



Fractionation of proteins and carbohydrates from crude microalgae extracts using an ionic liquid based-aqueous two phase system



Edgar Suarez Garcia^{a,1,*}, Catalina A. Suarez Ruiz^{a,1}, Tewodros Tilaye^a, Michel H.M. Eppink^a, Rene H. Wijffels^{a,b}, Corjan van den Berg^a

^a Bioprocess Engineering, AlgaePARC, Wageningen University and Research, PO Box 16, 6700 AA Wageningen, The Netherlands

^b Nord University, Faculty of Biosciences and Aquaculture, N-8049 Bodø, Norway

ARTICLE INFO

Keywords:

Crude protein
Partitioning
Ionic liquid
Phase diagram
Tie lines

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.

1. 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 [1]. Because of their sustainability, techno-functionality and broad range of applications, algae proteins have been in the spotlight of numerous studies [2]. However, microalgae proteins are often present intracellularly or forming complexes with pigments and polysaccharides and thus, their recovery and purification still represents a challenge [3]. Several processes have been developed for the extraction and fractionation of proteins from microalgae. pH-shifting, filtration and adsorption are commonly reported [4]. 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 [5]. Although large scale applications are reported [6], 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 [7]. Ionic liquid-based ATPS have been studied by several authors for the extraction of lipids proteins [8]. High extraction efficiencies and partition coefficients 3–4 times higher can be achieved in comparison with traditional polymer-salt systems [9].

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) [7]. In the case of proteins, hydrophobicity, isoelectric point, molecular weight and conformation play a critical role [10]. 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

* Corresponding author.

E-mail address: edgar.suarezgarcia@wur.nl (E. Suarez Garcia).

¹ These authors contributed equally.

Nomenclature

Letter	Definition [Units]
<i>a</i>	activity [–]
<i>CP</i>	crude protein [–]
<i>EE</i>	extraction efficiency [%]
<i>g</i>	interaction energy [J mol ⁻¹]
<i>k</i>	partition coefficient protei [–]
<i>Q</i>	penalty term [–]
<i>R</i>	universal gas constant [J mol ⁻¹ K ⁻¹]
<i>T</i>	system temperature [K]
<i>x</i>	mole fraction [mol mol ⁻¹]

Greek characters

α	non-randomness (NRTL model) [–]
τ	adjustable interaction parameter [–]
γ	activity coefficient [–]

Subscripts

<i>i, k, l</i>	component, parameters and tie lines respectively [–]
<i>ij</i>	refers to binary interaction between components <i>i</i> and <i>j</i>
<i>g</i>	free glucose [–]
<i>T, B</i>	top and bottom phase respectively [–]
<i>exp, calc</i>	experimental and calculated value respectively [–]
<i>p</i>	protein [–]

thermodynamic models have been successfully implemented for systems containing polymers [11], salts [12] and ILs [13]. 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 [12] and multicomponent Wilson model [14]. For polymer-polymer systems, modifications of the Pitzer's model [15], Flory Huggins theory [16] and UNIQUAC model [17], 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 [8,18], 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-phycoerythrin* from *Spirulina* strains [19], *B-phycoerythrin* from *Porphyridium cruentum* [20] and proteins from *Chlorella pyrenoidosa* [21] and *Chlorella sorokiniana* [22]. Combination of several disintegration-extraction methods have also been described. Lee and co-workers [23] 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. [24] 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 [25]. *T. suecica* has been traditionally used in aquaculture [26] and recently it has been highlighted due to the techno-functional properties of its proteins [27]. 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 [13], and because of its simplicity compared with models like UNIQUAC or UNIFAC [28]. To our knowledge, this is the first attempt to describe the partitioning of crude microalgae proteins in ATPS containing ILs using thermodynamic models.

2. Experimental section

2.1. 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.

2.2. 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 [29]. *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 [29]. Both photo-bioreactor systems were located in AlgaePARC (Wageningen, The Netherlands). After harvesting, biomass was stored at 4 °C until further use.

2.3. 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. [29]. The milled suspension was then centrifuged at 14000 rpm and 20 °C 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 cm² and a cut-off of 1000 kDa (Pellicon® XL Ultrafiltration Biomax®). The process was run at constant transmembrane pressure until a 5 × 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 4000g and 20 °C; MilliQ® water was used as feed for the second and third run. The resulting retentate, regarded as crude protein (CP), was stored at –20 °C until further use.

