	5.21	17.62
38.08	4.83	6.91	16.37
37.04	5.08	7.63	15.85
35.87	5.28	8.35	15.30
34.85	5.46	8.73	15.02
33.67	5.74	9.09	14.81
32.35	6.11	9.30	14.63
31.13	6.23	63.10	1.18
30.20	6.49	61.88	1.59
29.19	6.70	60.74	1.89
28.18	7.08	59.57	2.15
27.00	7.22	71.04	0.62
26.26	7.43	69.73	0.77
25.47	7.70	68.32	0.90
24.68	7.89	67.20	1.03
23.85	8.31	66.10	1.12
22.89	8.64	65.14	1.26
22.01	8.84	64.10	1.37

21.14	9.14	63.03	1.48
20.43	9.34	62.28	1.60
19.67	9.63	61.29	1.73
18.97	9.88	60.32	1.88
18.43	10.09	59.41	2.02
17.80	10.35	58.57	2.13
17.26	10.58	57.80	2.23
16.72	10.77	57.22	2.35
16.22	10.98	56.57	2.44
15.76	11.19	56.04	2.48
3.52	56.48	55.72	2.56

Table S7.4. Tie Lines (TL) information: weight fraction compositions of phase formers for TLs at top (T) and bottom (B) phases. Tie line length is also presented.

Tie line	PPG 400 $_{T}$ (w/w %)	PPG 400 $_{B}$ (w/w %)	Ch DHp $_{T}$ (w/w %)	Ch DHp $_{B}$ (w/w %)	H_2O $_{T}$ (w/w %)	H_2O $_{B}$ (w/w %)	Tie Line Length (TLL)
1	69.9	6.6	1.1	17.3	29	76.1	65.4
2	77.8	3.7	0.4	22.3	21.8	74	77.3
3	80	2.6	0.2	25.9	19.8	71.5	81.6
4	81	1.3	0.1	46.7	18.9	52	92.4

Table S7.5. Composition of mixture points used for partitioning experiments (Vr=1) with the respective TLLs and cloud point temperature (Tc) of the top phase in presence of microalgae biomolecules.

TLL	PPG 400 (w/w%)	Ch DHp (w/w%)	Tc (°C)
65.4	37.8	9.2	25
77.3	39.2	11.8	28
81.6	39.8	13.7	31
92.4	39.3	24.6	<80

Chapter 8

Neochloris oleoabundans biorefinery: integration of cell disruption and purification steps using aqueous biphasic systems-based in surface-active ionic liquids

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Abstract

In this work, an approach to integrate the downstream processing of bioactive compounds present in the microalgae cells by combining the use of tensioactive compounds and aqueous biphasic systems (ABS) is proposed. For this purpose, several aqueous solutions using solvents with and without tensioactive nature were investigated on their capacity to disrupt the microalgae cells as well as to extract the different classes of biomolecules, namely pigments (chlorophylls *a* and *b*, and lutein), proteins and carbohydrates. Cationic tensioactive compounds were selected due to their high ability to simultaneously extract the different classes of compounds present in the *Neochloris oleoabundans* biomass. To fractionate pigments, proteins and carbohydrates extracted from the microalgae, ABS formed by polyethylene glycol (PEG 8000) and sodium polyacrylate (NaPA 8000) were used, with the solvent selected to disrupt the cells acting as electrolyte. This allowed to tune the biomolecule's partition reaching a selective fractionation. This approach provided the simultaneous extraction of different biomolecules (pigments, protein and carbohydrates) from the cells and, the subsequent origin of two fractions, one rich in proteins (extraction efficiencies of 100%) and carbohydrates (extraction efficiency of 80%) and the second concentrated in pigments (e.g. lutein, extraction efficiency of 98%). The further isolation of the biomolecules from the ABS forming solvents is proposed aiming at the development of an integrated downstream process, including the cell disruption/compounds extraction, the fractionation, and the isolation of the biomolecules.

Keywords: tensioactive compounds, microalgae disruption, aqueous biphasic systems, purification, multi-product approach

Introduction

Microalgae have attracted an increased attention, not only by their diversity, but also by the wide range of commodity and specialty products possible to obtain (298-300). They have been considered as a sustainable source of valuable biomolecules, such as pigments, lipids, proteins, carbohydrates and vitamins. A multi-product biorefinery aims at the complete valorization of the microalgal biomass, obtaining the maximum amount of products with commercial value with a minimal waste (129, 301). One of the main challenges to accomplish an efficient biorefinery is the development of sustainable and efficient extraction and fractionation processes allowing the recovery of the various biomolecules, while maintaining their structural integrity and properties (276, 302). The downstream processing of a multi-product biorefinery is currently complex and not cost-effective. It is responsible for more than 50 % of the total production costs (6, 129). Its complexity is defined by the use of several unit operations to obtain each bioproduct with the purity demanded. When dealing with bioactive compoundsfrom plants and algae, for example, the main unit operations used are the harvesting, cell disruption, product extraction, the products separation from the contaminant compounds and, when needed, the product isolation from the solvents.

In the specific case of microalgae, the selection of cell disruption and extraction technologies is currently focusing single-product approaches, making the process difficult to integrate in a multi-product (biorefinery) scenario (303). Mechanical (e.g. bead milling, pulse electric field and high pressure homogenization) or non-mechanical (e.g. microwave, acid/alkali treatment and enzymes) technologies have been investigated and applied for cell disruption (7). However, they are not yet appropriately explored and optimized (304). Bead milling, for example, is considered an efficient and mild cell disruption technology for microalgae. Nevertheless, it is of high energetic demand and complexity due to the formation of a stable emulsion because of the breakage of cells in very small particles (241, 305), which is a major concern for the dowsntream process performance, especially from high lipid containing microalgae (241).

After cell disruption, depending on the product of interest, two approaches are typically followed. Water-soluble products (e.g. proteins and carbohydrates) present in the supernatant are separated by aqueous-based technologies, such as filtration or chromatographic methods, and more recently aqueous biphasic systems (ABS). Then, hydrophobic molecules (e.g. pigments and lipids) remaining in the solid phase are extracted using organic solvent-based methods (306), and so, complex unit operations have to be applied to obtain pure biomolecules from microalgae (6). In this work, the integration of cell disruption and extraction in a single unit operation allied to the selective separation of different fractions will be investigated.

Tensioactive compounds are amphiphilic compounds composed of a hydrophobic and hydrophilic part presenting a high affinity for hydrophobic and hydrophilic compounds. This characteristic makes them able to disrupt and/or permeabilize different cells, including microalgae (307). The impact of tensioactive compounds on microalgae cells (characterized by a thick cell wall), has been evaluated (222, 308, 309), where the main results evidence the sinergistic effect on the extraction yields by their ability to disrupt the microalgae cells (310). Meanwhile, ionic liquids (ILs) have also attracted the attention of researchers. Their solvation

ability for a wide range of components is well-known and the possibility of controlling their structure and hydrophilic-lipophilic balance may lead to the design of new ILs with hydrotropic or tensioactive properties (306, 311, 312). These have already successfullly used as disrupting agents by their capacity to permeabilize the microalgae cells (100, 130, 249, 313, 314).

In this study, we investigated the combined ability of a set of aqueous solutions using (ionic and non-ionic) tensioactive compounds and ILs to disrupt microalgae cells and extract some of the most abundant biomolecules present, namely pigments (chlorophylls *a* and *b*, lutein), proteins and carbohydrates. The biomolecules extraction was integrated with ABS to separate the distinct fractions obtained after cell disruption. ABS are formed by two immiscible aqueous phases, providing a mild environment to the biomolecules. Innumerable types of chemicals can form ABS (e.g. polymer, salts, ILs, surfactants and carbohydrates), providing flexibility to this technology. ABS formed using two polymers is one of the most studied systems, because they provide a gentle and biocompatible environment for labile biomolecules. Among the polymeric-based ABS, the one formed by polyethylene glycol (PEG) and sodium polyacrylate (NaPA) presents advantages, such as its low viscosity, fast separation rate and phase separation, high water content, relatively low costs, and possibility of recycling and reusing the phase formers (315-317). These systems have been used in the separation of several biomolecules such as proteins, enzymes, DNA and antibiotics (318-320). Furthermore, the addition of salts, ILs and tensioactive compounds as electrolytes has been explored to induce a faster separation of the phases, minimizing the polymers concentration, and enhancing the separation' selectivity (321, 322). This approach provides a large number of possible ABS and the appropriate electrolyte selection allows the manipulation of the biomolecules partition by modifying the phases' polarities, the water solvation and/or the ions' compartmentalization (316, 323-325).

Considering their advantages, different polymeric-based systems comprising PEG 8000 + NaPA 8000 + electrolytes were characterized and investigated on the fractionation of the microalgal biomolecules. The electrolytes considered (also used as disruption agents) were selected based on their ability to disrupt the cells and to extract the biomolecules to water. Then, the ability of polymeric ABS based in dodecyl sulfate sodium salt (SDS), 1-butyl-3-methylimidazolium chloride ([C_4mim]Cl), tributyl-1-tetradecylphosphonium ([$P_{4,4,4,14}$]Cl) and 1-dodecyltrimethylammonium bromide ([$N_{1,1,1,12}$]Br), to separate proteins from carbohydrates, and pigments (lutein and chlorophylls) was tested. After the appropriate electrolyte selection and operational optimization, an integrated process including cell disruption, extraction and purification of the biomolecules and recycling of the main solvents is successfully proposed.

Experimental

Materials

The ILs 1-ethyl-3-methylimidazolium chloride, [C_2mim]Cl (99 wt%); 1-butyl-3-methylimidazolium chloride, [C_4mim]Cl (99 wt%); 1-hexyl-3-methylimidazolium chloride, [C_6mim]Cl (98 wt%); 1-methyl-1-propylpiperidinium chloride, [C_3mpip]Cl (99 wt%); 1-butylpyridinium chloride, [C_4py]Cl (98 wt%); 1-butyl-1-methylpyrrolidinium chloride, [C_4mpyr]Cl (99 wt%); 1-butyl-3-methylimidazolium bromide, [C_4mim]Br (98 wt%); 1-butyl-3-methylimidazolium trifluoroacetate,

$[C_4mim][CF_3CO_2]$ (97 wt%); 1-butyl-3-methylimidazolium trifluoromethanesulfonate, $[C_4mim][CF_3SO_3]$ (99 wt%); 1-butyl-3-methylimidazolium thiocyanate, $[C_4mim][SCN]$ (98 wt%); 1-butyl-3-methylimidazolium acetate, $[C_4mim][CH_3CO_2]$ (98 wt%); 1-butyl-3-methylimidazolium dicyanamide, $[C_4mim][N(CN)_2]$ (98 wt%), tributyl-1-tetradecylphosphonium, $[P_{4,4,4,14}]Cl$ (97 wt%), 1-methyl-3-octylimidazolium chloride $[C_8mim]Cl$ (99 wt%); 1-decyl-3-methylimidazolium chloride, $[C_{10}mim]Cl$ (98 wt%); 1-dodecyl-3-methylimidazolium chloride, $[C_{12}mim]Cl$ (98 wt%); 1-methyl-3-tetradecylimidazolium chloride, $[C_{14}mim]Cl$ (98 wt%) as well as 1-hexadecyl-3-methylimidazolium chloride, $[C_{16}mim]Cl$ (98 wt%), were supplied by IoLiTec.

Tetrabutylphosphonium chloride, $[P_{4,4,4,4}]Cl$ (95 wt%) and tetrabutylphosphonium bromide, $[P_{4,4,4,4}]Br$ (> 95 wt%), were provided by Cytec. Tetrabutylammonium chloride, $[N_{4,4,4,4}]Cl$ (97 wt%), Decyltrimethylammonium chloride, $[N_{1,1,1,10}]Cl$ (98 wt%) was acquired from TCI. 1-dodecyltrimethylammonium bromide, $[N_{1,1,1,12}]Br$ (99 wt%); tetradecyltrimethylammonium bromide, $[N_{1,1,1,14}]Br$ (98 wt%) and hexadecylpyridinium chloride monohydrate, $[C_{16}py]Cl$ (99 wt%) were provided by Sigma-Aldrich. 1-hexyl)trimethylammonium bromide, $[N_{1,1,1,6}]Br$ (98 wt%) was purchased at Alfa Aesar. Their chemical structures as well as their critical micelle concentration (CMC) data are shown in Table S1 from ESI.

Lab grade Brij L4, Brij 93, Tween 80, Genapol X-080, Genapol C100, Merpol HCS were acquired from Sigma-Aldrich, and ACROS Organics supplied Tween 20 and Triton X-114. Dioctyl sulfosuccinate sodium salt, AOT (96 wt%) and sodium dodecyl-benzenesulfonate, SDBS (tech grade) were acquired from Sigma-Aldrich, while the dodecyl sulfate sodium salt, SDS (99%) was acquired from ACROS Organics. Polyethylene glycol (PEG 8000 $g.mol^{-1}$, purum), sodium polyacrylate (NaPA 8000 $g.mol^{-1}$; 45 wt% in water), and standard molecules D(+)-glucose (99.6 pure%), lutein (96% pure), chlorophyll *a* (95% pure) and chlorophyll *b* (95% pure) were supplied by Sigma-Aldrich.

Microalgae cultivation and harvesting

Neochloris oleoabundans (*N. 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 pH 8.0 and controlled temperature at 30 °C, under saline conditions using artificial sea water on Bold's Basal medium (143) composed of NaCl: 24.5 $g.L^{-1}$; $MgCl_2$: 9.8 $g.L^{-1}$; $CaCl_2$: 0.53 $g.L^{-1}$; K_2SO_4: 0.85 $g.L^{-1}$; $NaSO_4$: 3.2 $g.L^{-1}$; $NaHCO_3$: 0.8 $g.L^{-1}$. 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 MilliQ® water to obtain a biomass concentration of ~25$g_{DW}.kg^{-1}$. The algal cells were lyophilized and stored at -20 °C until further use.

Simultaneous cell disruption and extraction of biomolecules

A cell disruption method using aqueous solutions of non-tensioactive and tensioactive compounds was here studied. Several ILs with different cations (imidazolium, ammonium, phosphonium, pyrrolidinium, pyridinium, piperidinium), anions, alkyl chain lengths, as well as some conventional surfactants (non-ionic, cationic and anionic), were screened to disrupt/permeabilize the microalgae cell walls. Extractions with pure methanol (100% HPLC grade, supplied by CHEM-LAB) and MilliQ water were performed as controls.

Microalgae cells were continuously mixed with the different aqueous solutions using a solid-liquid ratio (SLR) of 0.025 (mass dry biomass/ volume of solvent) at 50 rpm during 1 hour in an orbital mixer and at room temperature. The final concentration (C) of all solvents was studied in aqueous solution at a concentration of 250 mM. SDBS was studied at 100 mM, described as its limit of water solubility. The cell suspension was centrifuged at 13000 × g for 10 min in a VVR microstar 17 centrifuge. The supernatant was separated from the pellet and both fractions were further analysed. The solutions presenting the best cell disruption performance were selected based on the release of cell components (proteins, pigments and carbohydrates). The cell wall structure damage after treatment was analysed by Scanning Electron Microscopy (SEM). The solid-liquid ratio (SLR) and the solvent concentration (C) were the parameters evaluated regarding the extraction performance of the different classes of biomolecules. At least three individual samples for each set of conditions were prepared, for which the amount of the different classes of biomolecules was quantified.

The performance of the extractions is discussed in terms of extraction yield ($Yield_{MB}$), which is the mass of a biomolecule present in the supernatant ($m_{MB,supernatant}$) divided by the mass of biomass used ($m_{biomass}$) as represented by Eq 8.1. The extraction efficiency $EE_{MB}(\%)$ of each class of biomolecules was calculated considering $m_{MB,i}$, which represents the mass content of the biomolecule initially composing the cells (Eq. 8.2).

$$Yield_{MB} = \frac{m_{MB,supernatant}}{m_{biomass}} \quad (8.1)$$

$$EE_{MB}(\%) = \frac{m_{MB,supernatant}}{m_{MB,i}} \quad (8.2)$$

After selecting the best solvents to extract the different classes of biomolecules, specifically proteins, carbohydrates and carotenoids (with emphasis on lutein), a surface response methodology (SRM) was applied to analyse the influence of the various parameters on the extraction. The concentration of the tensioactive compound (C) in mM and the SLR were the parameters studied by implementing a 2^2 factorial planning (Table S8.2 in ESI). In each factorial planning, the central point was experimentally assessed at least in triplicate. The obtained results were statistically analysed with a confidence level of 95%. The adequacy of the model was determined by evaluating the lack of fit, the regression coefficient (R^2), and the F-value obtained from the analysis of variance (ANOVA). The Statsoft Statistica 10.0© software was used for all statistical analyses and to represent the response surfaces and contour plots.

Phase diagrams of the ABS

Various tensioactives were selected based on their ability to disrupt the cells. These compounds were used as electrolytes in the formation of ABS composed of PEG 8000 and NaPA 8000. Some of the binodal curves applied in this work were adopted from literature (322). For the ABS using $[P_{4,4,4,14}]Cl$ and $[N_{1,1,1,12}]Br$ as electrolytes, the phase diagrams were determined in this work (322),(326).

