



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



C.A. Suarez Ruiz^{a,*}, M. Martins^b, J.A.P. Coutinho^b, R.H. Wijffels^{a,c}, M.H.M. Eppink^a,
C. van den Berg^a, S.P.M. Ventura^{b,*}

^a Bioprocess Engineering, AlgaePARC, Wageningen University, P.O. Box 16, 6700 AA Wageningen, the Netherlands

^b CICECO - Aveiro Institute of Materials, Department of Chemistry, University of Aveiro, 3810-193 Aveiro, Portugal

^c Nord University, N-8049 Bodø, Norway

HIGHLIGHTS

- The recovery of various biomolecules from *N. oleoabundans* was studied.
- The aqueous solution of [P_{4,4,4,14}]Cl was selected as the best solvent.
- PEG 8000 + NaPA 8000 + [P_{4,4,4,14}]Cl + water ABS had the highest performance.
- A single step including cell disruption and biomolecules extraction was developed.
- Proteins, carbohydrates, chlorophylls and lutein were separated.

ARTICLE INFO

Keywords:

Tensioactive compounds
Microalgae disruption
Aqueous biphasic systems
Purification
Multi-product approach

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.

1. 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 [1-3]. 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 [4,5]. 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

* Corresponding authors.

E-mail addresses: catalina.suarezruiz@wur.nl (C.A. Suarez Ruiz), spventura@ua.pt (S.P.M. Ventura).

<https://doi.org/10.1016/j.cej.2020.125683>

Received 21 December 2019; Received in revised form 5 May 2020; Accepted 27 May 2020

Available online 01 June 2020

1385-8947/ © 2020 Elsevier B.V. All rights reserved.

biomolecules, while maintaining their structural integrity and properties [6,7]. 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 [5,8]. Its complexity is defined by the use of several unit operations to obtain each bioproduct with the purity demanded. When dealing with bioactive compounds from 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 [9]. 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 [10]. However, they are not yet appropriately explored and optimized [11]. 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 [12,13], which is a major concern for the downstream process performance, especially from high lipid containing microalgae [12].

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 [14], and so, complex unit operations have to be applied to obtain pure biomolecules from microalgae [8]. 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 [15]. The impact of tensioactive compounds on microalgae cells (characterized by a thick cell wall), has been evaluated [16-18], where the main results evidence the synergistic effect on the extraction yields by their ability to disrupt the microalgae cells [19]. 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 [14,20,21]. These have already successfully used as disrupting agents by their capacity to permeabilize the microalgae cells [22-26].

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 [27-29]. These systems have been used in the separation of several biomolecules such as proteins, enzymes, DNA and antibiotics [30-32]. 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 [33,34]. 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 [28,35-37].

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 ([C₄mim]Cl), tributyl-1-tetradecylphosphonium ([P₄, 4, 4, 1,4]Cl) and 1-dodecyltrimethylammonium bromide ([N₁, 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.

2. Experimental

2.1. Materials

The ILs 1-ethyl-3-methylimidazolium chloride, [C₂mim]Cl (99 wt%); 1-butyl-3-methylimidazolium chloride, [C₄mim]Cl (99 wt%); 1-hexyl-3-methylimidazolium chloride, [C₆mim]Cl (98 wt%); 1-methyl-1-propylpiperidinium chloride, [C₃mpip]Cl (99 wt%); 1-butylpyridinium chloride, [C₄py]Cl (98 wt%); 1-butyl-1-methylpyrrolidinium chloride, [C₄mpyr]Cl (99 wt%); 1-butyl-3-methylimidazolium bromide, [C₄mim]Br (98 wt%); 1-butyl-3-methylimidazolium trifluoroacetate, [C₄mim][CF₃CO₂] (97 wt%); 1-butyl-3-methylimidazolium trifluoromethanesulfonate, [C₄mim][CF₃SO₃] (99 wt%); 1-butyl-3-methylimidazolium thiocyanate, [C₄mim][SCN] (98 wt%); 1-butyl-3-methylimidazolium acetate, [C₄mim][CH₃CO₂] (98 wt%); 1-butyl-3-methylimidazolium dicyanamide, [C₄mim][N(CN)₂] (98 wt%), tributyl-1-tetradecylphosphonium, [P₄, 4, 4, 1,4]Cl (97 wt%), 1-methyl-3-octylimidazolium chloride [C₈mim]Cl (99 wt%); 1-decyl-3-methylimidazolium chloride, [C₁₀mim]Cl (98 wt%); 1-dodecyl-3-methylimidazolium chloride, [C₁₂mim]Cl (98 wt%); 1-methyl-3-tetradecylimidazolium chloride, [C₁₄mim]Cl (98 wt%) as well as 1-hexadecyl-3-methylimidazolium chloride, [C₁₆mim]Cl (98 wt%), were supplied by IoLiTec.

Tetrabutylphosphonium chloride, [P₄, 4, 4, 4]Cl (95 wt%) and tetrabutylphosphonium bromide, [P₄, 4, 4, 4]Br (greater than 95 wt%), were provided by Cytec. Tetrabutylammonium chloride, [N₄, 4, 4, 4]Cl (97 wt%), Decyltrimethylammonium chloride, [N₁, 1, 1, 10]Cl (98 wt%) was acquired from TCI. 1-dodecyltrimethylammonium bromide, [N₁, 1, 1, 12]Br (99 wt%); tetradecyltrimethylammonium bromide, [N₁, 1, 1, 14]Br (98 wt%) and hexadecylpyridinium chloride monohydrate, [C₁₆py]Cl (99 wt%) were provided by Sigma-Aldrich. 1-hexyltrimethylammonium bromide, [N₁, 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, Mersol HCS were acquired from Sigma-Aldrich, and ACROS Organics supplied Tween 20 and Triton X-114. Diocetyl 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. Fig. S1

2.2. 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 [38] composed of NaCl: 24.5 g.L⁻¹; MgCl₂: 9.8 g.L⁻¹; CaCl₂: 0.53 g.L⁻¹; K₂SO₄: 0.85 g.L⁻¹; NaSO₄: 3.2 g.L⁻¹; NaHCO₃: 0.8 g.L⁻¹. The microalgae were harvested (80 Hz, 3000 × g, 0.75 m³.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 ~ 25g_{DW}.kg⁻¹. The algal cells were lyophilized and stored at -20 °C until further use.

2.3. 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 h 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 (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. (2)).

$$Yield_{MB} = \frac{m_{MB, supernatant}}{m_{biomass}} \quad (1)$$

$$EE_{MB} (\%) = \frac{m_{MB, supernatant}}{m_{MB, i}} \quad (2)$$

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 2² factorial planning (Table S2 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 (R²), 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.

2.4. 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 [34]. 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 [34,39].

2.5. 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 [34]. 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. 3–5.

$$K_{MB} = \frac{C_{MB, top}}{C_{MB, bottom}} \quad (3)$$

$$EE_{MB} (\%) = \frac{m_{MB, phase}}{m_{MB, initial}} \times 100 \quad (4)$$

$$S_{MBi/MBj} = \frac{K_{MBi}}{K_{MBj}} \quad (5)$$

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.

2.6. 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.

2.6.1. 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 [40]. 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 min [41]. To recover the pigments, the samples were centrifuged at $1800 \times g$ 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 $5 \mu\text{m}$ 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 $1 \text{ mL}\cdot\text{min}^{-1}$ and using an injection volume of $10 \mu\text{L}$. DAD was set at 665, 649 and 454 nm (max. absorption wavelengths) [40]. Each sample was analysed at least two times. The column oven and the autosampler were operated at a controlled temperature of 30°C .

2.6.2. 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 [42].

The total mass content of proteins in the microalgae cells ($m_{\text{PROTEINS},i}$) was determined after the extraction of the proteins following the protocol developed in the Bioprocess Engineering group, at Wageningen University [43]. 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 beaten 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 min.

2.6.3. 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 [44]. The calibration curve was prepared using D(+)-glucose as analytical standard.