2.4. 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 [30]. 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 [31], 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 [32]. 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 °C. Free glucose was determined as described below.

2.5. 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®.

2.6. 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. [33]. 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 $\sim 0.3 \text{ mg g}^{-1}$. 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.

2.7. 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.

2.8. 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 °C 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.

2.9. Partition coefficients and extraction efficiencies

The partition coefficient for proteins (k_p) between top (T) and bottom (B) phases was estimated according to Eq. (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. (2). $EE\%$ indicates the extent of extraction of a compound in the top phase compared to the total feed.

$$k_p = \frac{x_{p,T}}{x_{p,B}} \quad (1)$$

$$EE\% = \frac{C_T * V_T}{C_B * V_B + C_T * V_T} * 10 \quad (2)$$

where C stands for concentration and V stands for volume in each phase.

2.10. 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.

2.11. 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 [13]. 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 [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. [34] In this approach, equality of activities for component i in both phases (Eq. (3)) and mass conservation equations are coupled with the activity coefficient model.

$$(\gamma_i x_i)_{Top} = (\gamma_i x_i)_{Bottom} \quad (3)$$

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 [33] was used to estimate the corresponding interaction parameters τ_{ij} via model regression. First, the non-randomness parameter α was fixed between 0.1 and 0.3 [28]. The three remaining parameters were correlated following the solution algorithm proposed by Haghtalab and Paraj [35] 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 $\sim 0.3 \text{ mg g}^{-1}$ was used. This assumption has been already implemented by Hartounian et al. [17] and Perez et al. [12], and allow us to treat LLE and protein partitioning separately.
- iii. The protein partition coefficient (k_p) is calculated as follows:

$$k_p = \frac{x_p^T}{x_p^B} = \frac{\gamma_p^B}{\gamma_p^T} \quad (4)$$

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_p^T and x_p^B 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_p^T and x_p^B , and by an optimization procedure adopted by Perez et al. [12]. In this procedure (Appendix B), the interaction parameters are obtained by minimization of the following objective function:

3. 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 [33]. 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 [9,10]. 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 [36]. 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 Fig. 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 [33] to six tie lines and the corresponding model estimations are shown in Fig. 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 [13]

3.1. Protein partitioning

The equilibrium data and interaction parameters for the system Iol-Cit (Fig. 1 and Table 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. (5). 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 (Fig. 1).

$$TLL = \left(\frac{\Delta x_1^2}{\Delta x_2^2} \right)^{1/2} \quad (5)$$

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 Fig. 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. [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 [33]. Partition coefficient results are in agreement with studies on protein partitioning in ATPS containing the same ionic liquid [9,10,33]. Dreyer et al. [10] 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) [37] and BSA (pI ~ 4.7) [9], 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 [38] 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 [39].

Fig. 2B displays the partition coefficient of total protein from the CP derived from *N. oleoabundans* and *T. suecica*. Although a slight

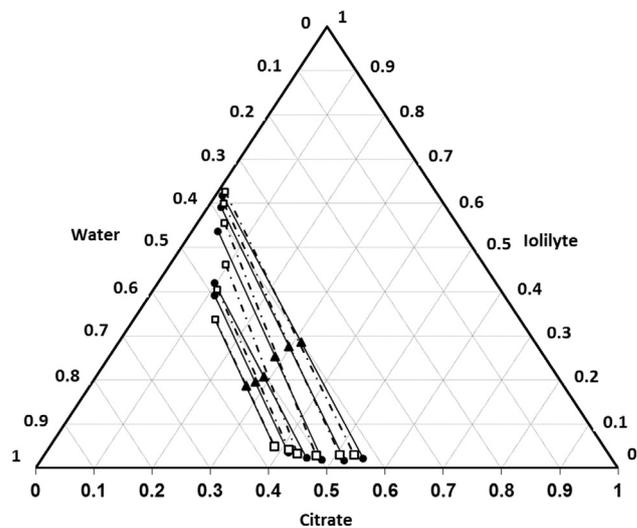


Fig. 1. Phase diagram (% w/w) for the system Iolilyte (1) – Citrate (2) – Water (2). Experimental data (filled symbols and solid line for tie lines, ▲ for feed compositions) and model estimation (open symbols and dashed line for tie lines).