Partition of biomolecules in the ABS

The partition of the biomolecules extracted from the biomass was evaluated in polymeric-based ABS, being the tensioactive used in the cells' disruption applied as electrolyte. The ABS were prepared gravimetrically by weighing the appropriate amount of each phase component and supernatant (after disruption/extraction process) in graduated centrifuge tubes. The mixture point adopted for the biomolecules partition was 20 wt% PEG 8000 + 5.0 wt% NaPA 8000 and 0.1 wt% of each electrolyte (supernatant after extraction process). The methods described in literature were adopted (322). The quantification of each biomolecule was performed in triplicate, being the results reported as the average of three independent assays plus their respective standard deviations. The partition coefficients of microalgal biomolecules (K_{MB}), extraction efficiencies in the top and bottom phases (EE_{MB} (%)) and Selectivity values (S) were determined as represented by Eqs. 8.3-8.5.

$$K_{MB} = \frac{C_{MB,top}}{C_{MB,bottom}} \qquad (8.3)$$

$$EE_{MB}(\%) = \frac{m_{MB,phase}}{m_{MB,initial}} \times 100 \qquad (8.4)$$

$$S_{MBi/MBj} = \frac{K_{MBi}}{K_{MBj}} \qquad (8.5)$$

where $C_{MB,top}$ and $C_{MB,bottom}$ are the concentration of the specific biomolecule in the top and bottom phases, respectively, $m_{MB,phase}$ is the biomolecule mass in each phase and $m_{MB,initial}$ is the biomolecule mass in the initial crude extraction obtained after the cells disruption.

Quantification of biomolecules

Possible interferences of the polymers, non-tensioactive and tensioactive solvents investigated were eliminated using blank controls, represented by the same mixtures, however without the presence of the corresponding biomolecules (carbohydrates, lutein, chlorophylls or proteins). This approach was followed in all quantification methods employed.

Pigments analysis

The pigments in the crude extract obtained after the cell disruption and present in each ABS phase were quantified by UV-Vis spectroscopy, using a Synergy HT spectrometer microplate reader between 200 and 750 nm. Calibration curves were prepared for lutein, chlorophylls *a* and *b* in the aqueous solutions selected and in methanol using analytical standards. OriginPro 8.0 was used for the spectral deconvolution of the peaks at the maximum absorption wavelengths of the pigments in all samples (327). The initial mass content of pigments

composing the cells ($m_{PIGMENTS,i}$) was determined by the complete extraction of the pigments from bead milled microalgae with methanol, followed by immersion in an ultrasound bath for 5 minutes (284). To recover the pigments, the samples were centrifuged at 1800 ×g for 10 min. This procedure was repeated until a white pellet was obtained.

To confirm the type of pigments present it was applied HPLC-DAD (Shimadzu, model PROMINENCE) using an analytical method developed and validated in CICECO - Aveiro Institute of Materials. The analyses were performed with an analytical C18 reversed-phase column (250 × 4.60 mm), kinetex 5 µm C18 100 A, from Phenomenex. The mobile phase consisted of 90% of methanol and 10% of acetonitrile. The separation was conducted in isocratic mode, at a flow rate of 1 mL.min^{-1} and using an injection volume of 10 µL. DAD was set at 665, 649 and 454 nm (max. absorption wavelengths) (327). Each sample was analysed at least two times. The column oven and the autosampler were operated at a controlled temperature of 30 °C.

Protein analysis

Proteins were quantified by the BCA Protein Assay Reagent Kit (Thermo Scientific), using Bovine Serum Albumin (BSA) as standard. Absorbance at 562 nm was measured by UV-Vis spectroscopy, using a Synergy HT spectrometer microplate reader. For an accurate quantification of proteins, the samples (standards and blanks) were treated with acetone to allow the precipitation of contaminants (285).

The total mass content of proteins in the microalgae cells ($m_{PROTEINS,i}$) was determined after the extraction of the proteins following the protocol developed in the Bioprocess Engineering group, at Wageningen University (230). The lyophilized cells were suspended in 1 mL of lysis buffer (60 mM of Tris and 2% of SDS, pH 9) in lysing matrix E tubes (6914–500, MP Biomedicals Europe). The samples were bead beated for 3 cycles of 60 s, at 6500 rpm, and pausing 120 s between each cycle (Precellys 24, Bertin Technologies). Finally, the cell suspension was heated at 100 °C for 30 minutes.

Carbohydrate analysis

The carbohydrates in the samples were quantified by UV-Vis spectroscopy, using a Synergy HT spectrometer microplate reader after the reaction of the carbohydrates with acidic phenol measured at 483 nm (108). The calibration curve was prepared using D(+)-glucose as analytical standard.

The total carbohydrate mass content ($m_{CARBOHYDRATES,i}$) was determined after the total extraction by acid hydrolysis of the lyophilized cells as described by Postma et al. (328). 1 mL of 2.5 M HCl was added to 1 mg of lyophilized biomass and incubated during 3 hours at 100 °C. After cooling down, the samples were neutralized using 1 mL of 2.5 M NaOH and centrifuged (1200 ×g, 10 min, at room temperature). The supernatant of each sample was used for the analysis of the carbohydrates.

For control, the cells were directly suspended in water without any pre-treatment. Starch samples were measured as positive controls.

Lipids analysis

The total fatty acid (TFA) content in the cells $m_{LIPIDS,i}$ was determined as described by Remmers et al. (232). 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) were used as polar lipid and TAG internal standards, respectively. To determine the total amount of lipids in the samples, 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 $mL.min^{-1}$. The column used was a Supelco Nucol^{tm} 25357, 30 m x 530 µm x 1.0 µm. Total fatty acid composition and content were calculated by taking the sum of all fatty acids determined.

Scanning Electron Microscopy

To visualize the cell disrupting effect of the solutions selected, Scanning Electron Microscopy (SEM) and fluorescence microscopy were used. Lyophilized cells, before and after treatment with the aqueous solutions of alternative tensioactive solvents, were fixed with glutaraldehyde at room temperature. 50 µL of glutaraldehyde [2 % (v/v)] was added to 0.5 mL of each sample. The samples were treated for 2 h and afterwards washed three times with distilled water. The pellets were lyophilized, and the samples were deposited on carbon conductive tape and coated with a carbon layer before observation by SEM, using a Hitachi SU-70 microscope operating at 15 kV.

The initial microalgae cells and pellets after the treatment with the aqueous solutions were suspended in 0.01 M phosphate-buffered saline (pH 7.2) and observed using a fluorescence microscope (Axio Imager 2, Zeiss). The emission window was set at 500-750 nm to collect the fluorescence images. Image processing was performed using ZEN v2.3 blue edition software (Carl Zeiss Microscopy GmbH).

Results and discussion

Solvent screening applied in cells' disruption and biomolecules extraction

The biomass was firstly characterized. These cells are composed of 377 ± 8 $mg.g_{dry\ biomass}^{-1}$ of lipids, 283 ± 7 $mg.g_{dry\ biomass}^{-1}$ of proteins, 287 ± 9 $mg.g_{dry\ biomass}^{-1}$ of carbohydrates, 5.7 ± 0.6 $mg.g_{dry\ biomass}^{-1}$ of lutein, 3.7 ± 0.7 $mg.g_{dry\ biomass}^{-1}$ of chlorophyll *a* and 1.2 ± 0.5 $mg.g_{dry\ biomass}^{-1}$ of chlorophyll *b*. The lipids were measured to characterize the biomass, although their purification is been focused in a complementary work under preparation.

The study of an alternative process to disrupt the cell wall and extract the different classes of biomolecules was carried using aqueous solutions of ILs (tensioactive and non-tensioactive) as well as conventional surfactants. The screening was performed using a SLR (mass of dry

biomass/volume of solvent) of 0.025, at room temperature, in the absence of light and for 1 h. For these experiments, the concentration of ILs and surfactants was fixed at 250 mM.

ILs composed of different cations and anions (Table S8.1 from ESI) were tested in order to understand the influence of their structure on the cells' disruption and consequent extraction of biomolecules.

Generally, it was observed that tensioactive ILs and common surfactants extracted more biomolecules than the non-tensioactive ones. The results from Fig. 8.1 show that aqueous solutions (250 mM) of non-tensioactive ILs behave no better than water. The extraction process using the alternative solvents has released 53 $mg.g_{dry\ biomass}^{-1}$ of proteins, 0.14 $mg.g_{dry\ biomass}^{-1}$ of lutein and 0.12 $mg.g_{dry\ biomass}^{-1}$ of chlorophylls (chlorophyll *a* + chlorophyll *b*). This suggests that water has penetrated the cell walls and other spaces in the cell, solubilising some proteins (121). The same behaviour was not found for the pigments considering their hydrophobic nature and consequent low affinity for water.

The influence of the hydrophilic/hydrophobic character of the ILs on the extraction of proteins was determined by comparing the effect of non-tensioactive ILs sharing the same anion (Cl^-). As previously reported (99, 292), the more hydrophilic the ILs are, the stronger their capacity to extract water-soluble proteins. The order for imidazolium-based ILs is $[C_2mim]Cl \approx [C_4mim]Cl > [C_6mim]Cl$ and for tetrabutylphosphonium-based ILs is $[P_{4,4,4,4}]Cl > [P_{4,4,4,4}]Br$, the latest having the lowest capacity to extract proteins. In other words, while the alkyl chain length of the IL cation increases, their hydrophilicity decreases, and with it, their interaction with (hydrophilic) proteins. On the contrary, there is a clear advantage of using the most hydrophobic solvents to extract the pigments, i.e. lutein and chlorophylls. This behaviour is in accordance with literature, since when solvents with tensioactive nature are used, the disruption (222, 329), or induced permeation (330, 331) of cell membranes is magnified.

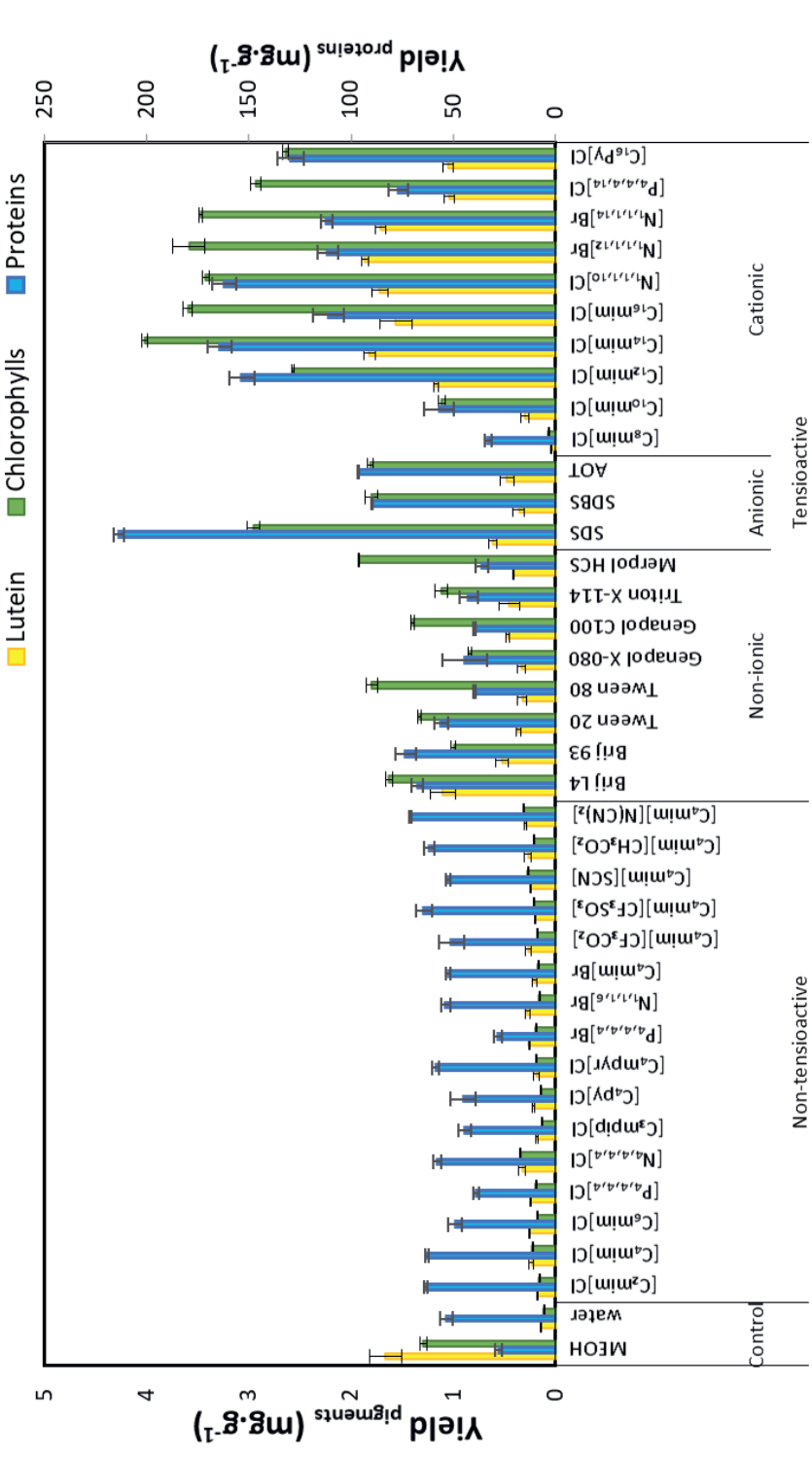

Fig.8.1. Extraction yield of pigments (lutein and chlorophylls a and b) and proteins from N. oleoabundans using aqueous solutions of non-tensioactive and tensioactive compounds (non-ionic, anionic and cationic). Error bars indicate standard deviations.

Actually, two main mechanisms have been discussed regarding the use of tensioactive compounds to extract biomolecules from biomass; (i) the solubilization of the biomolecules composing the cell membranes (310), (ii) the cell disruption considering the structural similarity between the tensioactive solvents and the membrane phospholipids. Even the combination of both factors has been considered. Moreover, and based in our results, ionic tensioactive compounds have a higher capacity to disrupt the cells and extract the biomolecules than non-ionic tensioactive compounds. The anionic surfactant SDS was found to be the solvent with the highest capacity to extract proteins from the cells (213.68 mg of proteins extracted *per* g of dry biomass), which is justified by the strong electrostatic interactions forming protein-surfactant complexes (332, 333), while SDS extracts poorly the lutein. Pigments like lutein, were better extracted by using cationic tensioactives. The main results suggest that, by increasing the cation alkyl chain length up to C_{14}, the extraction yields of lutein and chlorophylls increased, since the longer the chain length, the stronger the solvent ability to disrupt the cell wall (334) and to interact with/solubilize the pigments. Since the mechanism acting seems to be the cells disruption, the amount of proteins released from the cells increases with the elongation of the alkyl chain. Comparing the yields of extraction of pigments with methanol, here adopted as the conventional solvent for comparison purposes, maximum amounts of lutein and chlorophylls were obtained by applying cationic tensioactive ILs, respectively up to 1.9 $mg_{lutein}.g_{dry\ biomass}^{-1}$ against 1.7 $mg_{lutein}.g_{dry\ biomass}^{-1}$ for methanol and up to 4 $mg_{chlorophylls}.g_{dry\ biomass}^{-1}$ vs. 1.3 $mg_{chlorophylls}.g_{dry\ biomass}^{-1}$ for methanol.

Cationic tensioactive ILs for the disruption of cells and release of biomolecules

After the identification of the aqueous solutions of cationic tensioactive ILs as the most efficient solvents to disrupt the cells and to extract the biomolecules of interest, their impact on the extraction of high value pigments (lutein and chlorophylls), proteins and carbohydrates was evaluated (Fig. 8.2). For that, aqueous solutions of tensioactive (cationic) ILs and SDS at 250 mM were tested and their effects compared through the extraction efficiency and selectivity parameters. SDS was selected as reference in this work due to its general applicationas disruptive agent (335).

In general, all tensioactive compounds tested have demonstrated the ability to disrupt or to permeabilize the cells. SDS have extracted 77% of the proteins composing the cells, with a higher efficiency being obtained when compared with the one described for the cationic tensioactives. However, the green colour characterizing the aqueous extract changed to brown colour after applying SDS (and previously SDBS) with chlorophylls *a* and *b* being undetected by HPLC (Figure S8.1-B from the ESI), which implies their partial or complete degradation (336-339). In the end, the main results obtained for the extraction of proteins follow the order $[C_{16}mim]Cl \approx [C_{14}mim]Cl$ - 60%, $[C_{12}mim]Cl$ - 55%, $[N_{1,1,1,12}]Br \approx [N_{1,1,1,14}]Br$ - 40% and $[P_{4,4,4,14}]Cl$ - 37%. Like chlorophylls, lutein was efficiently extracted by the cationic tensioactive compounds tested, as shown in the HPLC analysis (Figure S8.1C from ESI).

Despite the lowest values of proteins extraction efficiency obtained for the cationic tensioactive ILs when compared with SDS, no apparent degradation of the biomolecules was detected. In addition, SDS demonstrated lower capacity to solubilize the pigments, the most hydrophobic

biomolecules. In summary, cationic tensioactive compounds were selected as the most efficient, these extracting more than 81% of chlorophylls, 60% of proteins, 33% of lutein, and 25% of total carbohydrates, this considering their maximum content in the cell.