The total carbohydrate mass content ($m_{\text{CARBOHYDRATES},i}$) was determined after the total extraction by acid hydrolysis of the lyophilized cells as described by Postma et al. [45]. 1 mL of 2.5 M HCl was added to 1 mg of lyophilized biomass and incubated during 3 h at 100°C . After cooling down, the samples were neutralized using 1 mL of 2.5 M NaOH and centrifuged ($1200 \times g$, 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.

2.6.4. Lipids analysis

The total fatty acid (TFA) content in the cells $m_{\text{LIPIDS},i}$ was determined as described by Remmers et al. [46]. 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 $20 \text{ mL}\cdot\text{min}^{-1}$. The column used was a Supelco Nucotm 25357, $30 \text{ m} \times 530 \mu\text{m} \times 1.0 \mu\text{m}$. Total fatty acid composition and content were calculated by taking the sum of all fatty acids determined.

2.7. 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. $50 \mu\text{L}$ 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).

3. Results and discussion

3.1. Solvent screening applied in cells' disruption and biomolecules extraction

The biomass was firstly characterized. These cells are composed of $377 \pm 8 \text{ mg}\cdot\text{g}_{\text{dry biomass}}^{-1}$ of lipids, $283 \pm 7 \text{ mg}\cdot\text{g}_{\text{dry biomass}}^{-1}$ of proteins, $287 \pm 9 \text{ mg}\cdot\text{g}_{\text{dry biomass}}^{-1}$ of carbohydrates, $5.7 \pm 0.6 \text{ mg}\cdot\text{g}_{\text{dry biomass}}^{-1}$ of lutein, $3.7 \pm 0.7 \text{ mg}\cdot\text{g}_{\text{dry biomass}}^{-1}$ of chlorophyll *a* and $1.2 \pm 0.5 \text{ mg}\cdot\text{g}_{\text{dry biomass}}^{-1}$ 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 S1 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. 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 $53 \text{ mg}\cdot\text{g}_{\text{dry biomass}}^{-1}$ of proteins, $0.14 \text{ mg}\cdot\text{g}_{\text{dry biomass}}^{-1}$ of lutein and $0.12 \text{ mg}\cdot\text{g}_{\text{dry biomass}}^{-1}$ of chlorophylls (chlorophyll *a* + chlorophyll *b*). This suggests that water has penetrated the cell walls and other spaces in the cell, solubilising some proteins [47]. 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 [48,49], the more hydrophilic the ILs are, the stronger their capacity to extract water-soluble proteins. The order for imidazolium-based ILs is $[\text{C}_2\text{mim}]\text{Cl} \approx [\text{C}_4\text{mim}]\text{Cl} > [\text{C}_6\text{mim}]\text{Cl}$ and for tetrabutylphosphonium-based ILs is $[\text{P}_{4,4,4,4}]\text{Cl} > [\text{P}_{4,4,4,4}]\text{Br}$, 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 [17,50], or induced

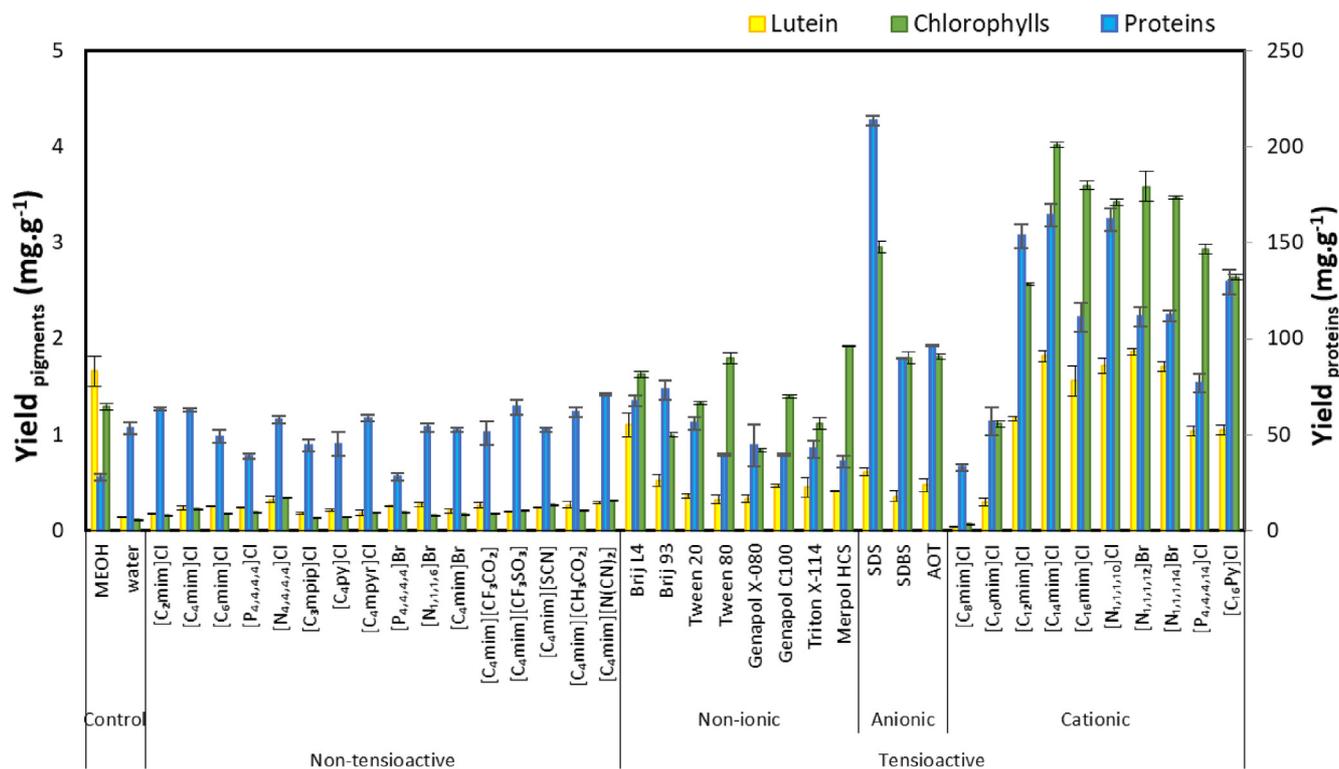


Fig. 1. Extraction yield of pigments (lutein and chlorophylls *a* and *b*) and proteins from *N. oleoabundans* using aqueous solutions of non-tensioactive and tensioactive compounds (non-ionic, anionic and cationic). Error bars indicate standard deviations.