Table 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).

IP	6 TL	4 TL	IP	BSA	Rubisco	CP <i>N. oleoabundans</i>	CP <i>T. suecica</i>
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.

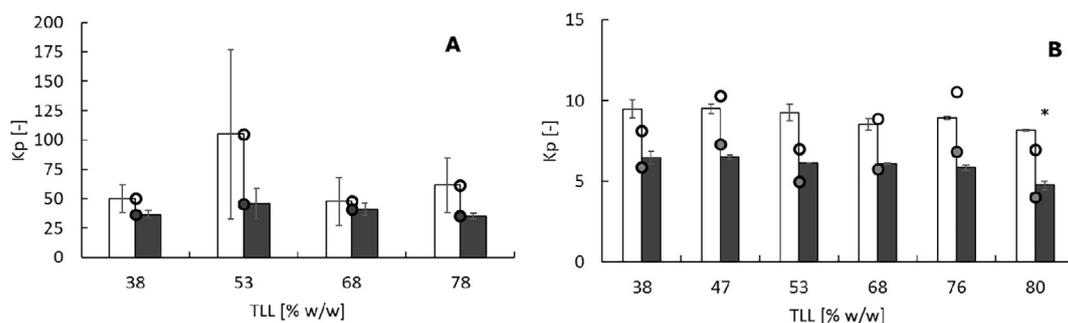


Fig. 2. Partition coefficients as function of tie line length in the system Iolilyte (1) – citrate (2) – water (3). Experimental data is shown in bars and model estimations with markers. (A) Rubisco (open marker and bars) and BSA (filled bars and marker). (B) Crude protein from *N. oleoabundans* (open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

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 [42,43]. 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% [9]), 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 Fig. 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 [40] 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 and 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. [41] noted a slight effect on protein yield when the TLL varied from 20 to 33%, while Patil and Raghavarao [42] observed an increase in protein yield from 90 to 97% when the TLL changed from 13 to 33%. In

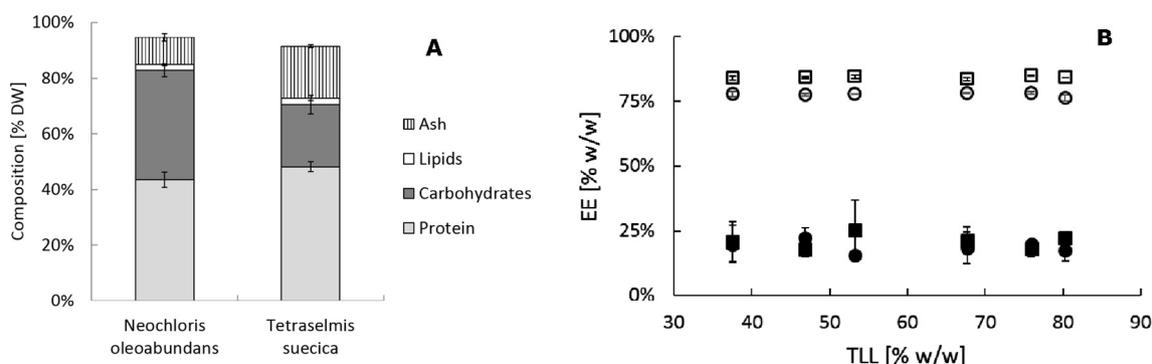


Fig. 3. (A) Overall composition (% dw) of crude protein extract from *T. suecica* and *N. oleoabundans*. Error bars indicate standard deviation. Experiments run in duplicate. (B) Extraction efficiencies for proteins (open symbols) and free glucose (filled symbols) for *T. suecica* (circles) and *N. oleoabundans* (squares).

both cases, c-phycoyanin was extracted from *Spirulina platensis* in a system containing Polyethylene glycol and phosphate. Suarez Ruiz et al. [33] found a small increase in *EE* for Rubisco (91–98%) in the system Iol-Cit for TLL in the range 37–80%.

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 Fig. 3A. The protein content reached 43.5 ± 2.7 and $48.1 \pm 1.8\%$ (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 [44]. As expected, a higher value of ash was observed for the marine strain *T. suecica* ($18.5 \pm 0.5\%$ dw) compared to *N. oleoabundans*, which was grown in fresh water. A maximum lipid content of $\sim 2\%$ dw was found for both strains; these are probably prosthetic groups (e.g., porphyrins) and lipoproteins [45].