Although some aqueous solutions of cationic tensioactives, namely $[N_{1,1,1,12}]Br$, are able to enhance the extraction efficiency of total carbohydrates, the extraction efficiency is still low and similar to the extraction efficiency reached using water, which may be explained by the low solubility of starch (most abundant polysaccharide in microalgae) in water. Starch may remain in the solid fraction together with other cell wall components due to its low solubility in water, as already reported when cell disruption is carried by mechanical techniques (340),(341).

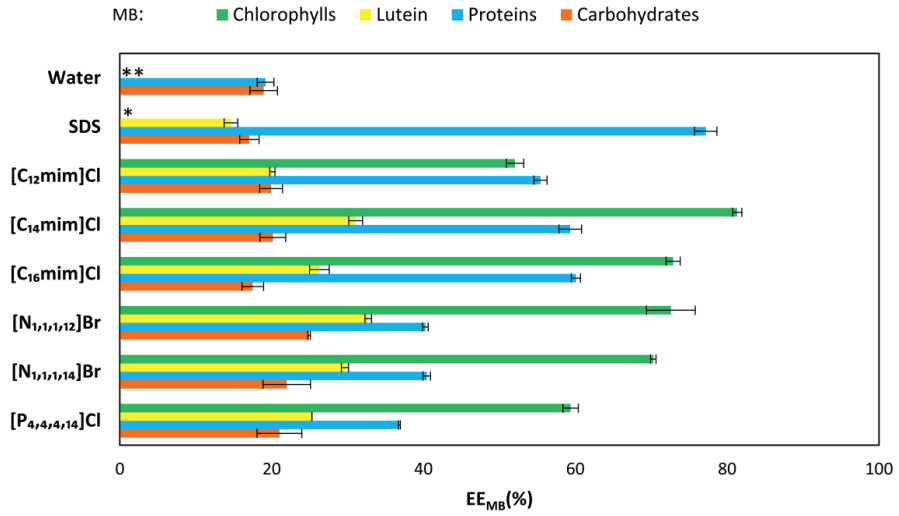

Fig. 8.2. Extraction efficiency of the microalgae biomolecules, EE_{MB} (%), determined for pigments (lutein, chlorophylls a and b), proteins and carbohydrates using aqueous solutions of cationic tensioactive compounds. Error bars indicate the standard deviations. *Chlorophylls were not detected in the supernatant when using SDS. ** No pigments (chlorophylls and lutein) were detected in the supernatant after using water as solvent.

To prove the effect of the tensioactive compounds applied as solvents and/or disrupting agents, after the biomolecules' extraction, the solid biomass fraction was analysed by SEM and fluorescence microscopy (Figure S8.2 from ESI). Here, the initial freeze-dried biomass treated with water (A, B) and the biomass residues after applying aqueous solutions of SDS (C and D), $[N_{1,1,1,12}]Br$ (E and F), $[P_{4,4,4,14}]Cl$ (G and H) and $[C_{14}mim]Cl$ (I and J) were investigated. In general, the images demonstrated that aqueous solutions of tensioactive compounds (cationic and anionic) are able to partially disrupt the cell wall, consequently releasing the intracellular compounds. As already demonstrated (342), this process is helped by the freeze-drying step, which may explain the extraction of proteins and carbohydrates when using only water as solvent. Based on the SEM images, the degree of disruption is higher when using the cationic tensioactive compounds, when compared with the anionic ones. No intact cells were detected

in the solids after treatment with the quaternary ammonium, imidazolium and phosphonium (Figures S8.2E to S8.2J from ESI). Indeed, the cationic tensioactive compounds interact strongly with the microalgae cells, which possess a net negative surface charge, probably driving the most intensive disruption (309, 343). On the other hand, in addition to the lowest degree of disruption found for the anionic surfactant, the biomass fluorescence after the treatment with SDS was reduced, which can explain the absence of chlorophylls (Figure S8.1).

Optimization of the operational conditions by RSM

To optimize the extraction efficiencies obtained using aqueous solutions of cationic and anionic tensioactive compounds, identified as the most efficient systems extracting the intracellular content of the cells, the influence of two process variables, the SLR and the tensioactive compound concentration (C in mM) were studied, by applying a RSM (Table S8.2 in ESI). This methodology allows the simultaneous analysis of different parameters and their effect on the extraction efficiency of the biomolecules under study. The extraction efficiency of lutein, total proteins and total carbohydrates (response variables) was studied regarding the SLR and C as independent variables. The extraction efficiencies of the biomolecules (Table S8.3), the model equations (Table S8.4), as well as the parameters obtained in the statistical analysis are presented in Table S8.5. The accuracy and precision of the model equations were validated and the experimental and predicted values of the response variable (Table S8.3) were compared.

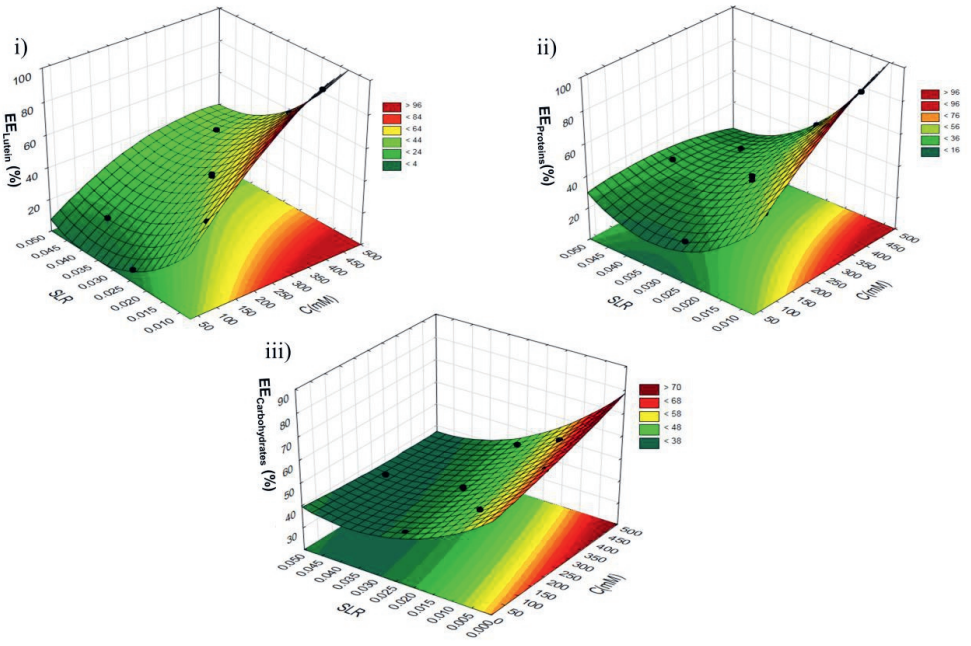

Fig. 8.3. Response surface plots representing the extraction efficiency [EE_{MB} (%)] of lutein (i), proteins (ii) and carbohydrates (iii) with the combined effects of solid-liquid ratio (SLR) and concentration, C (mM), using aqueous solutions of [$P_{4,4,4,14}$]Cl.

Fig. 8.3 depicts the response surface plots showing the effect of SLR and C on the extraction efficiency of lutein (i), total proteins (ii) and carbohydrates (iii) using an aqueous solution of $[P_{4,4,4,14}]Cl$. Both, C (mM) and SLR are statistically significant variables (Table S8.4 from ESI), with the latest being the most significant. The results indicate that decreasing the SLR, the extraction efficiency (%) increases for all the biomolecules. Meanwhile, by increasing the concentration of the IL the results demonstrated an increase in the extraction efficiency for the three types of biomolecules. As analysed, at optimum conditions, lutein and total proteins were completely extracted (100%) by the phosphonium-based aqueous solution, although at the same conditions, carbohydrates only reached a maximum extraction efficiency of 60%. Similar effects of SLR and C (mM) on the extraction of biomolecules were obtained applying the $[N_{1,1,1,12}]Br$-based aqueous solution (Figure S8.3) and $[C_{14}mim]Cl$ (data not reported).

Despite the results indicating the improvement of C the extraction efficiency, the use of high volumes of solvent can hinder the economic sustainability of the process, while the use of high concentrations of the tensioactive may increase the viscosity and the economic impact of the process. After the optimization, the SLR of 0.025 and the surfactant concentration of 250 mM were adopted for further studies.

Partition of microalgal biomolecules on the polymer-based ABS

After obtaining the aqueous extracts rich in carbohydrates, proteins and pigments, the fractionation of the biomolecules was carried by using polymer-based ABS. In this work, a specific type of polymer-based ABS (322) was applied, using as electrolytes SDS, $[C_{14}mim]Cl$, $[P_{4,4,4,14}]Cl$ and $[N_{1,1,1,12}]Br$.

The binodal curves based on polymers, PEG 8000 and NaPA 8000, using $[P_{4,4,4,14}]Cl$ and $[N_{1,1,1,12}]Br$ as electrolytes were experimentally determined in this work, while the binodal curves for systems using 0.1 wt% of SDS and $[C_{14}mim]Cl$ were adopted from literature (322). Figure S8.4 shows the binodal curves (weight fraction data (wt%) is represented in Table S8.9 from ESI), while the parameters obtained through the Merchuk equation are represented in Table S8.9 from ESI. As previously reported for other electrolytes (322), our results do not allow the identification of a clear trend regarding their ability to promote the ABS formation.

On Figure S8.4, it is also identified the mixture point used on the partition studies composed of 20 wt% of PEG 8000 + 5.0 wt% of NaPA 8000 and 0.1 wt% of crude extract with electrolyte, applied on the separation of pigments, proteins and carbohydrates. This mixture point allowed us to use less NaPA 8000 and more water, allowing to decrease the viscosity of the system, enhancing their biocompatibility by maintaining the pH of both phases ($7.06 \leq pH \leq 7.9$), independently of the electrolyte present (Table S8.10). For this study, the biomolecules were first extracted using the conditions previously optimized, namely SLR = 0.025 and C of 250 mM, for SDS (anionic), $[C_{14}mim]Cl$, $[P_{4,4,4,14}]Cl$ and $[N_{1,1,1,12}]Br$ (cationic) at room temperature and for 1 h of extraction.

Fig. 8.4A shows the partition coefficient logarithm function obtained for pigments, proteins and carbohydrates (Log K), while Fig. 8.4B depicts the extraction efficiencies (EE, %) obtained for

the top and bottom phases considering the systems studied. In general, the results indicate that the nature of the electrolytes tunes the partition preference of different biomolecules present in the crude extract. While the migration of pigments and proteins was found to be highly dependent on the electrolyte used, the carbohydrates partition showed to be independent of the electrolyte structure. The partition of carbohydrates between the two phases is similar ($-0.45 \leq \text{Log K} \leq 0.06$), although slightly maximized for the most hydrophilic phase, top (PEG 8000-rich) phase, as demonstrated by the EE up to 80% (Fig. 8.4B) (210, 322).

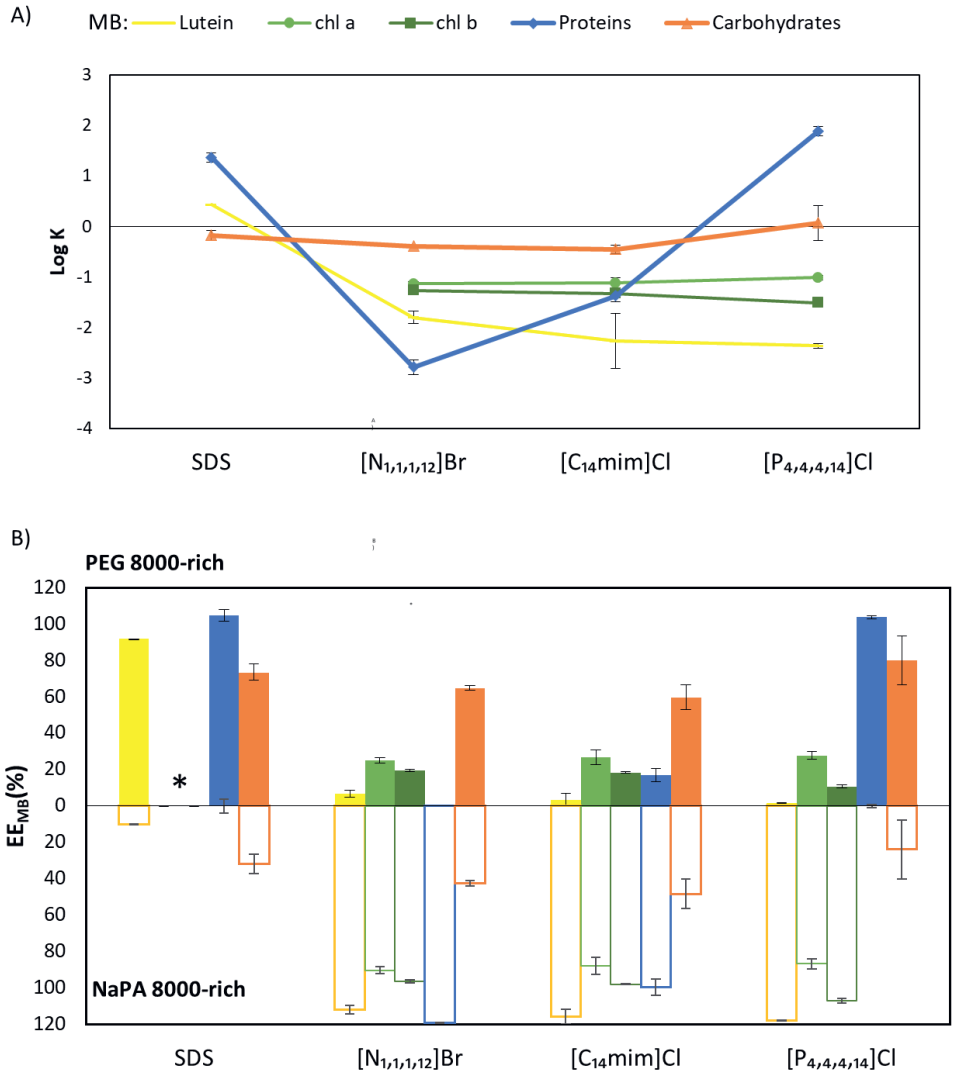

Fig. 8.4. Partition of biomolecules in polymeric-based ABS using 0.1 wt% of each electrolyte. A) partition coefficient logarithmic function (Log K) and B) EE_{MB} (%) in PEG 8000-rich phase (full columns) and NaPA 8000-rich phase (empty columns). *Chlorophylls a and b were not detected in the aqueous crude extract when using SDS.

Regarding the separation of pigments and proteins, the data show the big impact of the electrolyte nature on their partition profile. Pigments partition towards the bottom (NaPA 8000-rich) phase when using cationic electrolytes, and towards the top (PEG 8000-rich) phase when SDS (anionic surfactant) is applied. Therefore, and as previously reported (222) (209), the high affinity between the pigments, which are highly hydrophobic compounds, and the tensioactive compounds seems to justify this tendency. The maximum EE obtained for lutein, the most abundant pigment in *N. oleoabundans* (209), was up to 98% using $[P_{4,4,4,14}]Cl$. Although chlorophylls *a* and *b* are not detected when SDS is used, these are more concentrated in the bottom phase when tensioactive ILs are used as electrolytes.

Finally, and similarly to what occurred for pigments, the partition of proteins is also highly influenced by the electrolyte. For example, when applying the (anionic) SDS, 100% of proteins were recovered in the top (PEG 8000-rich) phase (Log K = 1.36). Nevertheless, when cationic ILs are used, proteins migrated towards the bottom (NaPA 8000-rich) phase (Log K =-2.79 and EE = 99.4 %). Due to the highest hydrophobic nature of $[P_{4,4,4,14}]Cl$ this seems to be the only exception ($EE_{protein}$ around 100% for PEG 8000-top phase).

Taking into account all the results and, since the aim of this work was to efficiently separate pigments from carbohydrates from proteins, the selectivity was calculated and reported in Table 8.1, evidencing even more the crucial role of the electrolytes to separate the different biomolecules. The selectivity of the compounds when using SDS is low, while the different cationic electrolytes have a significant influence on the selectivity. $[N_{1,1,1,12}]Br$ and $[C_{14}mim]Cl$ enhanced the separation of carbohydrates (PEG 8000-rich phase) from proteins and pigments (NaPA-rich phase), while $[P_{4,4,4,14}]Cl$, enhances the ability of the ABS to separate proteins and carbohydrates from pigments (*e.g.* lutein).

Table 8.1. Selectivity values (S) obtained for Proteins, carbohydrates (Carb) and pigments [specifically Lutein, chlorophyll a (Chl a) and chlorophyll b (Chl b)] using tensioactive compounds as electrolytes in PEG 8000-NaPA 8000-based ABS.