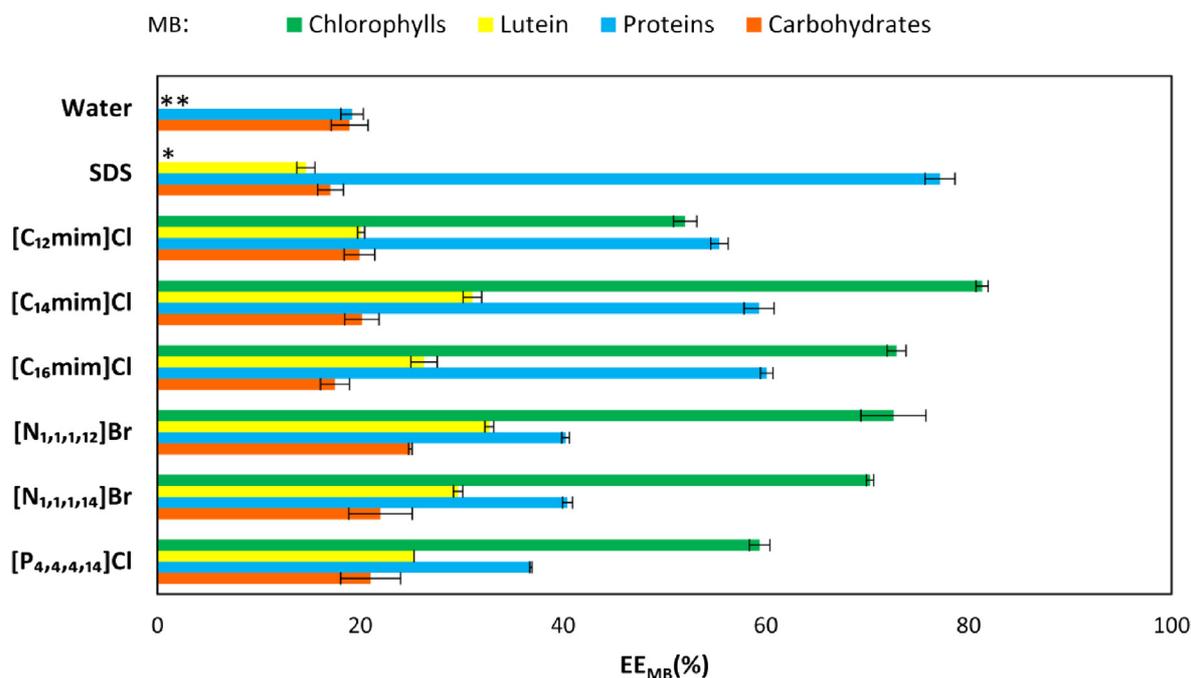


Fig. 2. Extraction efficiency of the microalgae biomolecules, EE_{MB} (%), determined for pigments (lutein, chlorophylls *a* and *b*), proteins and carbohydrates using aqueous solutions of cationic tensioactive compounds. Error bars indicate the standard deviations. *Chlorophylls were not detected in the supernatant when using SDS. ** No pigments (chlorophylls and lutein) were detected in the supernatant after using water as solvent.

permeation [51,52] of cell membranes is magnified.

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 [19], (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

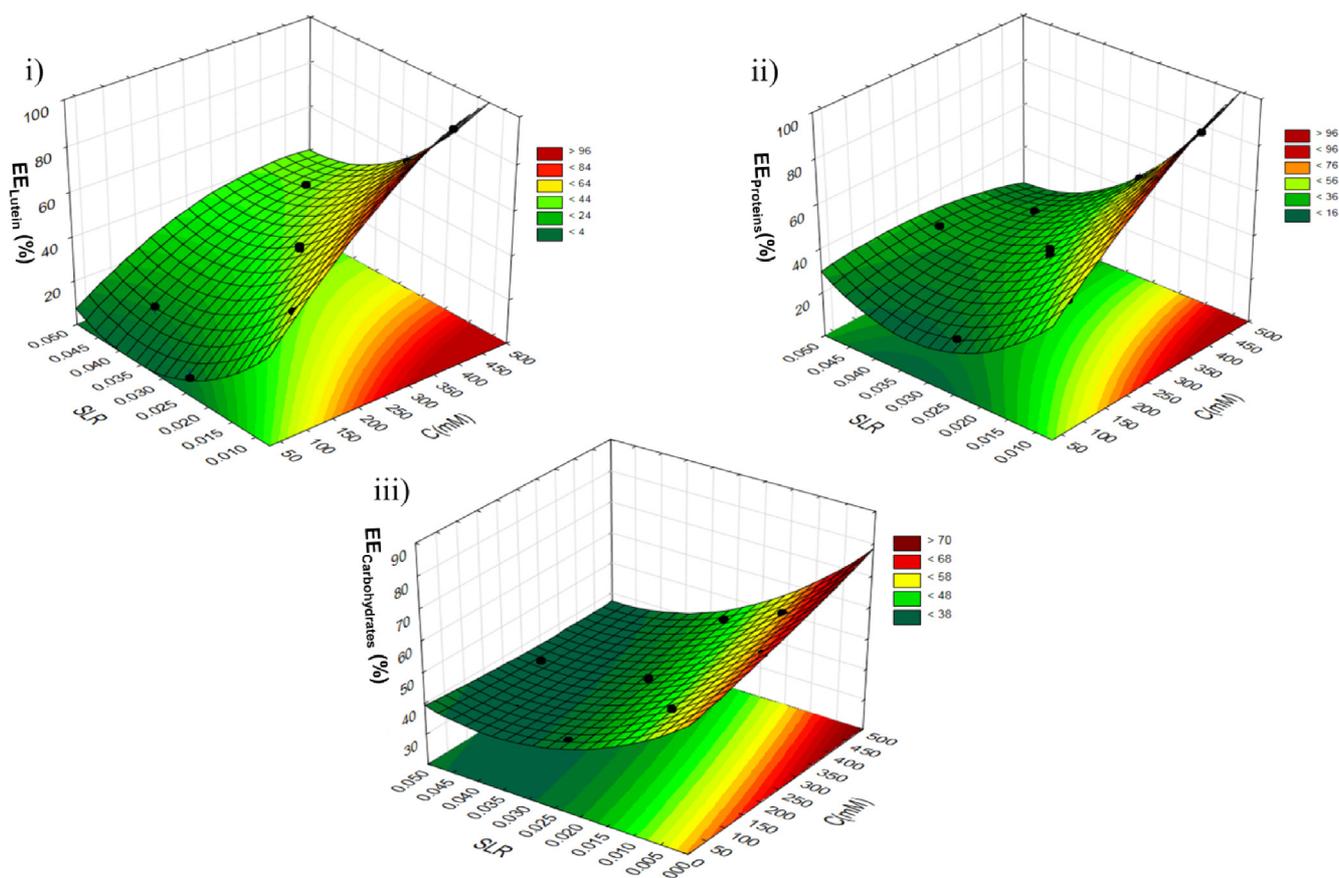


Fig. 3. Response surface plots representing the extraction efficiency [EE_{MB} (%)] of lutein (i), proteins (ii) and carbohydrates (iii) with the combined effects of solid-liquid ratio (SLR) and concentration, C (mM), using aqueous solutions of $[P_{4,4,4,14}]Cl$.

justified by the strong electrostatic interactions forming protein-surfactant complexes [53,54], 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 [55] 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 $1.9 \text{ mg}_{\text{lutein}} \cdot \text{g}_{\text{dry biomass}}^{-1}$ against $1.7 \text{ mg}_{\text{lutein}} \cdot \text{g}_{\text{dry biomass}}^{-1}$ for methanol and up to $4 \text{ mg}_{\text{chlorophylls}} \cdot \text{g}_{\text{dry biomass}}^{-1}$ vs. $1.3 \text{ mg}_{\text{chlorophylls}} \cdot \text{g}_{\text{dry biomass}}^{-1}$ for methanol.

3.2. 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. 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 applications as disruptive agent [56]).

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 (Fig. S1-B from the ESI), which implies their partial or complete degradation [57-60]. 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 (Fig. S1C 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 [61,62].