The carbohydrates content in the CP is notably high, in particular for *N. oleoabundans* ($\sim 40\%$ 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 [46] measured the sugar composition of 16 species of microalgae including the classes *Chlorophyceae* and *Prasinophyceae*, finding that glucose accounts for 55–85.3% of the total carbohydrates content. The fraction of free glucose to total carbohydrates in the CP is $21.49 \pm 0.55\%$ and $36.47 \pm 1.28\%$ for *N. oleoabundans* and *T. suecica* respectively.

The corresponding extraction efficiencies (*EE*) for total protein and free glucose, according to Eq. (2), are presented in Fig. 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 [8]; 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.

3.2. 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. [9] studied the stability and activity of Rubisco in Iolilyte 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 Fig. 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.

3.3. Protein partitioning estimations

The corresponding estimations of protein partitioning with the NRTL model are given in Fig. 2A for the reference proteins and in Fig. 2B for the microalgae CP. The respective interaction parameters are listed in Table 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 [12]. 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.

3.4. 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

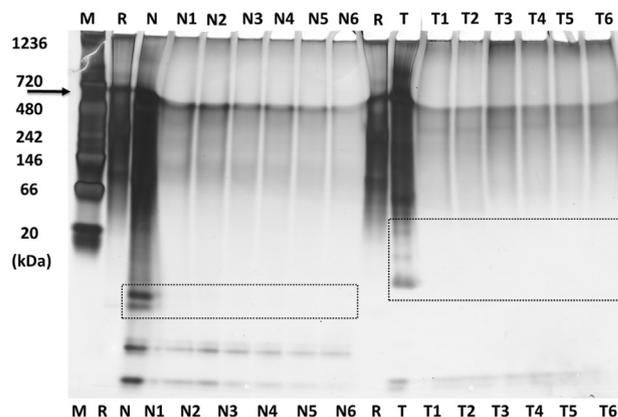


Fig. 4. Native gel electrophoresis for crude protein (CP) from *N. oleoabundans* (N) and *T. suecica* (T). M: protein marker. R: Rubisco (arrow at ~ 540 kDa). Numbers 1–6 refer to tie lines. Wells N and T refer to CP in water. Dotted squares indicate region in which protein bands are lost.

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 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.

4. 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 its flexibility and applicability in algae biorefinery.

Acknowledgements

This project was financed by the Dutch Technology Foundation TTW (former STW) under the project AlgaePro4You, nr. 12635 and within the TKI AlgaeParc Biorefinery program with financial support from the Ministry of Economic Affairs of the Netherlands in the framework of the TKI BioBased Economy under contract nr. TKIBE01009.

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:

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

where:

$$G_{ij} = \exp(-\alpha_{ij} \tau_{ij}) \quad (A1)$$

$$\tau_{ij} = \frac{g_{ij} - g_{jj}}{RT} = \frac{\Delta g_{ij}}{RT} \quad (A2)$$

Here, R is the universal gas constant ($\text{J mol}^{-1} \text{K}^{-1}$) 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 [28]. 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:

$$\ln \gamma_i = \frac{\sum_j x_j G_{ji} \tau_{ji}}{\sum_k x_k G_{ki}} + \sum_j \frac{x_j G_{ij}}{\sum_k x_k G_{kj}} \left(\tau_{ij} - \frac{\sum_m x_m G_{mj} \tau_{mj}}{\sum_k x_k G_{kj}} \right) \quad (A3)$$

Liquid-liquid equilibrium. The equilibrium condition implies equality in the chemical potentials of both phases and minimal Gibbs free energy [34]. For a two-phase system and n components:

$$a_i^T = a_i^B \quad (A4)$$

$$a_i = \gamma_i x_i \quad (A5)$$

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:

$$(A) \quad (A6)$$

If the molar compositions of both phases are known, Eqs. (A1)-(A4) 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 Appendices B and C.