Electrolyte	SDS	$[N_{1,1,1,12}]Br$	$[C_{14}mim]Cl$	$[P_{4,4,4,14}]Cl$
$S_{Proteins/Lutein}$	8.76	3.03	5.67	**17633.28**
$S_{Proteins/Chl\ a}$	-	0.90	0.92	**2507.07**
$S_{Proteins/\ Chl\ b}$	-	7.47	7.57	**45.09**
$S_{Carb/Proteins}$	0.03	8.32	8.22	**0.02**
$S_{Carb/Lutein}$	0.25	25.21	46.61	**317.12**
$S_{Carb/Chl\ a}$	-	7.47	7.57	**45.09**
$S_{Carb/Chl\ b}$	-	5.54	4.63	**14.14**

Integrated downstream processing of microalgae components

To develop a process feasible for industrial application, the integrated downstream process sketched in Fig. 8.5 was designed. This involves three main steps: the (I) cell disruption/biomolecules' extraction, (II) fractionation of the biomolecules released (proteins,

pigments and carbohydrates), and (III) polishing representing the isolation of the biomolecules purified from the phase formers, thus allowing the solvents recycling. As represented in Fig. 8.5, in step (I) the microalgae cells are disrupted using an aqueous solution of $[P_{4,4,4,14}]Cl$ at 250 mM, thus releasing from the biomass, 105 ± 2 $mg.g_{dry\ biomass}^{-1}$ of proteins, 60 ± 9 $mg.g_{dry\ biomass}^{-1}$ of carbohydrates, 2.90 ± 0.05 $mg.g_{dry\ biomass}^{-1}$ of chlorophylls, and 1.60 ± 0.06 $mg.g_{dry\ biomass}^{-1}$ of lutein. In step II), the biomolecules presented in the aqueous extract containing $[P_{4,4,4,14}]Cl$ were separated by applying a polymer-based ABS using this IL as electrolyte. This system was chosen due to its high selectivity to separate proteins ($EE_{Proteins}$ = 100%) and carbohydrates (EE_{Carb} = 80%) from pigments (e.g. lutein, EE_{Lutein} = 98%). Finally, an integrated unit considering the isolation of the biomolecules from the solvents was designed, allowing the solvents recycle, step III). Here, an ultrafiltration unit is proposed and applied to the PEG 8000-rich phase allowing the separation of proteins (*e.g.* Rubisco = 560 kDa) from carbohydrates (*e.g.* glucose = 180 Da). Subsequently, a second unit of ultrafiltration may be used to separate by size, the PEG 8000 from the carbohydrates. The recyclability of NaPA 8000 can be assured by back-extracting the pigments using a benign organic solvent industrially accepted and with a high affinity for hydrophobic pigments.

The process proposed in this work consideres not only the optimal disruption of microalgae cells and the extraction of different biomolecules, but also their fractionation and the recyclability of the solvents used. Besides the high yields of extraction and fractionation, the integration of these unit operations is the major advantage of using aqueous biphasic systems-based in surface-active ionic liquids in comparison with other previously reported methods (Table 8.2).

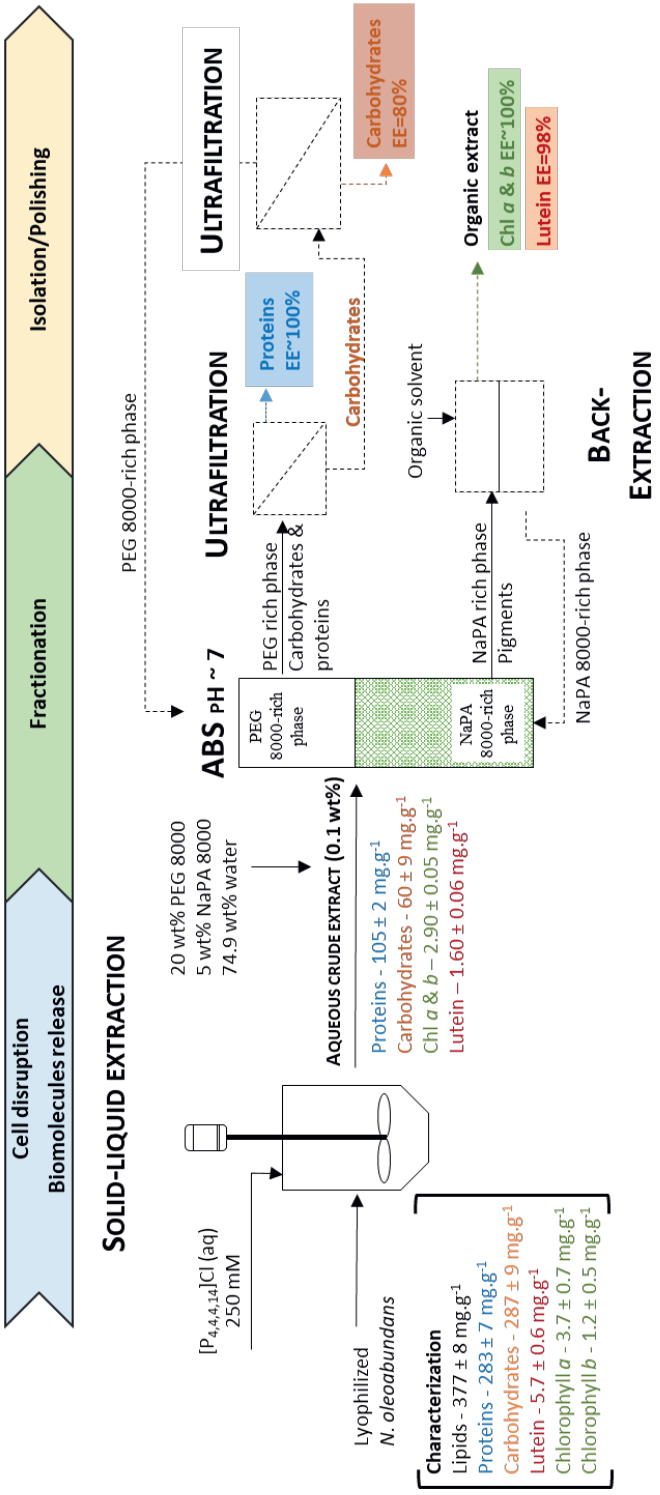

*Fig. 8.5. Schematic representation of the integrated process developed contemplating the three main steps, (I) biomolecules' extraction, (II) separation of the biomolecules released from the biomass, and (III) isolation of biomolecules purified from the phase forming solvents, allowing the solvents' recycling. Dashed lines represent steps that were not experimentally carried. **Note**: the units representing the amount of each class of compounds extracted are described for dry biomass ($mg.g_{dry\ biomass}^{-1}$).*

Table 8.2. Summary and comparison of studies on microalgae cell disruption/extraction/fractionation technologies.

Microalgae sp.	Main compounds	Extraction method	Yield	Reference
Neochloris oleoabundans	Carotenoids	Pressurized liquid extraction (using ethanol)	53.4 $mg_{carotenoids}/g_{extract}$	(125)
Chlorella pyrenoidosa	Lutein	Ultrasound-enhanced subcritical CO_2 extraction (with enzyme pretreatment) and Soxhlet extraction	1.24 $mg_{lutein}/g_{crude\ biomass}$	(344)
Haematococcus pluvialis	Lutein	Carbon dioxide (CO2) in supercritical fluid extraction - Ethanol as co-solvent	~7.15 $mg_{lutein}/g_{dry\ weight}$	(345)
Scenedesmus sp	Lutein	Microwave assisted binary phase solvent extraction method	11.92 $mg_{lutein}/g_{biomass}$	(346)
Chlorella vulgaris	Lutein	Ultrasound-assisted extraction and high performance counter-current chromatography	3.20 $mg_{lutein}/g_{dry\ weight}$	(347)
Phormidium autumnale	Chlorophyll *a*	Solid-liquid extraction	2.70 $\mu g_{chlorophyll\ a}/g_{dry\ weight}$	(348)
Dunaliella tertiolecta, Cylindrotheca closterium	Chlorophyll *a*	Microwave assisted extraction (MAE), cold and hot soaking and ultrasound-assisted extraction (UAE)	9.31 $mg_{chlorophyll\ a}/g_{dry\ weight}$and (hot soaking in acetone), 4.95 $mg_{chlorophyll\ a}/g_{dry\ weight}$ (UAE), 8.65$mg_{chlorophyll\ a}/g_{dry\ weight}$ (MAE)	(339)
Scenedesmus obliquus	Chlorophyll *a* and *b*	Supercritical fluid extraction with ethanol as co-solvent	0.85 $mg_{chlorophyll\ a}/\ g_{dry\ weight}$, 0.36 $mg_{chlorophyll\ b}/\ g_{dry\ weight}$	(349)
Chlorella vulgaris	Chlorophyll *a* and *b*	Ultrasound-assisted extraction	Max yield: 35.2 $mg_{chlorophylls}\ /g_{dry\ weight}$	(350)
Chlorella vulgaris	Lutein and chlorophyll *a* and *b*	Pressurized Liquid Extraction	3.7 $mg_{lutein}/g_{dry\ weight}$, 10.79 $mg_{chlorophyll\ a}/g_{dry\ weight}$ and 6.84 $mg_{chlorophyll\ b}/g_{dry\ weight}$	(351)

Chlorella sp.	Proteins	Hydrothermal pre-treatment combined with partial solvent delipidation and urea extraction	62% of total proteins (wt%)	(352)
Haematococcus pluvialis, Nannochloropsis oculata, Chlorella vulgaris, Porphyridium cruentum s and Arthrospira platensis	Proteins	High-pressure cell disruption > chemical treatment > ultrasonication > manual grinding.	Best yield achieved with high-pressure cell disruption: 41% to 90% (wt%)	(121)
Chlorella vulgaris.	Proteins	High-pressure cell disruption and alkaline treatment	52% (wt%)	(353)
Chlorella sp.	Carbohydrates	Conventional solvent extraction, fluidized bed extraction and Ultrasound assisted extraction	65–90 $mg_{glucose}/g_{dry\ weight}$ (CSE), 48 $mg_{glucose}/g_{dry\ weight}$ (FBE) and 368.5$mg_{glucose}/g_{dry\ weight}$ (UAE)	(179)
Chlorella vulgaris	Carbohydrates	Ionic liquid extraction	Glucan (starch) yield: 57.5% (45.1%) $g/g_{dry\ weight}$	(279)
Chlorococcum infusionum	Carbohydrates	Bead-beating followed by hydrolysis	89.6 % ($g/g_{dry\ weight}$)	(354)
Chlorella vulgaris	Lutein, chlorophyll *a* and *b* and proteins	Solvent extraction followed by alkaline pH extraction	5.4 $mg_{lutein}/g_{dry\ weight}$ and 15.4 $mg_{chlorophylls}/g_{\ dry\ weight}$ biomass, and 76% of total protein (wt%)	(355)
Nannochloropsis sp.	Chlorophyll *a* and *b*, carbohydrates and proteins	Pulsed electric field assisted extraction followed by basic extraction	~0.2 $mg_{chlorophylls}/g_{dry\ weight}$, ~30 $mg_{proteins}/g_{dry\ weight}$ and ~45 $mg_{carbohydrates}/g_{dry\ weight}$	(356)
Chlorella vulgaris	Proteins and carbohydrates	Combined bead milling and enzymatic hydrolysis	68% proteins, 74% carbohydrates(wt%)	(357)
Tetraselmis suecica	Proteins and carbohydrates	Bead milling	50.4% of proteins and 26.4% carbohydrates (wt%)	(358)
Nannochloropsis sp., Phaeodactylum tricornutum and Parachlorella kessleri	Proteins and carbohydrates	Pulsed electrical fields (PEF), high voltage electrical discharges (HVED), and ultrasonication (US)	PEF: 11.1-20.2 % carbohydrates, 1.8-4.1 % proteins; HVED: 23.5-43.7% carbohydrates, 3.2-5.3% proteins; US: 13.4-33.3 % carbohydrates, 6.4-11.4% proteins.(wt%)	(359)

Neochloris oleoabundans	Carbohydrates, proteins, pigments	Bead-milling and Multistep Aqueous biphasic systems	Yield fractionation: 82% proteins, 93% soluble sugars, 98% pigments (wt%)	(259)
Neochloris oleoabundans	Proteins, carbohydrates, lutein, chlorophyll *a* and *b*	ABS based in surface-active ionic liquids.	Yield extraction: 105 $mg_{proteins}/g_{dry\ weight}$, 60 $mg_{carbohydrates}/g_{dry\ weight}$, 2.90 $mg_{chlorophyll\ a\ and\ b}/g_{\ dry\ weight}$, 1.60 $mg_{lutein}/g_{\ dry\ weight.}$ Yield fractionation: 100% proteins, 80% carbohydrates, 100% chlorophyll *a* and *b* and 98% lutein (wt%)	This work

Conclusions

The extraction and separation of proteins, carbohydrates, chlorophylls *a* and *b* and lutein, was here successfully achieved. In this study, aqueous solutions of a cationic tensioactive compounds were tested and $[P_{4,4,4,14}]Cl$ (250 mM) was selected as being the most performant. In this work, an integrated process was designed, allowing to simultaneously carry the cell disruption and the biomolecules' extraction. This strategy permits to avoid the need for more complex processes where different units need to be adopted to perform these steps individually. Here, the separation of different biomolecules was tested by applying PEG 8000 + NaPA 8000-based ABS using $[P_{4,4,4,14}]Cl$ as electrolyte. A careful optimization considering the different profiles of partition obtained for proteins and pigments (electrolyte independent), and carbohydrates (electrolyte dependent), led to the definition of the integrated downstream process. This process allowed the cell disruption, the biomolecules' extraction, the combined fractionation of proteins and carbohydrates from pigments and the proper isolation of the biomolecules from the phase forming solvents, thus allowing their recycling.

Conflicts of interest: There are no conflicts of interest to declare.

Acknowledgements

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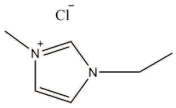
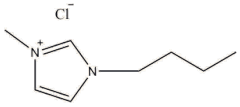
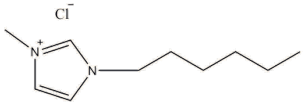
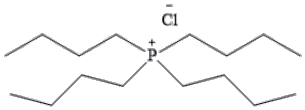
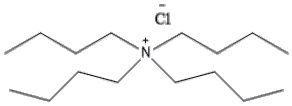
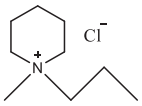
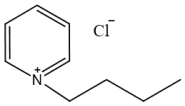
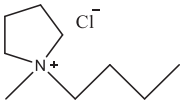
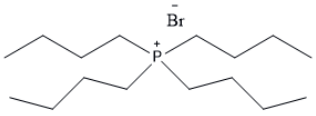
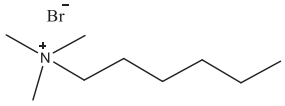
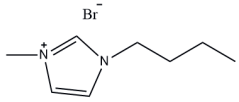
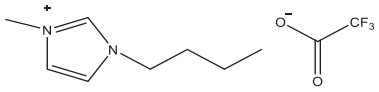
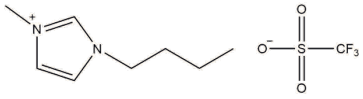
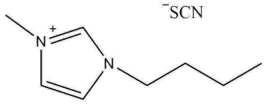
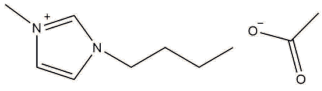
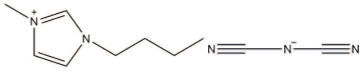
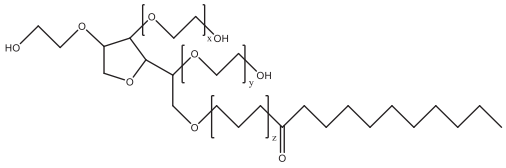
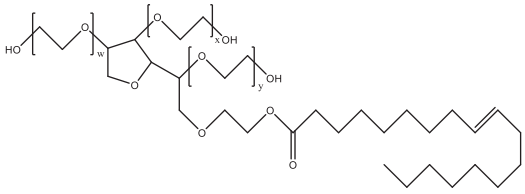
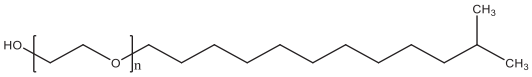
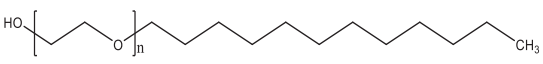
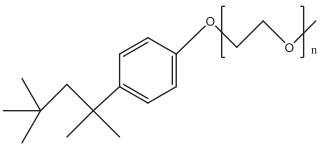
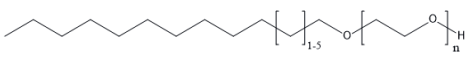
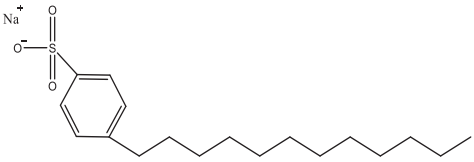
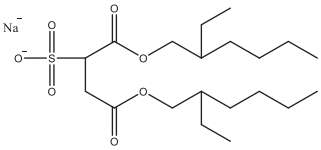
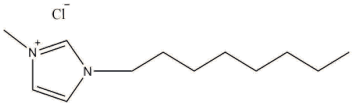
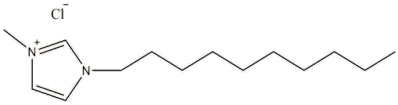
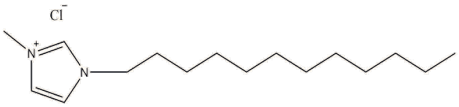
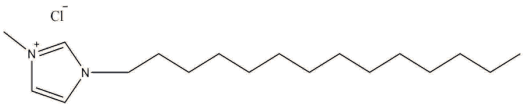
Supplementary material

Tables

Table S8.1. Chemical structures of the investigated compounds, as well as their critical micelle concentration (CMC). n.d. no data available.