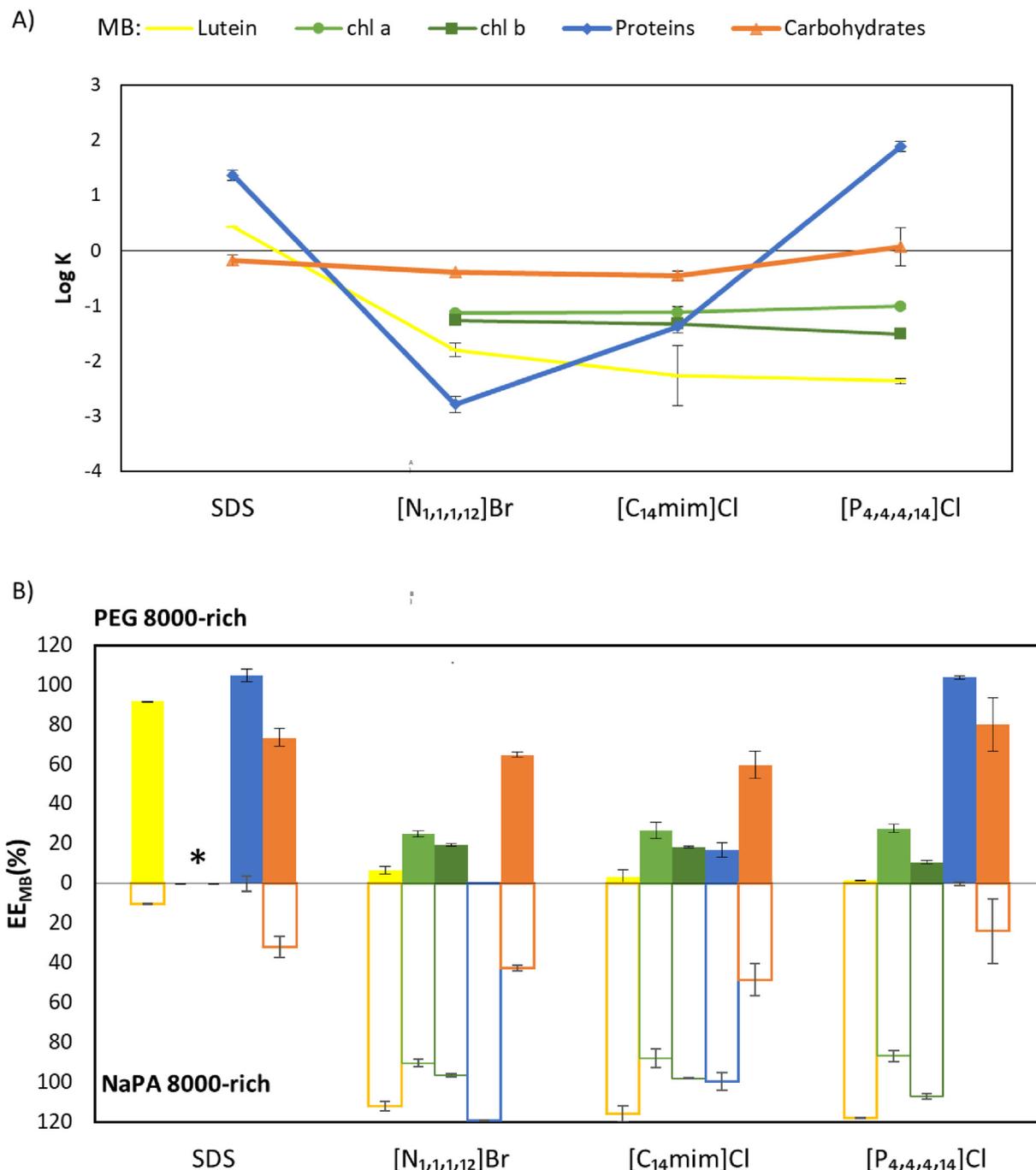


Fig. 4. Partition of biomolecules in polymeric-based ABS using 0.1 wt% of each electrolyte. A) partition coefficient logarithmic function (Log K) and B) EE_{MB} (%) in PEG 8000-rich phase (full columns) and NaPA 8000-rich phase (empty columns). *Chlorophylls *a* and *b* were not detected in the aqueous crude extract when using SDS.

Table 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
$S_{\text{Proteins/Lutein}}$	8.76	3.03	5.67	17633.28
$S_{\text{Proteins/Chl a}}$	–	0.90	0.92	2507.07
$S_{\text{Proteins/Chl b}}$	–	7.47	7.57	45.09
$S_{\text{Carb/Protein}}$	0.03	8.32	8.22	0.02
$S_{\text{Carb/Lutein}}$	0.25	25.21	46.61	317.12
$S_{\text{Carb/Chl a}}$	–	7.47	7.57	45.09
$S_{\text{Carb/Chl b}}$	–	5.54	4.63	14.14

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 (Fig. S2 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 [C₁₄mim]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 [63], this process is helped by the freeze-drying step, which may explain the extraction of proteins and carbohydrates when using only

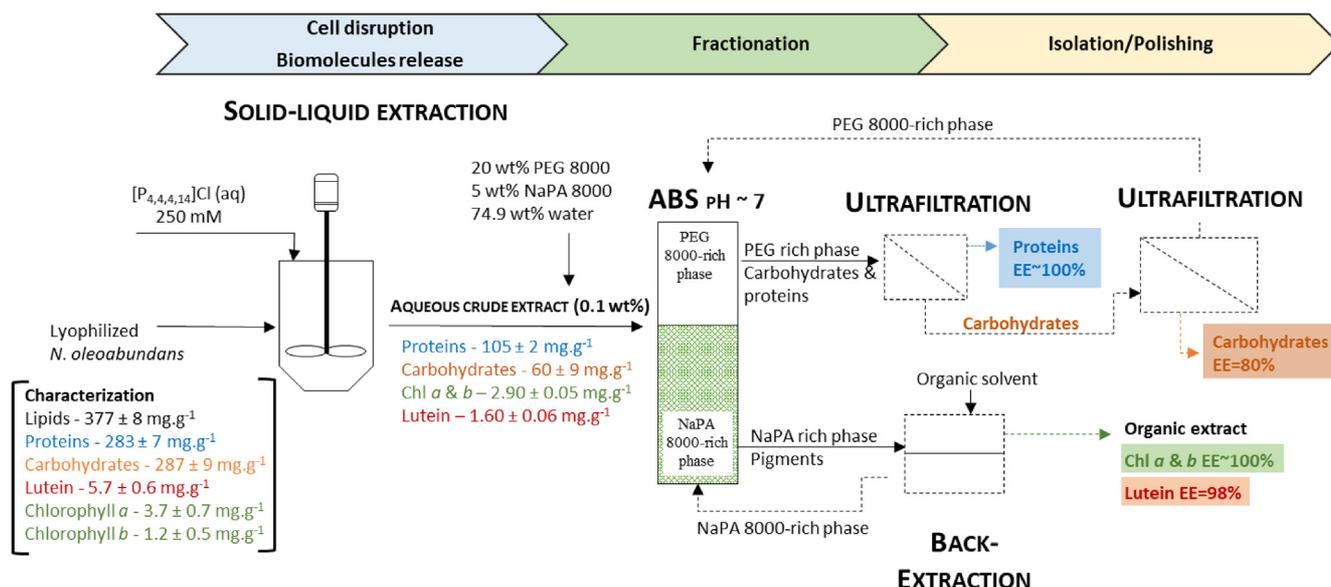


Fig. 5. Schematic representation of the integrated process developed contemplating the three main steps, (I) biomolecules' extraction, (II) separation of the biomolecules released from the biomass, and (III) isolation of biomolecules purified from the phase forming solvents, allowing the solvents' recycling. 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 ($\text{mg.g}_{\text{dry biomass}}^{-1}$).

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 (Figs. S2E to S2J 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 [18,64]. 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 (Fig. S1).

3.3. 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 S2 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 S3), the model equations (Table S4), as well as the parameters obtained in the statistical analysis are presented in Table S5. The accuracy and precision of the model equations were validated and the experimental and predicted values of the response variable (Table S3) were compared.

Fig. 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 S4 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 (Fig. S3) 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.

3.4. 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 [34] 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 [34]. Fig. S4 shows the binodal curves (weight fraction data (wt%) is represented in Table S9 from ESI), while the parameters obtained through the Merchuk equation are represented in Table S9 from ESI. As previously reported for other electrolytes [34], our results do not allow the identification of a clear trend regarding their ability to promote the ABS formation.

On Fig. S4, 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 ($7.06 \leq \text{pH} \leq 7.9$), independently of the electrolyte present (Table S10). 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), $[C_{14}mim]Cl$,

Table 2
Summary and comparison of studies on microalgae cell disruption/extraction/fractionation methods.