Appendix B. Computational algorithms

The solution method presented in this research is adopted from Haghtalab and Paraj [35] and involves the estimation of the interaction parameters starting from known experimental compositions (Fig. 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

$$OF_1 = \sum_l^6 \sum_i^3 [(x_{li} \gamma_{li}^T - (x_{li} \gamma_{li}^B)^2] + Q \sum_k^6 \tau_k^2 \quad (B1)$$

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 [35]. 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.

$$OF_2 = \sqrt{\frac{1}{6N} \sum_l^N \sum_i^3 \sum_k^2 (x_{ik}^{calc} - x_{ik}^{exp})^2} \quad (B2)$$

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} .

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 Fig. 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 .

$$OF_3 = \left(\sum_{l=1}^6 \frac{(k_{p,exp} - k_{p,calc})^2}{6} \right)^{1/2} \quad (B3)$$

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. [12]. The criterion for minimization (ϵ) was set to 10^{-6} .

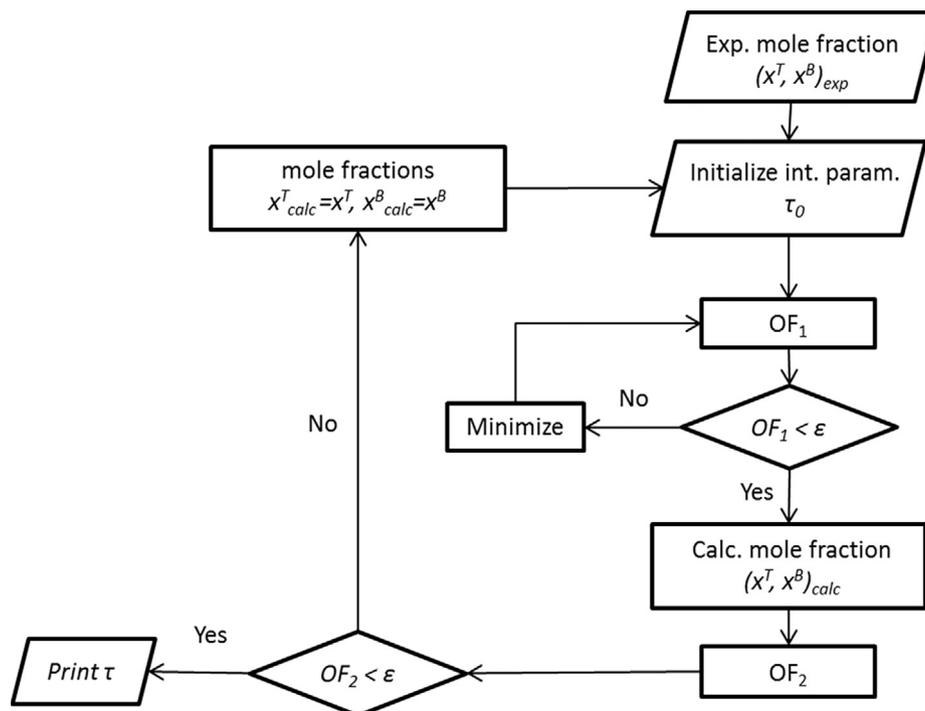


Fig. B1. Solution algorithm proposed by Haghtalab et al. [35] for estimating liquid-liquid interaction parameters.

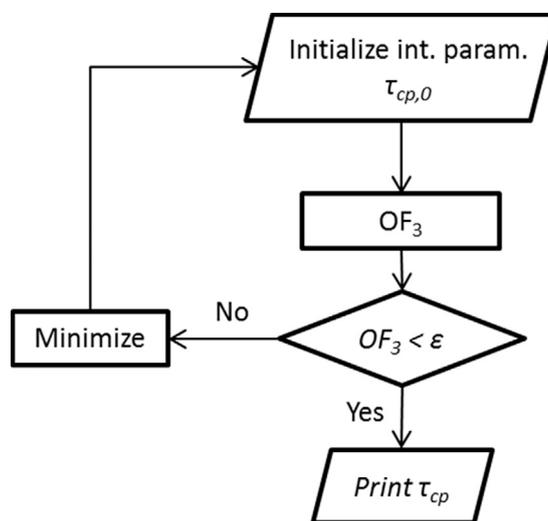


Fig. B2. Computational algorithm for estimating interaction parameters of crude proteins (CP).