Compound	Chemical structure	CMC (mM)	Type of compound
[C_2mim]Cl		---	Non-tensioactive ionic liquids
[C_4mim]Cl		---	Non-tensioactive
[C_6mim]Cl		---	Non-tensioactive
[$P_{4,4,4,4}$]Cl		---	Non-tensioactive
[$N_{4,4,4,4}$]Cl		---	Non-tensioactive
[C_3mpip]Cl		---	Non-tensioactive
[C_4py]Cl		---	Non-tensioactive
[C_4mpyr]Cl		---	Non-tensioactive
[$P_{4,4,4,4}$]Br		---	Non-tensioactive

$[N_{1,1,1,6}]Br$		---	Non-tensioactive
$[C_4mim]Br$		---	Non-tensioactive
$[C_4mim][CF_3CO_2]$		---	Non-tensioactive
$[C_4mim][CF_3SO_3]$		---	Non-tensioactive
$[C_4mim][SCN]$		---	Non-tensioactive
$[C_4mim][CH_3CO_2]$		---	Non-tensioactive
$[C_4mim][N(CN)_2]$		---	Non-tensioactive
Brij L4		n.d.	Non-ionic tensioactive
Brij 93		n.d.	Non-ionic tensioactive
Tween 20		0.06	Non-ionic tensioactive
Tween 80		0.012	Non-ionic tensioactive

Genapol X-080		0.15	Non-ionic tensioactive
Genapol C100		0.0755	Non-ionic tensioactive
Triton X-114		0.2	Non-ionic tensioactive
Merpol HCS		n.d.	Non-ionic tensioactive
SDS		8.0	Anionic tensioactive
SDBS		0.63	Anionic tensioactive
AOT		2.66	Anionic tensioactive
[C_8mim]Cl		220	Cationic tensioactive
[C_{10}mim]Cl		55	Cationic tensioactive
[C_{12}mim]Cl		15	Cationic tensioactive
[C_{14}mim]Cl		4	Cationic tensioactive

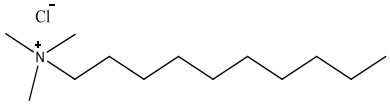
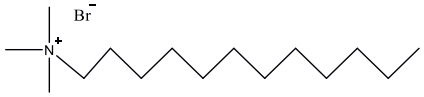
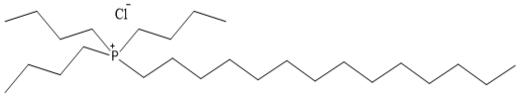
$[C_{16}mim]Cl$		1.26	Cationic tensioactive
$[N_{1,1,1,10}]Cl$		70	Cationic tensioactive
$[N_{1,1,1,12}]Br$		15.60	Cationic tensioactive
$[N_{1,1,1,14}]Br$		3.80	Cationic tensioactive
$[P_{4,4,4,14}]Cl$		4.69	Cationic tensioactive
$[C_{16}py]Cl$		0.96	Cationic tensioactive

Table S8.2. Design matrix for the surface response design for a 2^2 factorial planning.

Experiment	X_1	X_2
1	-1	-1
2	1	-1
3	-1	1
4	1	1
5	-1.41	0
6	1.41	0
7	0	-1.41
8	0	1.41
9	0	0
10	0	0
11	0	0

Table S8.3. RSM design: Experimental data using C (mM) and SLR as independent variables and respective experimental and theoretic results of biomolecule extraction efficiencies (EE%) using $[P_{4,4,4,14}]Cl$ and the respective relative deviation.

Run	C (mM)	SLR	EE_{Lutein} (%) Experimental values	EE_{Lutein} (%) Theoretic values	Residues
1	100	0.010	49.09	49.09	0.00
2	400	0.010	100.00	100.73	-0.73
3	100	0.040	14.25	11.96	2.29
4	400	0.040	43.57	42.01	1.56
5	39	0.025	6.35	7.66	-1.31
6	462	0.025	64.98	65.25	-0.27
7	250	0.004	100.00	99.16	0.84
8	250	0.046	29.16	31.58	-2.41
9	250	0.025	45.71	45.18	0.53
10	250	0.025	45.13	45.18	-0.05
11	250	0.025	44.72	45.18	-0.46

Run	C (mM)	SLR	$EE_{Carbohydrates}$ (%) Experimental values	$EE_{Carbohydrates}$ (%) Theoretic values	Residues
1	100	0.010	52.67	51.53	1.14
2	400	0.010	60.35	59.75	0.60
3	100	0.040	36.18	36.45	-0.26
4	400	0.040	36.68	37.48	-0.80
5	39	0.025	39.06	39.61	-0.55
6	462	0.025	46.36	46.14	0.21
7	250	0.004	61.68	62.84	-1.16
8	250	0.046	37.34	36.51	0.83
9	250	0.025	42.75	42.09	0.65
10	250	0.025	41.06	42.09	-1.03
11	250	0.025	42.47	42.09	0.38

Run	C (mM)	SLR	$EE_{Proteins}$ (%) Experimental values	$EE_{Proteins}$ (%) Theoretic values	Residues
1	100	0.010	51.22	55.82	-4.60
2	400	0.010	92.79	94.38	-1.59
3	100	0.040	19.06	23.39	-4.34
4	400	0.040	29.30	30.63	-1.33
5	39	0.025	24.61	22.91	1.70
6	462	0.025	52.63	55.20	-2.56
7	250	0.004	100.00	96.83	3.17
8	250	0.046	31.82	29.02	2.80
9	250	0.025	41.84	39.05	2.79
10	250	0.025	39.47	39.05	0.41
11	250	0.025	42.61	39.05	3.56

Table S8.4. Regression coefficients of EE% regarding the predicted second-order polynomial model for the *N. oleoabundans* biomolecules obtained from the RSM design using $[P_{4,4,4,14}]Cl$.

Lutein: Y = 52.1 + 0.3 × C – 3254.9 × SLR + 45137.9 × SLR² - 2.4 × C × SLR

	Regression Coefficients	**Standard error**	***t*-student (5)**	***p*-value**
Interception	52.1	3.988	13.069	0.00005
C	0.3	0.021	14.239	0.00003
C^2	0.0	0.000	-5.622	0.00247
SLR	-3254.9	206.209	-15.785	0.00002
SLR^2	45137.9	3469.101	13.011	0.00005
C × SLR	-2.4	0.411	-5.843	0.00208

Carbohydrates: Y = 59.45 + 0.04 × C - 1244.9 × SLR + 16439.36 × SLR² - 0.8 × SLR × C

	Regression Coefficients	**Standard error**	***t*-student (5)**	***p*-value**
Interception	59.45	2.029	29.301	0.000000
C	0.04	0.007	5.330	0.001777
SLR	-1244.90	118.999	-10.461	0.000045
SLR^2	16439.36	1975.337	8.322	0.000163
C × SLR	-0.80	0.244	-3.[	0.017123

Proteins: Y = 71.64 + 0.16 × C - 3400.98 × SLR + 53360.7 × SLR² - 3.48 × C × SLR

	Regression Coefficients	**Standard error**	***t*-student (5)**	***p*-value**
Interception	71.64	7.238	9.899	0.000061
C	0.16	0.024	6.899	0.000458
SLR	-3400.98	424.469	-8.012	0.000202
SLR^2	53360.70	7046.007	7.573	0.000275
C × SLR	-3.48	0.872	-3.993	0.007178

Table S8.5. ANOVA data for the extraction of *N. oleoabundans* biomolecules obtained from the RSM design using $[P_{4,4,4,14}]Cl$.

Lutein				
	Sum Squares	**Degrees of Freedom**	**Mean Square**	**F_{cal}**
Regression	**8932.185**	**5.000**	**1786.4**	**523.4**
Error	**17.065**	**5**	**3.4**	**R^2=0.99**
Total SS	**8949.250**	**10**		
Carbohydrates				
	Sum Squares	**Degrees of Freedom**	**Mean Square**	**F_{cal}**
regression	834.705	4.000	208.7	172.6
Error	7.2553	6	1.2	**R^2=0.99**
Total SS	841.961	10		
Proteins				
	Sum Squares	**Degrees of Freedom**	**Mean Square**	**F_{cal}**
regression	6784.956	4.000	1696.2	110.3
Error	92.312	6	15.4	**R^2=0.91**
Total SS	6877.268	10		

Table S8.6. RSM design, experimental data using C (mM) and SLR as independent variables and respective experimental and theoretic results of biomolecule extraction efficiencies (EE%) using $[N_{1,1,1,12}]Br$ and the respective relative deviation.

Run	C (mM)	SLR	EE_{Lutein} (%) Experimental values	EE_{Lutein} (%) Theoretic values	Residues
1	100	0.010	72.651	64.187	8.464
2	400	0.010	102.934	108.704	-5.771
3	100	0.040	29.149	20.641	8.508
4	400	0.040	78.899	84.626	-5.727
5	39	0.025	4.902	16.375	-11.473
6	462	0.025	101.588	92.869	8.719
7	250	0.004	107.044	108.390	-1.346
8	250	0.046	59.307	60.715	-1.408
9	250	0.025	77.071	77.601	-0.530
10	250	0.025	78.528	77.601	0.927
11	250	0.025	77.236	77.601	-0.365
Run	**C (mM)**	**SLR**	**$EE_{Carbohydrates}$ (%) Experimental values**	**$EE_{Carbohydrates}$ (%) Theoretic values**	**Residues**
1	100	0.010	34.227	35.117	-0.890
2	400	0.010	43.547	43.553	-0.006
3	100	0.040	31.077	32.432	-1.355
4	400	0.040	32.644	33.115	-0.471
5	39	0.025	32.075	30.764	1.311
6	462	0.025	37.251	37.193	0.058
7	250	0.004	43.124	42.769	0.355
8	250	0.046	34.531	33.517	1.015
9	250	0.025	35.652	37.106	-1.454
10	250	0.025	36.115	37.106	-0.991
11	250	0.025	39.535	37.106	2.429
Run	**C (mM)**	**SLR**	**$EE_{Proteins}$ (%) Experimental values**	**$EE_{Proteins}$ (%) Theoretic values**	**Residues**
1	100	0.010	69.580	66.800	2.779
2	400	0.010	61.203	66.843	-5.639
3	100	0.040	32.673	25.228	7.445
4	400	0.040	38.620	39.594	-0.974
5	39	0.025	26.248	33.127	-6.879
6	462	0.025	48.347	43.284	5.063
7	250	0.004	87.642	85.241	2.401
8	250	0.046	32.506	36.722	-4.217
9	250	0.025	43.754	45.830	-2.076
10	250	0.025	44.730	45.830	-1.100
11	250	0.025	49.027	45.830	3.197

Table S8.7. Regression coefficients of the predicted second-order polynomial model for the *N. oleoabundans* biomolecules EE% obtained from the RSM design using [$N_{1,1,1,12}$]Br.

Lutein				
	Regression Coefficients	**Standard error**	***t*-student (5)**	***p*-value**
Interception	51.690	19.850	2.604	0.048
C(mM)	0.380	0.100	3.737	0.013
$C(mM)^2$	0.000	0.000	-2.975	0.031
SLR	-2444.840	1026.470	-2.382	0.063
SLR^2	15540.290	17268.480	0.900	0.409
C(mM) × SLR	2.160	2.040	1.058	0.338
Carbohydrates				
	Regression Coefficients	**Standard error**	***t*-student (5)**	***p*-value**
Interception	30.469	3.704	8.226	0.000
C(mM)	0.072	0.019	3.743	0.013
$C(mM)^2$	0.000	0.000	-2.170	0.082
SLR	-119.263	191.553	-0.623	0.561
SLR^2	2317.623	3222.547	0.719	0.504
C(mM) × SLR	-0.861	0.381	-2.259	0.073
Proteins				
	Regression Coefficients	**Standard error**	***t*-student (5)**	***p*-value**
Interception	88.970	13.930	6.388	0.001
C(mM)	0.070	0.070	0.964	0.379
$C(mM)^2$	0.000	0.000	-1.407	0.218
SLR	-3238.530	720.160	-4.497	0.006
SLR^2	33872.680	12115.350	2.796	0.038
C(mM) × SLR	1.590	1.430	1.110	0.317

Table S8.8. ANOVA data for the extraction of *N. oleoabundans* biomolecules obtained from the RSM design using [$N_{1,1,1,12}$]Br.

Lutein					
	Sum Squares	**Degrees of Freedom**	**Mean Square**	**F_{cal}**	***p*-value**
Lack of fit	421.560	3	140.520	220.708	0.004
Pure Error	1.273	2	0.637	**R^2=0.956**	
Total	9702.999	10			
Carbohydrates					
	Sum Squares	**Degrees of Freedom**	**Mean Square**	**F_{cal}**	***p*-value**
Lack of fit	5.7276	3	1.90919	0.424	0.757
Pure Error	8.9976	2	4.49878	**R^2=0.917**	
Total	176.8056	10			
Proteins					
	Sum Squares	**Degrees of Freedom**	**Mean Square**	**F_{cal}**	***p*-value**
Lack of fit	192.391	3	64.130	8.149	0.111
Pure Error	15.738	2	7.869	**R^2=0.936**	
Total	3273.870	10			

Table S8.9. Parameters obtained through the Merchuk equation with the respective standard deviations (std) along with the weight fraction data (wt%) for the quaternary systems formed by NaPA 8000 + PEG 8000 + H_2O + electrolyte.

0.1 wt% [$N_{1,1,1,12}$]Br A ± std= 16.0067 ± 3.1242 B ± std=-0.2979 ± 0.089683 C ± std=7.3307x^{-5} ± 6.1088x^{-5}		**0.1 wt% [$P_{4,4,4,14}$]Cl** A ± std= 15.2771 ± 4.0172 B ± std=-0.2924 ± 0.12052 C ± std=4.1546x^{-5} ± 2.0619x^{-5}	
wt% NaPA	**wt% PEG**	**wt% NaPA**	**wt% PEG**
4.6104	14.0380	3.9307	16.4813
5.2725	10.7168	5.5195	10.7883
6.9630	7.3361	4.7140	14.3858
4.5166	13.3407	6.8847	6.8062
7.3161	6.9921	9.1697	3.1086
9.7264	2.7176	3.3578	17.6388
3.3151	17.0918		

Table S8.10. pH±0.01 values of top and bottom phases of the polymer-based ABS using electrolytes.

	pH	
Electrolyte	**top-phase**	**bottom-phase**
SDS	7.59(322)	7.08(322)
[$N_{1,1,1,12}$]Br	7.87	7.06
[C_{14}mim]Cl	7.64(322)	7.07(322)
[$P_{4,4,4,14}$]Cl	7.66	7.07

Table S8.111. Partition coefficient values of microalgae biomolecules (K_{MB}) extracted using polymeric-based ABS with electrolyte.

Electrolyte	**K_{Lutein}**	**$K_{Chl\ a}$**	**$K_{Chl\ b}$**	**K_{Carb}**	**$K_{Proteins}$**
SDS	2.660 ± 0.001	3.298 ± 0.0001	2.529 ± 0.001	0.700 ± 0.100	23.000 ± 5.000
[$N_{1,1,1,12}$]Br	0.016 ±0.005	0.054 ± 0.002	0.073 ± 0.002	0.400 ±0.020	0.050 ± 0.090
[C_{14}mim]Cl	0.008 ± 0.008	0.047 ± 0.002	0.077 ± 0.002	0.350 ± 0.070	0.040 ± 0.010
[$P_{4,4,4,14}$]Cl	0.004 ± 0.0005	0.030 ± 0.003	0.097 ± 0.003	1.400 ± 0.800	76.000 ± 6.000

Figures

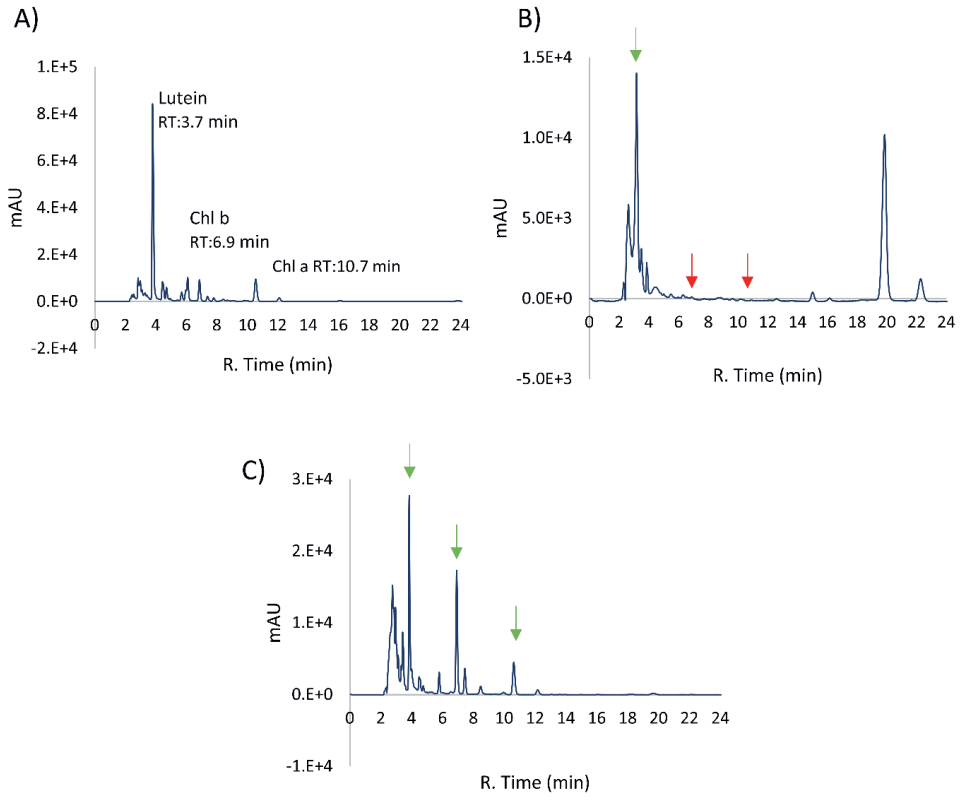


Figure S8.1. RP-HPLC chromatograms at 450 nm of the pigments (lutein, chlorophyll a and chlorophyll b) extracted from *N. oleoabundans* using methanol (A) and aqueous solutions of SDS (B) and [$P_{4,4,4,14}$]Cl (C). Green arrows refer to biomolecules detected and red arrows to biomolecules not detected by RP-HPLC chromatography.