Microalgae sp.	Main compounds	Extraction method	Yield	Reference
<i>Neochloris oleabundans</i> <i>Chlorella pyrenoidosa</i>	Carotenoids Lutein	Pressurized liquid extraction (using ethanol) Ultrasound-enhanced subcritical CO ₂ extraction (with enzyme pretreatment) and Soxhlet extraction	53.4 mg _{carotenoids} ·g _{extract} ⁻¹ 1.24 mg _{lutein} ·g _{carotenoid biomass} ⁻¹	[67] [68]
<i>Haematococcus pluvialis</i>	Lutein	Carbon dioxide (CO ₂) in supercritical fluid extraction -Ethanol as co-solvent	~7.15 mg _{lutein} ·g _{dry weight} ⁻¹	[69]
<i>Scenedesmus sp</i> <i>Chlorella vulgaris</i>	Lutein Lutein	Microwave assisted binary phase solvent extraction method Ultrasound-assisted extraction and high-performance counter-current chromatography	11.92 mg _{lutein} ·g _{biomass} ⁻¹ 3.20 mg _{lutein} ·g _{dry weight} ⁻¹	[70] [71]
<i>Phormidium autumnale</i> <i>Dunaliella tertiolecta</i> , <i>Cylindrocapsa closterium</i>	Chlorophyll <i>a</i> Chlorophyll <i>a</i>	Solid-liquid extraction Microwave assisted extraction (MAE), cold and hot soaking and ultrasound-assisted extraction (UAE)	2.70 µg _{chlorophyll a} ·g _{dry weight} ⁻¹ 9.31 mg _{chlorophyll a} ·g _{dry weight} ⁻¹ and (hot soaking in acetone), 4.95 mg _{chlorophyll a} ·g _{dry weight} ⁻¹ (UAE), 8.65 mg _{chlorophyll a} ·g _{dry weight} ⁻¹ (MAE)	[72] [60]
<i>Scenedesmus obliquus</i> <i>Chlorella vulgaris</i> <i>Chlorella vulgaris</i>	Chlorophyll <i>a</i> and <i>b</i> Chlorophyll <i>a</i> and <i>b</i> Lutein and chlorophyll <i>a</i> and <i>b</i>	Supercritical fluid extraction with ethanol as co-solvent Ultrasound-assisted extraction Pressurized Liquid Extraction	0.85 mg _{chlorophyll a} ·g _{dry weight} ⁻¹ , 0.36 mg _{chlorophyll b} ·g _{dry weight} ⁻¹ Max yield: 35.2 mg _{chlorophylls} ·g _{dry weight} ⁻¹ 3.7 mg _{lutein} ·g _{dry weight} ⁻¹ , 10.79 mg _{chlorophyll a} ·g _{dry weight} ⁻¹ and 6.84 mg _{chlorophyll b} ·g _{dry weight} ⁻¹	[73] [74] [75]
<i>Chlorella sp.</i>	Proteins	Hydrothermal pre-treatment combined with partial solvent delipidation and urea extraction	62% of total proteins (wt%)	[76]
<i>Haematococcus pluvialis</i> , <i>Nannochloropsis oculata</i> , <i>Chlorella vulgaris</i> , <i>Porphyridium cruentum s</i> and <i>Arthrospira platensis</i> <i>Chlorella sp.</i>	Proteins Proteins Carbohydrates	High-pressure cell disruption > chemical treatment > ultrasonication > manual grinding. High-pressure cell disruption and alkaline treatment Conventional solvent extraction, fluidized bed extraction and Ultrasound assisted extraction	Best yield achieved with high-pressure cell disruption: 41% to 90% (wt%) 52% (wt%) 65–90 mg _{glucose} ·g _{dry weight} ⁻¹ (CSE), 48 mg _{glucose} ·g _{dry weight} ⁻¹ (FBE) and 368.5 mg _{glucose} ·g _{dry weight} ⁻¹ (UAE)	[47] [77] [78]
<i>Chlorella vulgaris</i> <i>Chlorococcum infusatum</i> <i>Chlorella vulgaris</i>	Carbohydrates Carbohydrates Lutein, chlorophyll <i>a</i> and <i>b</i> and proteins	Ionic liquid extraction Bead-beating followed by hydrolysis Solvent extraction followed by alkaline pH extraction	Glucan (starch) yield: 57.5% (45.1% g _{dry weight} ⁻¹) 89.6% (g _{dry weight} ⁻¹) 5.4 mg _{lutein} ·g _{dry weight} ⁻¹ and 15.4 mg _{chlorophylls} ·g _{dry weight} ⁻¹ biomass, and 76% of total protein (wt%)	[79] [80] [81]
<i>Nannochloropsis sp.</i>	Chlorophyll <i>a</i> and <i>b</i> , carbohydrates and proteins	Pulsed electric field assisted extraction followed by basic extraction	~0.2 mg _{chlorophylls} ·g _{dry weight} ⁻¹ , ~30 mg _{proteins} ·g _{dry weight} ⁻¹ and ~45 mg _{carbohydrates} ·g _{dry weight} ⁻¹	[82]
<i>Chlorella vulgaris</i> <i>Tetraselmis suecica</i> <i>Nannochloropsis sp.</i> , <i>Phaeodactylum tricornutum</i> and <i>Parachlorella kessleri</i>	Proteins and carbohydrates Proteins and carbohydrates Proteins and carbohydrates	Combined bead milling and enzymatic hydrolysis Bead milling Pulsed electrical fields (PEF), high voltage electrical discharges (HVED), and ultrasonication (US)	68% proteins, 74% carbohydrates (wt%) 50.4% of proteins and 26.4% carbohydrates (wt%) 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%)	[83] [62] [84]
<i>Neochloris oleabundans</i> <i>Neochloris oleabundans</i>	Carbohydrates, proteins, pigments Proteins, carbohydrates, lutein, chlorophyll <i>a</i> and <i>b</i>	Bead-milling and Multistep Aqueous biphasic systems ABS based in surface-active ionic liquids.	Yield fractionation: 82% proteins, 93% soluble sugars, 98% pigments (wt%) Yield extraction: 105 mg _{proteins} ·g _{dry weight} ⁻¹ , 60 mg _{carbohydrates} ·g _{dry weight} ⁻¹ , 2.90 mg _{chlorophyll a} and <i>b</i> ·g _{dry weight} ⁻¹ , 1.60 mg _{lutein} ·g _{dry weight} ⁻¹ . Yield fractionation: 100% proteins, 80% carbohydrates, 100% chlorophyll <i>a</i> and <i>b</i> and 98% lutein (wt%)	[85] This work

[P_{4,4,4,14}]Cl and [N_{1,1,1,12}]Br (cationic) at room temperature and for 1 h of extraction.

Fig. 4A shows the partition coefficient logarithm function obtained for pigments, proteins and carbohydrates (Log K), while Fig. 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. 4B) [34,65].

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 [17,66], 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* [66], 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 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 [C₁₄mim]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).

3.5. Integrated downstream processing of microalgae components

To develop a process feasible for industrial application, the integrated downstream process sketched in Fig. 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. 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, $105 \pm 2 \text{ mg.g}_{\text{dry biomass}}^{-1}$ of proteins, $60 \pm 9 \text{ mg.g}_{\text{dry biomass}}^{-1}$ of carbohydrates, $2.90 \pm 0.05 \text{ mg.g}_{\text{dry biomass}}^{-1}$ of chlorophylls, and $1.60 \pm 0.06 \text{ mg.g}_{\text{dry biomass}}^{-1}$ 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 considers not only the optimal disruption of microalgae cells and the extraction of different biomolecules, but also their fractionation, compounds isolation and solvents recyclability. Besides the high yields of extraction and purification, the integration of these unit operations appears as the major advantage of using ABS-based in surface-active ILs when compared with other approaches, previously reported in literature and compiled in Table 2.

4. 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.

Declaration of Competing Interest

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

Acknowledgements

This work was 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. This work was developed within the scope of the project CICECO-Aveiro Institute of Materials, UIDB/50011/2020 & UIDP/50011/2020, financed by national funds through the Portuguese Foundation for Science and Technology/MCTES. The authors are grateful for the financial support of Portuguese Foundation for Science and Technology (FCT) for the doctoral grant of SFRH/BD/122220/2016 of M. Martins. S.P.M. Ventura acknowledges FCT for the contract IF/00402/2015.

Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.cej.2020.125683>.