References

- R.B. Draaisma, R.H. Wijffels, P.M. Slegers, L.B. Brentner, A. Roy, M.J. Barbosa, Food commodities from microalgae, *Curr. Opin. Biotechnol.* 24 (2013) 169–177, <http://dx.doi.org/10.1016/j.copbio.2012.09.012>.
- K. Samarakoon, Y.J. Jeon, Bio-functionalities of proteins derived from marine algae – a review, *Food Res. Int.* 48 (2012) 948–960, <http://dx.doi.org/10.1016/j.foodres.2012.03.013>.
- I.S. Chronakis, A.N. Galatanu, T. Nylander, B. Lindman, The behaviour of protein preparations from blue-green algae (*Spirulina platensis* strain Pacifica) at the air/water interface, *Colloids Surfaces A Physicochem. Eng. Asp.* 173 (2000) 181–192, [http://dx.doi.org/10.1016/S0927-7757\(00\)00548-3](http://dx.doi.org/10.1016/S0927-7757(00)00548-3).
- A.L. Lupatini, L.M. Colla, C. Canan, E. Colla, Potential application of microalga *Spirulina platensis* as a protein source, *J. Sci. Food Agric.* 97 (2017), <http://dx.doi.org/10.1002/jsfa.7987>.
- P.A.J. Rosa, A.M. Azevedo, S. Sommerfeld, W. Bäcker, M.R. Aires-Barros, Aqueous two-phase extraction as a platform in the biomanufacturing industry: economical and environmental sustainability, *Biotechnol. Adv.* 29 (2011) 559–567, <http://dx.doi.org/10.1016/j.biotechadv.2011.03.006>.
- A. Glyk, T. Scheper, S. Beutel, PEG – salt aqueous two-phase systems: an attractive and versatile liquid-liquid extraction technology for the downstream processing of proteins and enzymes, (2015) 6599–6616. doi: 10.1007/s00253-015-6779-7.
- S.P.M. Ventura, F.A. e Silva, M. V. Quental, D. Mondal, M.G. Freire, J.A.P. Coutinho, Ionic-liquid-mediated extraction and separation processes for bioactive compounds: Past, present, and future trends, *Chem. Rev.* (2017) *acs.chemrev*. 6b00550. doi:10.1021/acs.chemrev.6b00550.
- S.Y. Lee, I. Khoiroh, C.W. Ooi, T.C. Ling, P.L. Show, Recent advances in protein extraction using ionic liquid-based aqueous two-phase systems, *Sep. Purif. Rev.* (2017) 1–14, <http://dx.doi.org/10.1080/15422119.2017.1279628>.
- R.K. Desai, M. Streefland, R.H. Wijffels, M.H.M. Eppink, Extraction and stability of selected proteins in ionic liquid based aqueous two phase systems, *Green Chem.* 16 (2014) 2670, <http://dx.doi.org/10.1039/C3GC42631A>.
- S. Dreyer, P. Salim, U. Kragl, Driving forces of protein partitioning in an ionic liquid-based aqueous two-phase system, *Biochem. Eng. J.* 46 (2009) 176–185, <http://dx.doi.org/10.1016/j.bej.2009.05.005>.
- Y.-T. Wu, D.-Q. Lin, Z.-Q. Zhu, Thermodynamics of aqueous two-phase systems—the effect of polymer molecular weight on liquid–liquid equilibrium phase diagrams by the modified NRTL model, *Fluid Phase Equilib.* 147 (1998) 25–43, [http://dx.doi.org/10.1016/S0378-3812\(98\)00249-0](http://dx.doi.org/10.1016/S0378-3812(98)00249-0).
- B. Perez, L.P. Malpiedi, G. Tubío, B. Nerli, P. de Alcántara, Pessôa Filho, Experimental determination and thermodynamic modeling of phase equilibrium and protein partitioning in aqueous two-phase systems containing biodegradable salts, *J. Chem. Thermodyn.* 56 (2013) 136–143, <http://dx.doi.org/10.1016/j.jct.2012.07.017>.
- L.D. Simoni, Y. Lin, J.F. Brennecke, M.A. Stadtherr, Modeling liquid-liquid equilibrium of ionic liquid systems with NRTL, electrolyte-NRTL, and UNIQUAC, *Ind. Eng. Chem. Res.* 47 (2008) 256–272, <http://dx.doi.org/10.1021/ie070956j>.
- P.P. Madeira, X. Xu, Prediction of protein partition in polymer/salt aqueous two-phase systems using the modified Wilson model, *Biochem. Eng. J.* 24 (2005) 147–155, <http://dx.doi.org/10.1016/j.bej.2005.02.004>.
- D.Q. Lin, Y.T. Wu, L.H. Mei, Z.Q. Zhu, S.J. Yao, Modeling the protein partitioning in aqueous polymer two-phase systems: influence of polymer concentration and molecular weight, *Chem. Eng. Sci.* 58 (2003) 2963–2972, [http://dx.doi.org/10.1016/S0009-2509\(03\)00167-2](http://dx.doi.org/10.1016/S0009-2509(03)00167-2).
- A.D. Diamond, J.T. Hsu, Protein partitioning in PEG/dextran aqueous two-phase systems, *AIChE J.* 36 (1990) 1017–1024, <http://dx.doi.org/10.1002/aic.690360707>.