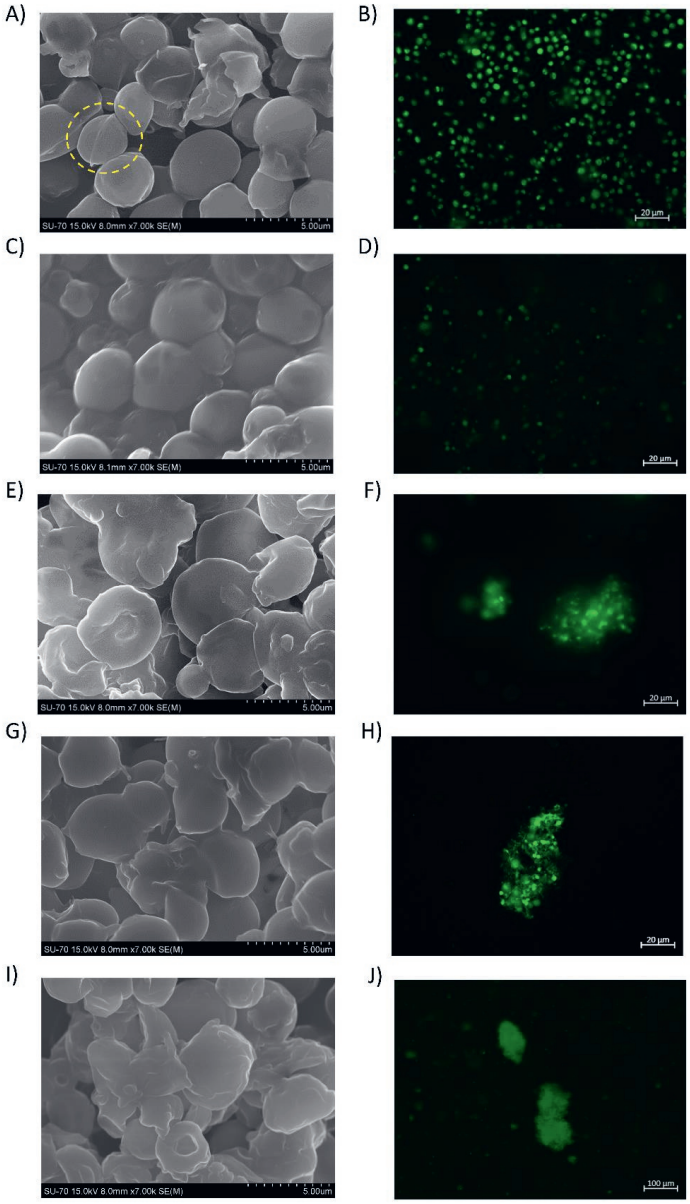


Figure S8.2. SEM images and confocal fluorescence (green auto fluorescence) images of microalgae biomass after extraction of biomolecules with water (A and B) and using aqueous solutions of SDS (C and D), $[N_{1,1,1,12}]Br$ (E and F), $[P_{4,4,4,14}]Cl$ (G and H) and $[C_{14}mim]Cl$ (I and J). Yellow circle refers to a complete, not disturbed *N. oleoabundans* cell.

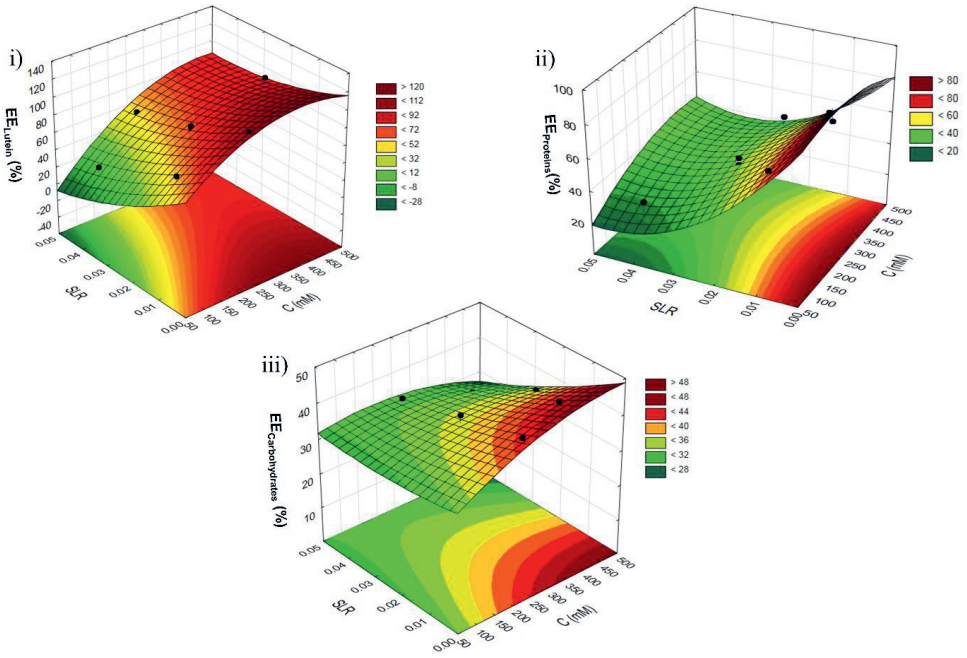


Figure S8.3. Response surfaces corresponding to the extraction efficiency - EE_{MB} (%) of lutein (i), proteins (ii) and carbohydrates (iii) with the combined effects of solid-liquid ratio (SLR) and concentration, C (mM), using the tensioactive compound $[N_{1,1,1,12}]Br$.

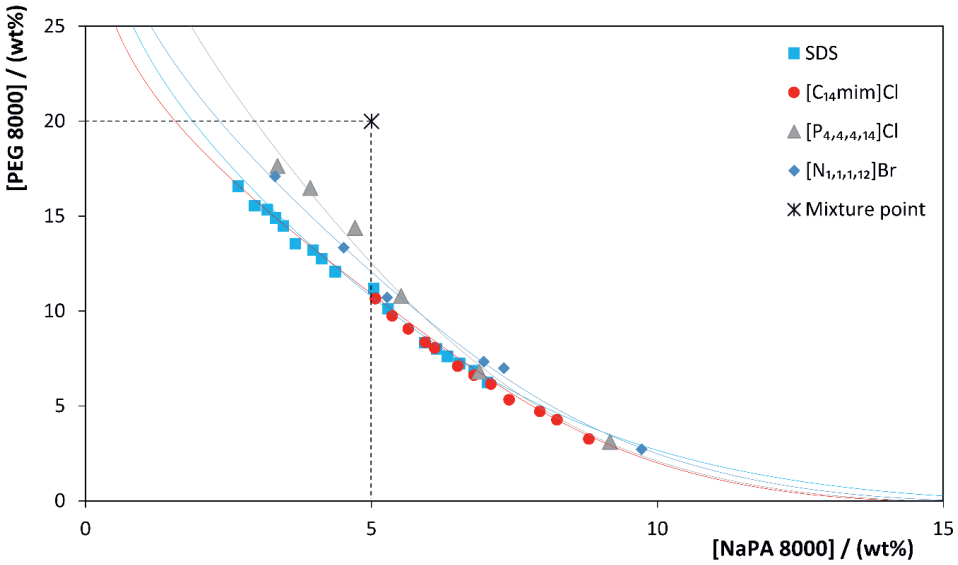


Figure S8.4. Binodal curves of the polymeric-based ABS composed of NaPA 8000 + PEG 8000 + 0.1 wt% of SDS, $[C_{14}mim]Cl$, $[P_{4,4,4,14}]Cl$, and $[N_{1,1,1,12}]Br$, as electrolytes. The asterisk represents the mixture point used in this work to test the partition of the different classes of biomolecules composing the microalgae cells. Experimental data for SDS and $[C_{14}mim]Cl$ were obtained from Santos et al.(322)

Chapter 9

General discussion: Integration and simplification of multiproduct microalgae biorefineries using aqueous solutions

Introduction

The increasing demand of bio-based products represents challenges for upstream and downstream processes (360). To develop a truly bio-based economy, biorefineries should aim to convert all biomass (e.g. microalgae) fractions into energy as well as products (food, feed, biomaterials and biochemicals), while minimizing unit operations and fossil energy inputs (35). Clean, green, sustainable and simplified processes able to extract and recover biomolecules effectively, selectively and in a mild manner, are needed for the development of an **integrated biorefinery.**

In the transition from oil refinery to biorefinery, solvent-based separation has become very important because it may be the most appropriate option for the fractionation of complex biomass streams (361). Aqueous solutions are of interest for biochemical separations as most organic solvents denature biomolecules (e.g. proteins). ATPS, which uses water as main component (continuous medium), is considered a safe, non-toxic and environmentally friendly method. These systems combine two incompatible hydrophilic solutes that form two phases above certain concentrations and conditions. Solutes such as polymers, salts, alcohols, surfactants, ionic liquids (ILs), sugars, amino acids and deep eutectic solvents can form ATPS (246, 362, 363). Furthermore, changes in temperature and pH can also trigger the phase separation of some systems.

Other separation methods based on water that have emerged include **Aqueous micellar two-phase systems (AMTPS)/ Cloud point extraction**, hydrotrope forming solutions, **Aqueous multiphase systems (MUPs)** and CO_2-switchable solvents. These techniques seem promising to apply in microalgae biorefinery where the isolation of different compounds (two or more) present in complex mixtures, and their simultaneous separation in a single step is needed (37, 129). A simplified and integrated microalgae biorefinery using few unit operations and separating most of the biomass components is desired. Hereby we discuss the opportunities and challenges for the development of integrated microalgal multiproduct biorefineries using aqueous solutions.

Aqueous solutions in microalgae biorefinery

To simplify and consequently reduce processing costs, aqueous systems have been studied to replace commonly used technologies in the different steps of the downstream processing of microalgae (from the harvest to the fractionation and polishing). However, studies focusing in the valorisation of different components are scarce.

Microalgae harvesting mediated by aqueous solutions

Harvesting of microalgae is traditionally done by centrifugation or filtration. Apart from these technologies, flocculation has been widely explored (364). This technology is employed as a simple method that may reduce the energy and costs involved in microalgae harvesting. However, chemical flocculation can result in contamination of the biomass and the medium, affecting the whole biorefinery process. Aqueous solution of thermo-responsive polymers have been investigated to harvest microalgae (Figure 9.1) (365) and since the medium can be recovered and reused, this is a promising method. The potential of this novel harvesting technology increases if the polymer does not affect the next steps of biorefinery (cell disruption

and fractionation) and can be further used or recycled. Flocculation has also been used to integrate microalgae harvesting and cell disruption. Cationic surfactants have been evaluated, however, medium contamination is still a disadvantage (309, 366, 367).

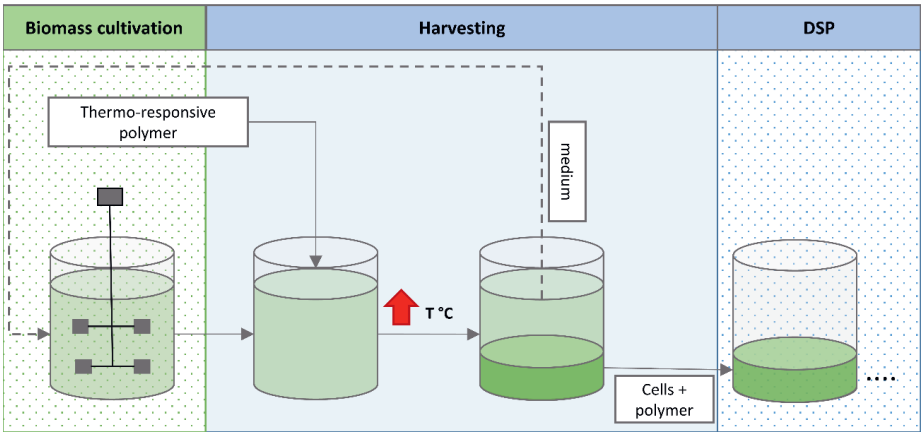


Figure 9.1. Schematic diagram of the harvesting process of microalgae using thermo-responsive polymers and further medium recycling (365).

Smirnova and collaborators investigated the use of cloud point extraction for the continuous recovery of hydrophobic products (free fatty acids) *in situ* from cell culture (331, 368, 369). Due to the low toxicity it may be applied for biorefineries with food applications. The successful design of this kind of process, which depends on the microalgae specie and product (mainly investigated to extract high-value compounds), can potentially solve low productivity in the commercialization of these microalgae products (370). The possibility of combining cell harvesting with the direct purification of target molecules is clearly an opportunity for the development of simplified downstream processes, although, it cannot be applied in all microalgae species and products.

ATPS as an integrated method could handle high loads of biomolecules overcoming challenges when harvesting high added-value products from dilute cultures (371). The influence of the solute (e.g. surfactant) on further DSP and the possibility to recycle the cultivation medium was not addressed in most of the studies, which is a requirement before applying this technology at large-scale (372). The design of reusable materials to harvest cells, the possibility to recycle the microalgae cultivation medium after harvesting and the integration of harvesting with further downstream processing steps could increase the environmental and economical sustainability of the whole process (309, 373).

Cell disruption and extraction of microalgae biomolecules mediated by aqueous solutions

Most microalgae species have strong a cell wall. This clear barrier hampers the accessibility to intracellular compounds. Cell disruption, therefore, is crucial in the development of a biorefinery process. To conserve the functionality and native state of the intracellular products, mild cell disruption techniques are required. The development of solvents has widened the application of aqueous systems to early stages in biorefinery (e.g. cell disruption). Surfactants,

ionic liquids and deep eutectic solvents are novel phase forming components that are promising due to their ability to solvate many materials, including rigid cell walls.

The ability of surfactants as cell disrupters and extraction agents of microalgae antioxidants (α-tocopherol, β-carotene and gallic acid) integrating two steps (cell disruption and fractionation) with a single ATPS step has been demonstrated (222). Thermo-responsive polymers and Ionic liquids have also been used to disrupt or permeabilize microalgae cells and enhance the recovery of different biomolecules (130, 374). Other switchable solvent systems, besides thermo-responsive solvents (commonly based on polymers and surfactants) were also recently investigated for the extraction of lipids from wet microalgae (272). These solvents switch their polarity upon exposure to CO_2 or temperature change. The number of process steps induced by the solvent recovery represents one of the major weakness of this process, since large part of the solvent penetrate the algae cells and becomes difficult to recover (375).

A circular extraction concept, in which both states of the switchable solvent were used to extract polar and non-polar compounds seem promising (376). They increased in this way the extraction and fractionation of biomolecules (lipids, carbohydrates and proteins), and therefore the usefulness of the solvent. CO_2 switchable solvents are commonly based on amines, which are not always environmentally benign and recovery of the solvent is therefore essential.

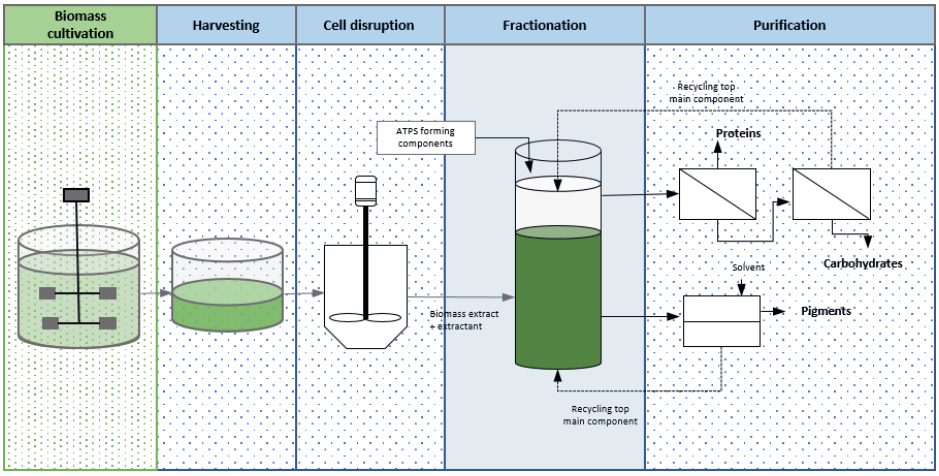


Figure 9.2. Schematic diagram of the process integrating cell disruption and purification steps using ATPS-based in surface-active ionic liquids for microalgae biorefinery.(377)

In order to simplify the microalgae biorefinery, we propose in this thesis (**Chapter 8**) to combine the use of tensioactive compounds and aqueous two phase systems (ATPS) (378). The tensioactive compound solution acts as cell disrupter and is also used in the formation of the ATPS. The process diagram is shown in Figure 9.2. This process avoids the need for more

complex processes where different unit operations need to be adopted to perform these steps individually.