References

- [1] S.P. Slocombe, J.R. Benemann, *Microalgal production for biomass and high-value products*, CRC Press, Boca Raton, 2017.
- [2] T. Suganya, M. Varman, H.H. Masjuki, S. Renganathan, *Macroalgae and microalgae as a potential source for commercial applications along with biofuels production: A*

- biorefinery approach, *Renew. Sustain. Energy Rev.* 55 (2016) 909–941.
- [3] M. Koller, A. Muhr, G. Brauneig, *Microalgae as versatile cellular factories for value-added products*, *Algal Research* 6 (2014) 52–63.
- [4] K.W. Chew, J.Y. Yap, P.L. Show, N.H. Suan, J.C. Juan, T.C. Ling, D.J. Lee, J.S. Chang, *Microalgae biorefinery: High value products perspectives*, *Bioresour. Technol.* 229 (2017) 53–62.
- [5] G.P. t Lam, M.H. Vermuë, M.H.M. Eppink, R.H. Wijffels, C. van den Berg, *Multi-Product Microalgae Biorefineries: From Concept Towards Reality*, *Trends Biotechnol.* (2017) 216–227.
- [6] S.P.M. Ventura, B.P. Nobre, F. Ertekin, M. Hayes, M. Garcíá-Vaquero, F. Vieira, M. Koc, L. Gouveia, M.R. Aires-Barros, A.M.F. Palavra, *Extraction of value-added compounds from microalgae*, in: C. Gonzalez-Fernandez, R. Muñoz (Eds.), *Microalgae-Based Biofuels and Bioproducts: From Feedstock Cultivation to End-Products*, Elsevier Inc., Amsterdam, 2017, pp. 461–483.
- [7] I. Gifuni, A. Pollio, C. Safi, A. Marzocchella, G. Olivieri, *Current Bottlenecks and Challenges of the Microalgal Biorefinery*, *Trends Biotechnol.* 37 (2018) 242–252.
- [8] J. Ruiz, G. Olivieri, J. de Vree, R. Bosma, P. Willems, J.H. Reith, M.H. Eppink, D.M. Kleinegris, R.H. Wijffels, M.J. Barbosa, *Towards industrial products from microalgae*, *Energy Environ. Sci.* 9 (2016) 3036–3043.
- [9] C. Dixon, L.R. Wilken, *Green microalgae biomolecule separations and recovery*, *Bioresources and Bioprocessing* 5 (2018) 1–4.
- [10] E. Günerken, E. d'Hondt, M. Eppink, L. Garcia-Gonzalez, K. Elst, R. Wijffels, *Cell disruption for microalgae biorefineries*, *Biotechnol. Adv.* 33 (2015) 243–260.
- [11] K.-Y. Show, D.-J. Lee, J.-H. Tay, T.-M. Lee, J.-S. Chang, *Microalgal drying and cell disruption—recent advances*, *Bioresour. Technol.* 184 (2015) 258–266.
- [12] S.Q. Law, S. Mettu, M. Ashokkumar, P.J. Scales, G.J. Martin, *Emulsifying properties of ruptured microalgae cells: Barriers to lipid extraction or promising biosurfactants?* *Colloids Surf., B* 170 (2018) 438–446.
- [13] E.M. Grima, M.J.I. González, A.G. Giménez, *Solvent extraction for microalgal lipids*, Springer, *Algae for biofuels and energy*, 2013, pp. 187–205.
- [14] R.K. Desai, H. Montillet, X. Li, B. Schuur, J.M. Kleijn, F.A. Leermakers, R.H. Wijffels, M.H. Eppink, *One-step mild biorefinery of functional biomolecules from microalgae extracts*, *React. Chem. Eng.* 3 (2018) 182–187.
- [15] Y.S. Lai, Y. Zhou, R. Martarella, Z. Wang, B.E. Rittmann, *Synergistic integration of Cl₂–Cl₆ cationic surfactants for flocculation and lipid extraction from *Chlorella* biomass*, *ACS Sustainable Chem. Eng.* 5 (2017) 752–757.
- [16] W.-C. Huang, J.-D. Kim, *Cationic surfactant-based method for simultaneous harvesting and cell disruption of a microalgal biomass*, *Bioresour. Technol.* 149 (2013) 579–581.
- [17] G. Ulloa, C. Coutens, M. Sánchez, J. Sineiro, J. Fábregas, F.J. Deive, A. Rodríguez, M.J. Núñez, *On the double role of surfactants as microalga cell lysis agents and antioxidants extractants*, *Green Chem.* 14 (2012) 1044–1051.
- [18] J.Y. Seo, R. Praveenkumar, B. Kim, J.-C. Seo, J.-Y. Park, J.-G. Na, S.G. Jeon, S.B. Park, K. Lee, Y.-K. Oh, *Downstream integration of microalga harvesting and cell disruption by means of cationic surfactant-decorated Fe₃O₄ nanoparticles*, *Green Chem.* 18 (2016) 3981–3989.
- [19] Y.S. Lai, F. De Francesco, A. Aguinaga, P. Parameswaran, B.E. Rittmann, *Improving lipid recovery from *Scenedesmus* wet biomass by surfactant-assisted disruption*, *Green Chem.* 18 (2016) 1319–1326.
- [20] M. Martins, C.W. Ooi, M.C. Neves, J.F. Pereira, J.A. Coutinho, S.P. Ventura, *Extraction of recombinant proteins from *Escherichia coli* by cell disruption with aqueous solutions of surface-active compounds*, *J. Chem. Technol. Biotechnol.* (2018).
- [21] A.F.M. Cláudio, M.C. Neves, K. Shimizu, J.N.C. Lopes, M.G. Freire, J.A. Coutinho, *The magic of aqueous solutions of ionic liquids: ionic liquids as a powerful class of catanionic hydrotropes*, *Green Chem.* 17 (2015) 3948–3963.
- [22] V.C.A. Orr, N.V. Plechkova, K.R. Seddon, L. Rehmann, *Disruption and Wet Extraction of the Microalgae *Chlorella vulgaris* Using Room-Temperature Ionic Liquids*, *ACS Sustainable Chem. Eng.* 4 (2016) 591–600.
- [23] R.K. Desai, M. Streefland, R.H. Wijffels, M.H.M. Eppink, *Novel astaxanthin extraction from *Haematococcus pluvialis* using cell permeabilising ionic liquids*, *Green Chem.* 18 (2016) 1261–1267.
- [24] M. Olkiewicz, M.P. Caporgno, J. Font, J. Legrand, O. Lepine, N.V. Plechkova, J. Pruvost, K.R. Seddon, C. Bengoa, *A novel recovery process for lipids from microalgae for biodiesel production using a hydrated phosphonium ionic liquid*, *Green Chem.* 17 (2015) 2813–2824.
- [25] C. Chiappe, A. Mezzetta, C.S. Pomelli, G. Jaquaniello, A. Gentile, B. Masciocchi, *Development of cost-effective biodiesel from microalgae using protic ionic liquids*, *Green Chem.* 18 (2016) 4982–4989.
- [26] M. Shankar, P.K. Chhotaray, A. Agrawal, R.L. Gardas, K. Tamilarasan, M. Rajesh, *Protic ionic liquid-assisted cell disruption and lipid extraction from fresh water *Chlorella* and *Chlorococcum* microalgae*, *Algal Research* 25 (2017) 228–236.
- [27] K.V.G. Barros, P.M. Souza, M.M. Freitas, E.X. Ferreira Filho, A.P. Junior, P.O. Magalhães, *PEG/NaPA aqueous two-phase systems for the purification of proteases expressed by *Penicillium restrictum* from Brazilian Savanna*, *Process Biochem.* 49 (2014) 2305–2312.
- [28] J.H. Santos, M.R. Almeida, C.I. Martins, A.C. Dias, M.G. Freire, J.A. Coutinho, S.P. Ventura, *Separation of phenolic compounds by centrifugal partition chromatography*, *Green Chem.* 20 (2018) 1906–1916.
- [29] H.O. Johansson, M. Ishii, M. Minaguti, E. Feitosa, T.C.V. Penna, A. Pessoa Jr, *Separation and partitioning of Green Fluorescent Protein from *Escherichia coli* homogenate in poly(ethylene glycol)/sodium-poly(acrylate) aqueous two-phase systems*, *Sep. Purif. Technol.* 62 (2008) 166–174.