- H. Hartounian, E.W. Kaler, S.I. Sandler, Aqueous two-phase systems. 2. Protein partitioning, *Ind. Eng. Chem. Res.* 33 (1994) 2294–2300, <http://dx.doi.org/10.1021/ie00034a007>.
- Z. Du, Y.L. Yu, J.H. Wang, Extraction of proteins from biological fluids by use of an ionic liquid/aqueous two-phase system, *Chem. – A Eur. J.* 13 (2007) 2130–2137, <http://dx.doi.org/10.1002/chem.200601234>.
- X. Luo, P. Smith, C.L. Raston, W. Zhang, Vortex fluidic device-intensified aqueous two phase extraction of C-phycoerythrin from *Spirulina maxima*, *ACS Sustain. Chem. Eng.* 4 (2016) 3905–3911, <http://dx.doi.org/10.1021/acssuschemeng.6b00756>.
- J. Benavides, M. Rito-Palomares, Simplified two-stage method to B-phycoerythrin recovery from *Porphyridium cruentum*, *J. Chromatogr. B Anal. Technol. Biomed. Life Sci.* 844 (2006) 39–44, <http://dx.doi.org/10.1016/j.jchromb.2006.06.029>.
- A.G. Waghmare, M.K. Salve, J.G. LeBlanc, S.S. Arya, Concentration and characterization of microalgae proteins from *Chlorella pyrenoidosa*, *Bioresour. Bioprocess.* 3 (2016) 16, <http://dx.doi.org/10.1186/s40643-016-0094-8>.
- W.N. Phong, C.F. Le, P.L. Show, J.-S. Chang, T.C. Ling, Extractive disruption process integration using ultrasonication and an aqueous two-phase system for protein recovery from *Chlorella sorokiniana*, *Eng. Life Sci.* 17 (2017), <http://dx.doi.org/10.1002/elsc.201600133>.
- 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, <http://dx.doi.org/10.1016/j.bej.2017.04.009>.
- V.C.A. Orr, N.V. Plechkova, K.R. Seddon, L. Rehmman, Disruption and wet extraction of the microalgae *Chlorella vulgaris* using room-temperature ionic liquids, *ACS Sustain. Chem. Eng.* 4 (2016) 591–600, <http://dx.doi.org/10.1021/acssuschemeng.5b00967>.
- H.A. Abu Hajar, R.G. Riefler, B.J. Stuart, Cultivation of the microalga *Neochloris oleoabundans* for biofuels production and other industrial applications (a review), *Appl. Biochem. Microbiol.* 53 (2017) 640–653, <http://dx.doi.org/10.1134/S0003683817060096>.
- R.M. Gladue, J.E. Maxey, Microalgal feeds for aquaculture, *J. Appl. Phycol.* 6 (1994) 131–141, <http://dx.doi.org/10.1007/BF02186067>.
- A. Schwenzfeier, P.A. Wierenga, M.H.M. Eppink, H. Gruppen, Effect of charged polysaccharides on the techno-functional properties of fractions obtained from algae soluble protein isolate, *Food Hydrocoll.* 35 (2014) 9–18, <http://dx.doi.org/10.1016/j.foodhyd.2013.07.019>.
- H. Renon, J.M. Prausnitz, Local compositions in thermodynamic excess functions for liquids mixtures, *AIChE J.* 14 (1968) 116–128.
- P.R. Postma, E. Suarez Garcia, C. Safi, K. Yonathan, G. Olivieri, M.J. Barbosa, R.H. Wijffels, M.H.M. Eppink, Energy efficient bead milling of microalgae: Effect of bead size on disintegration and release of proteins and carbohydrates, *Bioresour. Technol.* 224 (2016) 670–679, <http://dx.doi.org/10.1016/j.biortech.2016.11.071>.
- O. Lowry, N. Rosebrough, L. Farr, R. Randall, et al., Protein measurement with the folin phenol reagent, *J. Biol. Chem.* 193 (1951) 265–275.
- 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.
- J. Folch, M. Lees, G.H. Sloane, et al., A simple method for the isolation and purification of total lipides from animal tissues, *J. Biol. Chem.* 226 (1957) 497–509.
- C.A. Suarez Ruiz, C. van den Berg, R.H. Wijffels, M.H.M. Eppink, Rubisco separation using biocompatible aqueous two-phase systems, *Sep. Purif. Technol.* (2017) 1–8, <http://dx.doi.org/10.1016/j.seppur.2017.05.001>.
- F. Denes, P. Lang, Liquid – Liquid – Liquid Equilibrium, *Symp. A Q. J. Mod. Foreign Lit.* (2006) 877–890.
- A. Haghtalab, A. Paraj, Computation of liquid-liquid equilibrium of organic-ionic liquid systems using NRTL, UNIQUAC and NRTL-NRF models, *J. Mol. Liq.* 171 (2012) 43–49, <http://dx.doi.org/10.1016/j.molliq.2012.04.008>.
- A. Sinha, A. Singh, S. Kumar, S.K. Khare, Microbial mineralization of struvite: a