These studies, demonstrate the potential of aqueous systems in a multiproduct microalgae biorefinery. However, more research and development is needed focusing on the recovery of the extractants and in the large-scale production processes to understand if these technologies are economically feasible.

Fractionation and purification of microalgae biomolecules mediated by aqueous solutions

Aqueous solutions have been typically used in the last steps of DSP, namely fractionation and/or polishing of biomolecules. ATPS, in particular, has been used in the purification of hydrophilic compounds such as proteins. Nowadays, its application has been expanded to hydrophobic compounds with the use of a plethora of novel phase formers (e.g. ILs, amino acids, sugars, surfactants, thermo-responsive polymers and deep eutectic solvents (DES)) (23, 135, 379-382). The evaluation of ILs aqueous solutions and the less explored DES, to fractionate compounds from microalgae, is growing exponentially (86, 87, 256, 383-385) and the scale-up of these technologies for the production of high-value compounds such as the protein B-phycoerythrin using these solutions seems economically feasible (141). Nevertheless, microalgae have an enormous potential to produce a wide variety of products including commodities and high-value products (proteins, lipids, pigments, carbohydrates).

Table 9.1. Summary of microalgae fractionation and purification studies using aqueous solutions.

Microalgae sp.	**fractionation products**	**Cell disruption/Extraction method**	**Fractionation method**	**Reference**
Chlorella protothecoides	Lutein	Organic solvent extraction	ATPS	(145)
Spirulina platensis	C-phycocyanin	Ultrasound	Ionic liquid based-ATPS	(386)
Isochrysis galbana	fucoxanthin	Organic solvent extraction (ethanol)	ATPS	(142)
Porphyridium cruentum	B-phycoerythrin	Bead milling and isoelectric precipitation	ATPS	(141)
Isochrysis galbana	Proteins, Arabinans, and Glucans	Degreasing and organic solvent extraction	ATPS	(387)
Tetraselmis suecica	α-tocopherol, β-carotene and gallic acid	Surfactant based extraction	ATPS	(275)

Chlorella sorokiniana	Proteins	Electrolysis cell disruption	Liquid biphasic flotation	(388)
Acutodesmus obliquus.	Cinnamic acid	Cloud point extraction	cloud point extraction	(369)
Scenedesmus obliquus	fatty acids	Cloud point extraction	cloud point extraction	(331)
Dunaliella salina	*β*-carotene	Surfactant based extraction	ATPS	(389)
Chlorella vulgaris	Proteins	Osmotic shock through liquid biphasic flotation (LBF) system	osmotic shock through liquid biphasic flotation (LBF) system	(390)
Neochloris oleoabundans	Lipids, carbohydrates and proteins	Bead milling and IL-based extraction	bead milling and IL-based extraction	(281)
Neochloris oleoabundans	proteins and carbohydrates	Bead milling	ATPS	(210)
Neochloris oleoabundans	chlorophylls, lutein and proteins	Bead milling	ATPS	(209)
Neochloris oleoabundans	carbohydrates and starch	Bead milling	ATPS	(391)
Neochloris oleoabundans	lutein, chlorophylls, proteins, carbohydrates	Bead milling	ATPS	(259)

Studies using aqueous systems are often focused on the fractionation of a highly valuable single-compound (often excluding commodity products) from biomass as shown in table 9.1. However, the formation of two or more phases allows the simultaneous fractionation of components of different nature while using the same system (225, 387). We demonstrated in this thesis that two or more molecules can be fractionated from microalgae extracts using IL-based ATPS in a simple and efficient way (209) (210). It is a promising separation method even for non-soluble components such as lipids and starch (391). The main components from microalgae (lipids, pigments, carbohydrates and proteins) can be fully valorised with a multistep fractionation as proposed in **chapter 7** (Figure 9.3) (259), simplifying the microalgae downstream processing.

Another strategy proposed in this thesis focused first on valorising microalgae lipids (**chapter 6**). Lipids, being the most abundant molecules in *Neochloris oleoabundans* and other microalgae strains, are normally extracted for biofuels by chemical extraction using organic solvents, which makes difficult to valorise labile molecules in the biomass such as proteins. Aiming to develop a microalgae-based biorefinery to obtain as many bioproducts as possible, ATPSs and several aqueous solutions (ILs and polymers) were studied in their interaction with lipids. Polypropylene glycol 400 (PPG 400), a polymer used in the formation of ATPS, displaced a high amount of lipids (>70%) in an immiscible layer from disrupted microalgae. After recovering the lipids, the rest of biomolecules can be fractionated by ATPS.

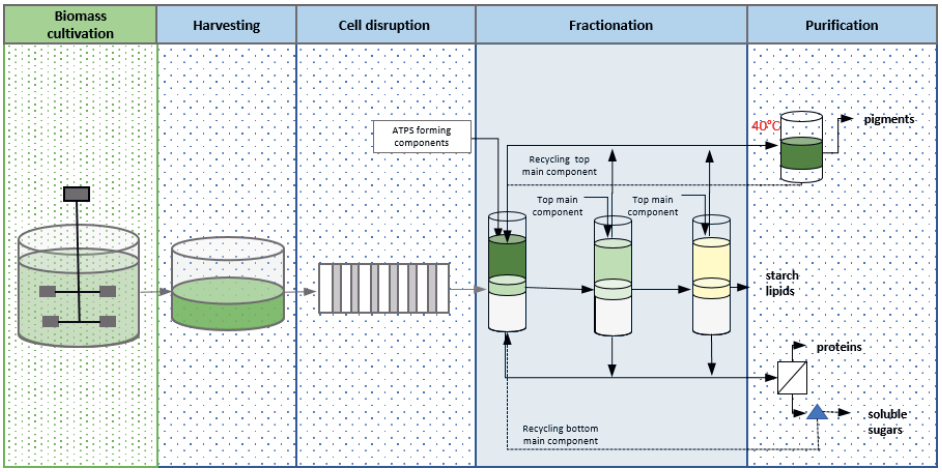


Figure 9.3. Schematic diagram of a multistep fractionation of several biomolecules from microalgae using IL-based ATPS.(259)

Integration of microalgae biorefinery using aqueous solutions: Opportunities

Microalgae biorefinery consists of harvesting, cell disruption, extraction and fractionation. These steps are often individually studied without taking into account the whole biorefinery chain. Technologies using aqueous solutions (e.g. ATPS) or water as main solvent could integrate the whole process to valorise most of the microalgae biomolecules. These solutions are recognized as efficient and mild to concentrate cells and biomolecules from one aqueous phase to another. They have proven their applicability in the whole biorefinery chain, from harvesting of cells to purification of the products. Glyk et al. (13) reviewed traditional ATPS composed of polymer-salt for the extraction and purification of proteins and enzymes. The scale up, recycling of phase forming components and the economic aspects of the technology were also addressed. They highlighted advantages of this liquid-liquid platform such as technical simplicity, process integration, easy scalability and continuous operation capability. It can solve current challenges of conventional DSP methods.

Mild conditions and the possibility of integrating several unit operations in one, are the most important advantages that technologies mediated by aqueous solutions have to reduce production costs. Therefore, these liquid-liquid platforms can provide advantages for the extraction of products from microalgal biomass (10, 129). Microalgae are one of the most

promising bio-based sources due to their huge diversity and thus diverse composition of biomolecules and bioactive compounds. However, large-scale microalgae production for bulk commodities is not yet economically feasible due to cultivation and biorefinery costs. The high biorefinery costs can be reduced by the valorisation of all microalgal fractions (bulk and high-value products). However, economic projections by Ruiz et. (6) show that a microalgae multi-product biorefinery using individual unit operations for cell harvesting and disruption, in addition to different unit operations to extract each cell compound (e.g. ATPS for proteins and organic solvents for lipids) is long and costly. Bulk commodities from microalgae have a low price and high value components cannot counterweight the cost in this way. In Figure S9.1 of the supporting material a flowsheet for the complete valorisation of microalgae fractions using conventional technologies is shown. The number of unit operations used demonstrates the complexity of the process.

It was reported that DSP (cell disruption and fractionation) accounts for 50-60% of the total production costs of microalgae biomass (as shown in Figure 9.4) (129). Large volumes to be processed and the small size of microalgal cells result in high capital expenditure and energy consumption to harvest microalgal cells. Other bottlenecks are high-energy consumption by mechanical cell disruption and high costs for the fractionation of functional proteins (32% of total costs). Furthermore, the use of volatile solvents that need distillation to be recovered increase the process costs.

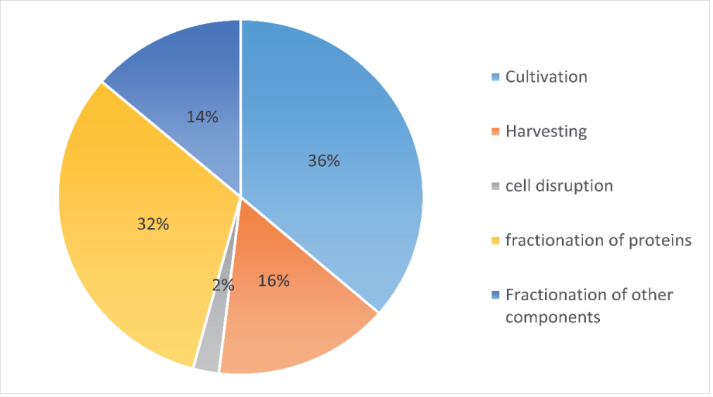


Figure 9.4. Cultivation and DSP % of production costs. Data from scenario calculated by Ruiz et al. (6) (100 Ha, location: Spain, closed flat panel reactor).

ATPS as a liquid-liquid extraction method does not require complex equipment and the phases form spontaneously due to their phase density differences and differences in chemical interaction with water. It has been estimated that the phase forming components account for more than 58% of the process cost when using ATPS (85). It is possible to reduce those costs by recycling the phase forming components, performing multistage extraction and using less expensive phase forming components (238, 392). ATPS is a proven tool to fractionate proteins in a mild manner but it can also fractionate other biomolecules (e.g. pigments and carbohydrates). If ATPS is used to fractionate hydrophobic and hydrophilic biomolecules

simultaneously, individual unit operations to fractionate pigments or sugars would not be needed and the production costs could be reduced.

Technologies using aqueous solutions (such as ATPS or cloud point extraction) have been applied mainly in the fractionation and purification of microalgal components, which are the last stages of DSP. This does not contribute to the development of a simplified biorefinery, since many unit operations are needed and often technologies used for harvesting and cell disruption (e.g. bead milling) affect the performance of fractionation technologies. Mechanical disruption, for example, although it is an efficient method, disrupts the cells in small particles causing emulsions (167). These emulsions are very stable and difficult to break, as consequence fractionation is affected and needs several steps to break the emulsion and separate the biomolecules. The use of aqueous solutions (of ILs for example) to break or permeabilize the cells in a milder manner would avoid the formation of these emulsions and reduce substantially the unit operations to follow (225). Therefore, the investigation of technologies using aqueous solutions and their applicability in the early stages of DSP (harvesting and cell disruption) is important. The design of double task aqueous phase systems (e.g. cell disruption and fractionation) is also a key factor in the development of a simplified microalgae biorefinery.

The efficiency of ATPS to fractionate biomolecules could reduce the costs of microalgae production (393). The yields obtained for different products using ATPS are shown in Table 9.2. Two or more DSP stages are integrated in a single ATPS and high yields were achieved in most of the cases (~60-100%). The yields were obtained at room temperature or mild temperatures (30-40 °C) and non-toxic, biodegradable and biocompatible aqueous solutions were used in most of the studies summarized in table 9.2.
ATPS and other technologies mediated by aqueous solutions have the potential to change the complexity and high price of the conventional microalgae biorefinery for multiple compounds, reducing the number of unit operations needed. In order to integrate these technologies in microalgae biorefineries, the whole process should be studied from harvesting to the purification, to understand how each step influences the other.

Table 9.2 Overview of studies integrating biorefinery stages mediated by aqueous solutions

Biorefinery stages	Technology	Yield %	Results	Ref
harvesting/recycling	Cloud point extraction (thermo-responsive polymers)	> 98%	Algal cell separation using *Chlorella protothecoide* T:~32 °C	(365)
Cultivation/harvesting/fractionation	Cloud point extraction (non-ionic surfactants)	-	Free fatty acids from *Acutodesmus obliquus*, good biocompatibility T:30-40 °C	(331)
cell disruption/fractionation	ATPS (non-ionic surfactant-salt)	60-99%	α-tocopherol, β-carotene, gallic acid from *Tetraselmis suecica*, high antioxidant activities	(222).
cell disruption/fractionation	Cloud point extraction (thermo-responsive polymer)	30%	Cell disruption of *Chlorella protothecoides*	(374)
cell disruption/fractionation	Switchable protic ionic liquids	88%	Lipids from *Scenedesmus obliquus T:* 80 C	(313)
Fractionation/partial purification	ATPS (polymer-salt)	98%	Phycobiliproteins and enzymes from red algae (*Caloglossa continua*)	(221)
Fractionation/partial purification	ATPS (polymer-salt)	84%	B-phycoerythrin from *Porphyridium cruentum*	(141)
Fractionation/partial purification	ATPS (polymer-IL)	> 61% and 97%	Proteins and pigments (Lutein) from *Neochloris oleoabundans*	(209)

Future perspectives

Aqueous solutions can be integrated in the biorefinery of microalgal extracellular compounds (Figure 9.5) and intracellular compounds (Figure 9.6). The first scheme (Figure 9.5) describes an ATPS for harvesting and product(s) fractionation in one-step. The possibility to combine cultivation, harvesting and product fractionation in one-step (Non-destructive extraction/milking) using one system is an attractive approach that can reduce costs of production. Recent analysis show that this approach has the potential of reducing the cost price of hydrocarbons from *Botryococcus braunii* from US$3.20L^{-1} and US$1.45 L^{-1} (394). Processes combining cultivation, harvesting and product fractionation using aqueous phase systems have been developed for the recovery of proteins *in situ* and for the simultaneous reaction (biocatalysis) and separation step of enzymes (13, 395).

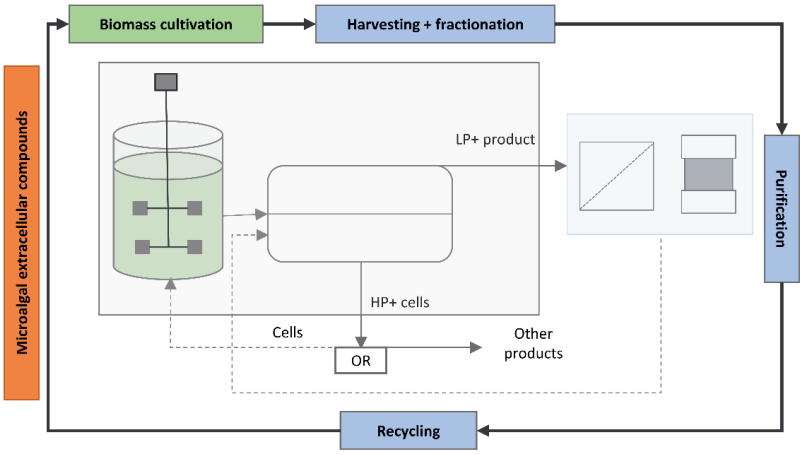


Figure 9.5. Schematic diagram of a proposed downstream process for extracellular compounds from microalgae using aqueous solutions. LP: light phase and HP: heavy phase

In the second scheme (Figure 9.6), harvesting, cell disruption and fractionation are integrated by using aqueous solutions of polymers and aqueous multiphase systems. The use of the same solutions (e.g. responsive polymers) along the different stages, could reduce the need of recycling steps in every DSP stage. Ultrafiltration/diafiltration and/or chromatography could be used for the polishing of the biomolecules and to recycle the phase forming components. The concentration and partial purification of biomolecules by ATPS enhance the yields and reduce the stages of ultrafiltration/diafiltration or chromatography necessary to obtain certain purity.(238) Furthermore, in this way we avoid the formation of an emulsion facilitating the fractionation of the biomolecules after cell disruption. This will allow the mild fractionation of the products. The use of aqueous phase systems with more than two phases could separate selectively different products in each phase. This will require strategic design of the systems and solutions to use, but it could decrease substantially the number of unit operations in the whole DSP.