- [30] H.O. Johansson, F.M. Magaldi, E. Feitosa, A. Pessoa Jr, *Protein partitioning in poly(ethylene glycol)/sodium polyacrylate aqueous two-phase systems*, *J. Chromatogr. A* 1178 (2008) 145–153.
- [31] J.F. Pereira, V.C. Santos, H.-O. Johansson, J.A. Teixeira, A. Pessoa Jr, *A stable liquid-liquid extraction system for clavulanic acid using polymer-based aqueous two-phase systems*, *Sep. Purif. Technol.* 98 (2012) 441–450.
- [32] H.-O. Johansson, T. Matos, J.S. Luz, E. Feitosa, C.C. Oliveira, A. Pessoa Jr, L. Bülow, F. Tjerneld, *Plasmid DNA partitioning and separation using poly(ethylene glycol)/poly(acrylate)/salt aqueous two-phase systems*, *J. Chromatogr. A* 1233 (2012) 30–35.
- [33] H.-O. Johansson, E. Feitosa, A.P. Junior, *Phase diagrams of the aqueous two-phase systems of poly(ethylene glycol)/sodium polyacrylate/salts*, *Polymers* 3 (2011) 587–601.
- [34] J.H. Santos, M. Martins, A.J. Silvestre, J.A. Coutinho, S.P. Ventura, *Fractionation of phenolic compounds from lignin depolymerisation using polymeric aqueous biphasic systems with ionic surfactants as electrolytes*, *Green Chem.* 18 (2016) 5569–5579.
- [35] J.H.P.M. Santos, F.A.E. Silva, J.A.P. Coutinho, S.P.M. Ventura, A. Pessoa, *Ionic liquids as a novel class of electrolytes in polymeric aqueous biphasic systems*, *Process Biochem.* 50 (2015) 661–668.
- [36] J.H. Santos, J.C. Flores-Santos, G.P. Meneguetti, C.O. Rangel-Yagui, J.A. Coutinho, M. Vitolo, S.P. Ventura, A. Pessoa Jr, *In situ purification of periplasmatic L-asparaginase by aqueous two phase systems with ionic liquids (ILs) as adjuvants*, *J. Chem. Technol. Biotechnol.* 93 (2018) 1871–1880.
- [37] A.M. Lopes, J.V.D. Molino, V.C. dos Santos-Ebinuma, A. Pessoa Jr., S.R. Valentini, J.F.B. Pereira, *Effect of electrolytes as adjuvants in GFP and LPS partitioning on aqueous two-phase systems: 1. Polymer-polymer systems*, *Sep. Purif. Technol.* 206 (2018) 39–49.
- [38] C.C.o.A.a.P. CCAP, *Bold's Basal Medium (BB)*, <https://www.ccap.ac.uk/pdfrecipes.htm>, 2015 (Accessed January 2017).
- [39] S.P.M. Ventura, C.M.S.S. Neves, M.G. Freire, I.M. Marrucho, J. Oliveira, J.A.P. Coutinho, *Evaluation of anion influence on the formation and extraction capacity of ionic-liquid-based aqueous biphasic systems*, *J. Phys. Chem. B* 113 (2009) 9304–9310.
- [40] A.C. Leite, A.M. Ferreira, E.S. Morais, I. Khan, M.G. Freire, J.A. Coutinho, *Cloud Point Extraction of Chlorophylls from Spinach Leaves Using Aqueous Solutions of Nonionic Surfactants*, *ACS Sustainable Chem. Eng.* 6 (2017) 590–599.
- [41] H.K. Lichtenthaler, [34] *Chlorophylls and carotenoids: Pigments of photosynthetic biomembranes*, *Methods Enzymol.* (1987) 350–382.
- [42] R.E. Brown, K.L. Jarvis, K.J. Hyland, *Protein measurement using bicinchoninic acid: elimination of interfering substances*, *Anal. Biochem.* 180 (1989) 136–139.
- [43] P.R. Postma, T.L. Miron, G. Olivieri, M.J. Barbosa, R.H. Wijffels, M.H.M. Eppink, *Mild disintegration of the green microalgae *Chlorella vulgaris* using bead milling*, *Bioresour. Technol.* 184 (2015) 297–304.
- [44] M. Dubois, K.A. Gilles, J.K. Hamilton, P.A. Rebers, F. Smith, *Colorimetric Method for Determination of Sugars and Related Substances*, *Anal. Chem.* 28 (1956) 350–356.
- [45] P. Postma, G. Pataro, M. Capitoli, M. Barbosa, R.H. Wijffels, M. Eppink, G. Olivieri, G. Ferrari, *Selective extraction of intracellular components from the microalga *Chlorella vulgaris* by combined pulsed electric field-temperature treatment*, *Bioresour. Technol.* 203 (2016) 80–88.
- [46] I.M. Remmers, A. Hidalgo-Ulloa, B.P. Brandt, W.A.C. Evers, R.H. Wijffels, P.P. Lamers, *Continuous versus batch production of lipids in the microalgae *Acutodesmus obliquus**, *Bioresour. Technol.* 244 (2017) 1384–1392.
- [47] C. Safi, A.V. Ursu, C. Laroche, B. Zebib, O. Merah, P.Y. Pontalier, C. Vaca-Garcia, *Aqueous extraction of proteins from microalgae: effect of different cell disruption methods*, *Algal Res* 3 (2014) 61–65.
- [48] M. Martins, F.A. Vieira, I. Correia, R.A.S. Ferreira, H. Abreu, J.A.P. Coutinho, S.P.M. Ventura, *Recovery of phycobiliproteins from the red macroalga: *Gracilaria* sp. using ionic liquid aqueous solutions*, *Green Chem.* 18 (2016) 4287–4296.
- [49] S.Y. Lee, P.L. Show, T.C. Ling, J.S. Chang, *Single-step disruption and protein recovery from *Chlorella vulgaris* using ultrasonication and ionic liquid buffer aqueous solutions as extractive solvents*, *Biochem. Eng. J.* 124 (2017) 26–35.
- [50] M. Manaargadoo-Catin, A. Ali-Cherif, J.L. Pognas, C. Perrin, *Hemolysis by surfactants - A review*, *Adv. Colloid Interface Sci.* 228 (2016) 1–16.
- [51] A.P. Gerola, P.F. Costa, F.H. Quina, H.D. Fiedler, F. Nome, *Zwitterionic surfactants in ion binding and catalysis*, *Curr. Opin. Colloid Interface Sci.* 32 (2017) 39–47.
- [52] P. Glembin, R. Racheva, M. Kerner, I. Smirnova, *Micelle mediated extraction of fatty acids from microalgae cultures: implementation for outdoor cultivation*, *Sep. Purif. Technol.* 135 (2014) 127–134.
- [53] S.I. Cheng, D.C. Stuckey, *Protein precipitation using an anionic surfactant*, *Process Biochem.* 47 (2012) 712–719.
- [54] M. le Maire, P. Champeil, J.V. Möller, *Interaction of membrane proteins and lipids with solubilizing detergents*, *Biochimica et Biophysica Acta (BBA)-Biomembranes* 1508 (2000) 86–111.
- [55] B. Yoo, B. Jing, S.E. Jones, G.A. Lamberti, Y. Zhu, J.K. Shah, E.J. Maginn, *Molecular mechanisms of ionic liquid cytotoxicity probed by an integrated experimental and computational approach*, *Sci. Rep.* 6 (2016) 19889.
- [56] R.E. Farrell, *R. Methodologies, A laboratory guide for isolation and characterization*, *RNA Methodologies* (1993) 76–82.
- [57] P. Matile, S. Hörtensteiner, H. Thomas, *Chlorophyll degradation*, *Annu. Rev. Plant Biol.* 50 (1999) 67–95.
- [58] D. Amir-Shapira, E.E. Goldschmidt, A. Altman, *Autolysis of chlorophyll in aqueous and detergent suspensions of chloroplast fragments*, *Plant Sci.* 43 (1986) 201–206.
- [59] B.R. Jovanic, S. Bojovic, B. Panic, B. Radenkovic, M. Despotovic, *The effect of detergent as polluting agent on the photosynthetic activity and chlorophyll content in bean leaves*, *health* 2 (2010) 395.
- [60] V. Pasquet, J.-R. Chérouvier, F. Farhat, V. Thiéry, J.-M. Piot, J.-B. Bérard, R. Kaas, B. Serive, T. Patrice, J.-P. Cadoret, *Study on the microalgal pigments extraction*

- process: Performance of microwave assisted extraction, *Process Biochem.* 46 (2011) 59–67.
- [61] M. Demuez, A. Mahdy, E. Tomás-Pejó, C. González-Fernández, M. Ballesteros, Enzymatic cell disruption of microalgae biomass in biorefinery processes, *Biotechnol. Bioeng.* 112 (2015) 1955–1966.
- [62] E.S. Garcia, J. van Leeuwen, C. Safi, L. Sijtsma, M. Eppink, R. Wijffels, C. van den Berg, Selective and energy efficient extraction of functional proteins from microalgae for food applications, *Bioresour. Technol.* 268 (2018) 197–203.
- [63] B. Cordero, D. Voltolina, Viability of mass algal cultures preserved by freezing and freeze-drying, *Aquacult. Eng.* 16 (1997) 205–211.
- [64] M.W. Tenney, W.F. Echelberger, R.G. Schuessler, J.L. Pavoni, Algal flocculation with synthetic organic polyelectrolytes, *Appl. Microbiol.* 18 (1969) 965–971.
- [65] E. Suarez Garcia, C.A. Suarez Ruiz, T. Tilaye, M.H.M. Eppink, R.H. Wijffels, C. van den Berg, Fractionation of proteins and carbohydrates from crude microalgae extracts using an ionic liquid based-aqueous two phase system, *Sep. Purif. Technol.* 204 (2018) 56–65.
- [66] C.A. Suarez Ruiz, D.P. Emmery, R.H. Wijffels, M.H.M. Eppink, C. van den Berg, Selective and mild fractionation of microalgal proteins and pigments using aqueous two-phase systems, *J. Chem. Technol. Biotechnol.* 93 (2018) 2774–2783.
- [67] M. Castro-Puyana, M. Herrero, I. Urreta, J.A. Mendiola, A. Gifuentes, E. Ibáñez, S. Suárez-Alvarez, Optimization of clean extraction methods to isolate carotenoids from the microalga *Neochloris oleoabundans* and subsequent chemical characterization using liquid chromatography tandem mass spectrometry, *Anal. Bioanal. Chem.* 405 (2013) 4607–4616.
- [68] X.-D. Fan, Y. Hou, X.-X. Huang, T.-Q. Qiu, J.-G. Jiang, Ultrasound-enhanced subcritical CO₂ extraction of lutein from *Chlorella pyrenoidosa*, *J. Agric. Food. Chem.* 63 (2015) 4597–4605.
- [69] A. Molino, S. Mehariya, A. Iovine, V. Larocca, G. Di Sanzo, M. Martino, P. Casella, S. Chianese, D. Musmarra, Extraction of astaxanthin and lutein from microalga *Haematococcus pluvialis* in the red phase using CO₂ supercritical fluid extraction technology with ethanol as co-solvent, *Mar. Drugs* 16 (2018) 432.
- [70] K.L. Low, A. Idris, N.M. Yusof, Novel protocol optimized for microalgae lutein used as food additives, *Food Chem.* 307 (2020) 125631.
- [71] T. Fábryová, J. Cheel, D. Kubáč, P. Hrouzek, L. Tůmová, J. Kopecký, Purification of lutein from the green microalgae *Chlorella vulgaris* by integrated use of a new extraction protocol and a multi-injection high performance counter-current chromatography (HPCCC), *Algal Research* 41 (2019) 101574.
- [72] D.B. Rodrigues, C.R. Menezes, A.Z. Mercadante, E. Jacob-Lopes, L.Q. Zepka, Bioactive pigments from microalgae *Phormidium autumnale*, *Food Res. Int.* 77 (2015) 273–279.
- [73] A.C. Guedes, M.S. Gião, A.A. Matias, A.V. Nunes, M.E. Pintado, C.M. Duarte, F.X. Malcata, Supercritical fluid extraction of carotenoids and chlorophylls a, b and c, from a wild strain of *Scenedesmus obliquus* for use in food processing, *J. Food Eng.* 116 (2013) 478–482.
- [74] W. Kong, N. Liu, J. Zhang, Q. Yang, S. Hua, H. Song, C. Xia, Optimization of ultrasound-assisted extraction parameters of chlorophyll from *Chlorella vulgaris* residue after lipid separation using response surface methodology, *J. Food Sci. Technol.* 51 (2014) 2006–2013.
- [75] K.H. Cha, H.J. Lee, S.Y. Koo, D.-G. Song, D.-U. Lee, C.-H. Pan, Optimization of pressurized liquid extraction of carotenoids and chlorophylls from *Chlorella vulgaris*, *J. Agric. Food. Chem.* 58 (2010) 793–797.
- [76] H. Karan, R. de Boeck, J. Roles, B. Hankamer, I.L. Ross, Hydrothermal pre-treatment coupled with urea solubilisation enables efficient protein extraction from microalgae, *Green Chem.* 21 (2019) 6361–6371.
- [77] A.-V. Ursu, A. Marcati, T. Sayd, V. Sante-Lhoutellier, G. Djelveh, P. Michaud, Extraction, fractionation and functional properties of proteins from the microalgae *Chlorella vulgaris*, *Bioresour. Technol.* 157 (2014) 134–139.
- [78] G. Zhao, X. Chen, L. Wang, S. Zhou, H. Feng, W.N. Chen, R. Lau, Ultrasound assisted extraction of carbohydrates from microalgae as feedstock for yeast fermentation, *Bioresour. Technol.* 128 (2013) 337–344.
- [79] V.C. Orr, L. Rehmman, Ionic liquids for the fractionation of microalgae biomass, *Curr. Opin. Green Sustainable Chem.* 2 (2016) 22–27.
- [80] A. Karemore, R. Sen, Downstream processing of microalgal feedstock for lipid and carbohydrate in a biorefinery concept: a holistic approach for biofuel applications, *RSC Adv.* 6 (2016) 29486–29496.
- [81] S. Kulkarni, Z. Nikolov, Process for selective extraction of pigments and functional proteins from *Chlorella vulgaris*, *Algal research* 35 (2018) 185–193.
- [82] O. Parniakov, F.J. Barba, N. Grimi, L. Marchal, S. Jubeau, N. Lebovka, E. Vorobiev, Pulsed electric field and pH assisted selective extraction of intracellular components from microalgae *Nannochloropsis*, *Algal Research* 8 (2015) 128–134.
- [83] R.S. Alavijeh, K. Karimi, R.H. Wijffels, C. van den Berg, M. Eppink, Combined bead milling and enzymatic hydrolysis for efficient fractionation of lipids, proteins, and carbohydrates of *Chlorella vulgaris* microalgae, *Bioresour. Technol.* 123321 (2020).
- [84] R. Zhang, N. Lebovka, L. Marchal, E. Vorobiev, N. Grimi, Comparison of aqueous extraction assisted by pulsed electric energy and ultrasonication: Efficiencies for different microalgal species, *Algal Research* 47 (2020) 101857.
- [85] C.A. Suarez Ruiz, J. Kwaijtaal, O.C. Peinado, C. Van Den Berg, R.H. Wijffels, M.H. Eppink, Multistep Fractionation of Microalgal Biomolecules Using Selective Aqueous Two-Phase Systems, *ACS Sustainable Chem. Eng.* 8 (2020) 2441–2452.