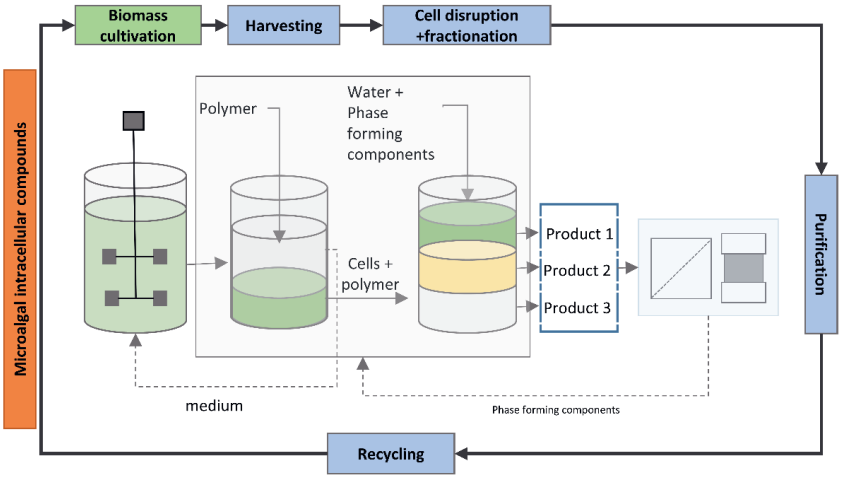


Figure 9.6. Schematic diagram of a proposed downstream process for intracellular compounds from microalgae using aqueous solutions.

Most of the studies using aqueous solutions for microalgae biorefinery focus on the extraction of lipids using ILs. ILs are capable of disrupting wet microalgae biomass faster than organic solvents (384). Some of the extractions were accomplished at high temperatures, which undervalue thermolabile compounds such as proteins, chlorophylls and carotenoids. Mild extraction conditions, therefore, are a prerequisite to achieve multiproduct biorefineries. Most of the ILs used to extract microalgal lipids are hydrophilic. Hydrophilic ILs are able to form ATPS that could facilitate the simultaneous extraction and fractionation of different microalgae compounds. Similarly, surface-active compounds (e.g. surfactants) are able to harvest and disrupt cells; moreover, they enhance the solubility of different compounds in water and are able to form aqueous phase systems. Aqueous solutions of hydrophilic ILs and polymers can permeabilize the cells at room temperature and displace the lipids in an immiscible layer. Since no volatile organic solvents are needed to recover the lipids, the cost of fractionation and recycling could be reduced (249). This approach will integrate disruption and fractionation in a single step. The rest of the products present in the biomass can be fractionated by forming and IL-based ATPS.

“Smart” solvents (water-soluble) are being designed, which allows more freedom in the development of separation technologies mediated by aqueous solutions (19). Especially the recycling of the solvent has been a concern in the last years and important achievements such as responsive polymers and switchable solvents have been realized as a solution to this challenge. Solvents based on natural compounds, which are biodegradable, biocompatible and cheap contribute also significantly to achieving “green” biorefineries. The development and techno-economic evaluation of these new biorefineries based on aqueous phase systems will be necessary to determine if these new routes are economically and environmentally sustainable in the production of microalgal products. However, taking into account previous economic evaluations, reducing the unit operations will decrease considerable the costs of production.

Conclusions

Separation technologies using aqueous solutions can simplify the complex microalgae biorefinery for the separation of multiple compounds if traditional DSP unit operations are applied. Furthermore, they are a sustainable, "green", mild and versatile technology when used in the correct manner. To develop these separation technologies for the valorisation of product from biomass, different aspects should be taken into account:

1. The development of these separation technologies must consider the whole DSP process and should not be developed as individual unit operations.
2. The development of separation processes is strongly coupled with the development in material science. The design of new materials can enhance the possibilities and quality of separation technologies.
3. The development of separation processes should consider costs but also the environmental sustainability of the process. Solvents synthesized with natural products, which are biocompatible and biodegradable should be preferred.
4. The development of separation processes must be done in a continuous/cyclic manner, enabling the development of solvent recycling and reuse.

Although efforts have been made to integrate the diverse technologies using aqueous solutions in microalgae biorefinery, studies still focus on separating only one product. It is clear that the whole valorisation of the biomass needs more research. Furthermore, the development of new and responsive solvents, synthetized from natural sources is important not only for microalgae biorefinery but also for other biorefineries. The recycling of the solvents must always accompany the development of downstream processes and it should not be seen as an independent process. This because a recycling process depend on the product(s) desired, the solvent and the started material.

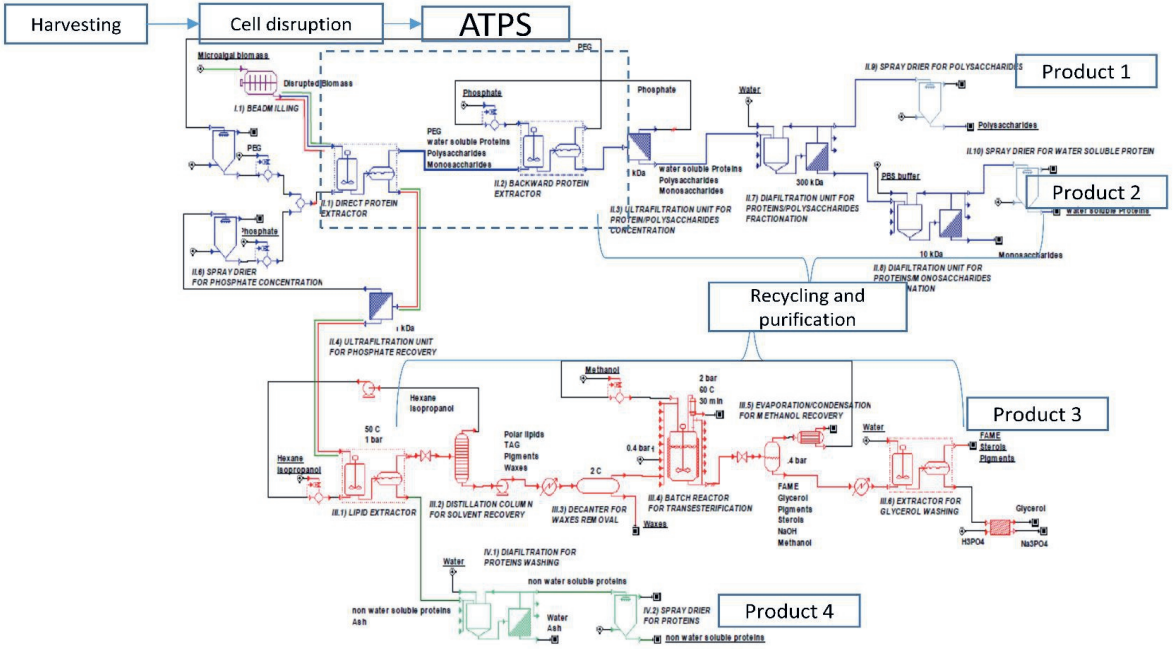


Figure S9.1. Flowsheet of complete biorefinery using conventional technologies and ATPS as fractionation technology. The figure was adapted from Ruiz et al. (6)

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Summary

Microalgae have plenty of potential as a sustainable resource due to their large diversity and high amount of valuable components (proteins, pigments, carbohydrates and lipids). However, their exploitation is currently not very efficient. Only a single product is commonly valorized in microalgae biorefinery, while the remaining fractions are wasted or devalued. The conventional microalgae biorefinery process consists of harvesting, cell disruption/extraction, fractionation, and purification. To be able to valorise all the fractions from microalgae, mild, efficient and selective technologies are needed, assuring that labile molecules, such as proteins do not lose their native conformation and functionality (**chapter 1**).

Aqueous two-phase system (ATPS) was adopted as a new approach in microalgae biorefinery for the extraction and fractionation of multiple microalgae products. These liquid-liquid extraction platforms are rich in water and are therefore favourable to separate, purify and concentrate multiple biomolecules. Furthermore, plenty of solutes can be used to form ATPS: polymers, salts, ILs, surfactants, carbohydrates and amino acids. This makes ATPS a versatile technology that can be useful for the separation of a plethora of biomolecules. In this thesis, ILs were specially explored, due to their solubility for a wide range of solutes and advantages compared to volatile solvents commonly used in biorefinery **(chapter 1).**

In **chapter 2** of this thesis the interactions between ionic liquids and Rubisco (an essential enzyme in microalgae) was a key point for the selection of the phase forming components after a broad screening. Three biocompatible ATPS were selected: a conventional polymer-salt ATPS and two IL-based ATPS (IL-salt and polymer-IL). Mixture points and parameters influencing phase formation in these ATPS were investigated in this chapter. The partitioning of Rubisco was studied depending on different parameters (nature and concentration of phase forming components and the pH of the system). Although the IL-salt ATPS was the most efficient system, the polymer-IL was able to retain the native conformation of Rubisco, also at higher concentrations.

In **chapter 3** we studied the equilibrium of an IL-based ATPS and the partitioning of proteins from two interesting microalgae strains containing high amount of proteins (*Neochloris oleoabundans* and *Tetraselmis suecica).* Their partitioning behaviour was compared with purified proteins (bovine serum Albumin (BSA) and Rubisco). It was proposed that the net charge of the protein significantly influence the partitioning of the proteins. The IL-based ATPS was able to mildly and efficiently separate proteins from sugars, demonstrating a simultaneous fractionation. The partitioning of proteins from crude microalgal extracts in IL-based ATPS was successfully represented using a thermodynamic model (Non Random Two Liquids model).

In **chapter 4,** After evaluating different ATPS, an IL-based ATPS (polymer-IL) proved to be an efficient platform to selectively and mildly fractionate microalgal proteins from pigments. These systems showed to be a versatile tool to fractionate biomolecules from *N. oleoabundans* cultivated in saline as well as in fresh water. The ATPS type and phase forming components strongly influenced the partitioning preference of the biomolecules, especially of proteins, due to electrostatic interactions between the protein and the IL. Phase forming components also

influence the integrity of proteins and pigments, which was considered in the proposed platform. The ability of the selected IL based ATPS to mildly fractionate two compounds simultaneously from a complex microalgae mixture, was an important step towards the development of a multiproduct microalgae biorefinery.

The partitioning of microalgal carbohydrates in ATPS was studied in **chapter 5.** *Neochloris oleoabundans* cultivated under stressed conditions accumulated a high amount of carbohydrates and lipids. This microalgae was used to investigate the partitioning of glucose and starch in different ATPS (included in previous chapters). ATPS were able to efficiently separate microalgal starch (interface) from free glucose (aqueous bottom phase) in a selective manner and the final carbohydrate fractions obtained were free of pigments. Starch and carbohydrate partitioning was mainly influenced by hydrogen bonding interactions.

Microalgal lipids (using *Neochloris oleoabundans* cultivated under stressed conditions) showed a low affinity for both aqueous phases of the ATPS studied (**chapter 6)**. Aiming to understand more in detail the behavior of lipids in ATPS, model lipids (commercial canola oil (representing TAGs) and partially purified yeast polar lipids (representing PL)) were used. Results demonstrated 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. To complete this study, the solubility of lipids in various solutions was studied to understand the mechanism that drive lipids to displace, which seems a good approach for the fractionation of lipids. We proposed the displacement of lipids (in an immiscible layer) using an aqueous solution of polypropylene glycol 400. The combination of this approach with a subsequent ATPS allows the fractionation of multiple microalgae biomolecules (lipids, proteins, pigments, carbohydrates) (based on **previous chapters**).

In **chapter 7,** a multistep approach using ATPS was proposed to fractionate most of the microalgae (*N. oleoabundans)* fractions avoiding the need of many unit operations. ATPS formed by polypropylene glycol and different cholinium-ILs were investigated based on their ability to partition the biomolecules. We observed that the anion of the ILs strongly influences the partitioning of the soluble proteins and other compounds. After this evaluation, an ATPS was selected and the multistep approach was designed. With this, the recovery efficiency of soluble biomolecules in the aqueous phases was enhanced and non-soluble components were concentrated in the interface. Furthermore, we proposed and proved the applicability of recycling routes.

In **chapter 8**, an integrated and multiproduct microalgae biorefinery approach was proposed. Several aqueous solutions (non-tensioactive and tensioactive) were studied on their capacity to disrupt the microalgae (*N. oleoabundans)* cells and to extract their biomolecules. The partitioning of the extracted biomolecules by ATPS (polymer-polymer) using the extractant component as electrolyte was optimized. This process allowed the cell disruption, the biomolecules' extraction, the combined fractionation of multiple biomolecules and the proper isolation of the biomolecules from the phase forming solvents, thus allowing their recycling.

Finally in the general discussion (**chapter 9),** we considered the opportunities and challenges for the development of simple and integrated multiproduct biorefineries mediated by aqueous solutions (including ATPS). Technologies using aqueous solutions are promising platforms and the results in this thesis highlight this potential.

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About the Author

Catalina Andrea Suarez Ruiz was born on 28 July 1987 in Medellin, Colombia. She followed secondary education at Las Bethlemitas in Medellin, Colombia and she studied Chemical Engineering at University of Antioquia.
Then, Catalina moved to Italy to follow a MSc. in Chemical and Sustainable processes Engineering with specialization in Biotechnology at Politecnico of Torino. She investigated the purification of microalgal proteins using supercritical carbon dioxide in the University of Twente, The Netherlands as part of her MSc. thesis.
She received her master degree in 2013 and in 2014 she started her PhD at Wageningen University, chair group Bioprocess Engineering. The research involved the application of aqueous two phase systems for the purification of microalgal components. The results of this PhD research are described in this thesis.

Catalina started working at Polpharma Biologics Utrecht in 2019 and currently works leading the downstream processing development team.

List of Publications

C. A. Suarez Ruiz, C. Van den Berg, R. H. Wijffels, & M. H. M. Eppink. Rubisco separation using biocompatible aqueous two-phase systems. *Separation and Purification Technology*, *196*, 254-261.

E. Suarez Garcia, **C. A. Suarez Ruiz**, T. Tilaye, M. H. M. Eppink, R. H. Wijffels & C. van den Berg (2018). Fractionation of proteins and carbohydrates from crude microalgae extracts using an ionic liquid based-aqueous two phase system. *Separation and Purification Technology*, *204*, 56-65.

C.A. Suarez Ruiz, D. P. Emmery, R. H. Wijffels, M. H. M. Eppink & C. van den Berg. (2018). Selective and mild fractionation of microalgal proteins and pigments using aqueous two-phase systems. *Journal of Chemical Technology & Biotechnology*, *93*(9), 2774-2783.

C. A. Suarez Ruiz, S. Zarate Baca, L. van den Broek, C. van den Berg, R. H. Wijffels & M. H. M Eppink. (2020). Selective fractionation of free glucose and starch from microalgae using aqueous two-phase systems. *Algal Research*, *46*, 101801.

C. A. Suarez Ruiz, J. Kwaijtaal, O. C. Peinado, C. van den Berg, R. H. Wijffels & M. H. M Eppink. (2020). Multistep Fractionation of Microalgal Biomolecules Using Selective Aqueous Two-Phase Systems. *ACS Sustainable Chemistry & Engineering*, *8*(6), 2441-2452.

C. A. Suarez Ruiz, M. Martins, J. A. P. Coutinho, R. H. Wijffels., M. H. M Eppink, C.Van den Berg & S. P. M. Ventura. (2020). *Neochloris oleoabundans* biorefinery: Integration of cell disruption and purification steps using aqueous biphasic systems-based in surface-active ionic liquids. *Chemical Engineering Journal*, *399*, 125683.

C.A. Suarez Ruiz, Oriol Cabau Peinado, C. van den Berg, R.H. Wijffels, M.H.M. Eppink. Efficient fractionation of lipids in a multi-product biorefinery by polymers and IL-based aqueous two-phase systems*.(Submitted)*

Overview of completed training activities

Discipline specific activities			
MODDE	Umetrics	Amsterdam	2015
Biorefinery for biomolecules*	VLAG	Wageningen	2015
Microalgae Process design	VLAG	Wageningen	2015
Advanced course Bioprocess design	VLAG	Delft	2015
Microalgae biorefinery	VLAG	Wageningen	2016
Advance course Downstream processing	VLAG	Delft	2016
Workshop Algae & Seaweed*	Wageningen University	Wageningen	2016
Continouos processing for biotherapeutic proteins	Nederlandse Biotechnologische Vereniging	Oss	2015
YAS Malta 2016*	YAS2016	Qawra	2016
ILSEPT 2017**	Elsevier	Kuala Lumpur	2017
General			
Advanced speaking skills	WUR	Wageningen	2016
Scientific Writing	WGS	Wageningen	2016
Presenting with impact	WGS	Wageningen	2016
WGS PhD Workshop Carousel 2015	WGS	Wageningen	2015
WGS PhD Workshop Carousel 2016	WGS	Wageningen	2016
WGS PhD Workshop Carousel 2017	WGS	Wageningen	2017
Scientific Publishing	WGS	Wageningen	2017
Information literacy for PhD including EndNote introduction	WUR Library	Wageningen	2016
Optional			
Biorefinery group meeting (Since 2015)	BPE	Wageningen	2015-2018
AlgaePARC biorefinery meeting	AlgaePARC biorefinery project	Wageningen	2014
AlgaePARC biorefinery meeting	AlgaePARC biorefinery project	Wageningen	2015
AlgaePARC biorefinery meeting	AlgaePARC biorefinery project	Wageningen	2015
AlgaePARC biorefinery meeting	AlgaePARC biorefinery project	Wageningen	2016
PhD days 2015	BPE	Wageningen	2015
PhD days 2016	BPE	Wageningen	2016
PhD days 2017	BPE	Wageningen	2017
BPE Meetings (Monday and Thursday) Since 2014	BPE	Wageningen	2014-2018
PhD trip (plan)	BPE	San Diego	2018
Teaching			
Separation process design		Wageningen	2015
Biorefinery		Wageningen	2016
Biorefinery		Wageningen	2017
Microalgae biorefinery		Wageningen	2016

*Poster presentation

** Oral presentation

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