- promising process to overcome phosphate sequestering crisis, *Water Res.* 54 (2014) 33–43, <http://dx.doi.org/10.1016/j.watres.2014.01.039>.
- [37] A.H. Martin, M. Nieuwland, G.A.H. De Jong, Characterization of heat-set gels from RuBisCO in comparison to those from other proteins, *J. Agric. Food Chem.* 62 (2014) 10783–10791.
- [38] S.C. Silvério, O. Rodríguez, J.A. Teixeira, E.A. Macedo, The effect of salts on the liquid-liquid phase equilibria of PEG600 + salt aqueous two-phase systems, *J. Chem. Eng. Data.* 58 (2013) 3528–3535, <http://dx.doi.org/10.1021/je400825w>.
- [39] A. Hartman, G. Johansson, P. Albertsson, Partition of Proteins in a Three-Phase System 81 (1974) 75–81.
- [40] A. Schwenzfeier, P.A. Wierenga, H. Gruppen, Isolation and characterization of soluble protein from the green microalgae *Tetraselmis* sp, *Bioresour. Technol.* 102 (2011) 9121–9127, <http://dx.doi.org/10.1016/j.biortech.2011.07.046>.
- [41] L. Zhao, Y. Liang Peng, J. mei Gao, W. min Cai, Bioprocess intensification: An aqueous two-phase process for the purification of C-phycoyanin from dry *Spirulina platensis*, *Eur. Food Res. Technol.* 238 (2014) 451–457, <http://dx.doi.org/10.1007/s00217-013-2124-5>.
- [42] G. Patil, K.S.M.S. Raghavarao, Aqueous two phase extraction for purification of C-phycoyanin, *Biochem. Eng. J.* 34 (2007) 156–164, <http://dx.doi.org/10.1016/j.bej.2006.11.026>.
- [43] A. Boaglio, G. Bassani, G. Picó, B. Nerli, Features of the milk whey protein partitioning in polyethyleneglycol-sodium citrate aqueous two-phase systems with the goal of isolating human alpha-1 antitrypsin expressed in bovine milk, *J. Chromatogr. B Anal. Technol. Biomed. Life Sci.* 837 (2006) 18–23, <http://dx.doi.org/10.1016/j.jchromb.2006.03.049>.
- [44] 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, <http://dx.doi.org/10.1016/j.algal.2013.12.004>.
- [45] A. Schwenzfeier, F. Lech, P.A. Wierenga, M.H.M. Eppink, H. Gruppen, Foam properties of algae soluble protein isolate: effect of pH and ionic strength, *Food Hydrocoll.* 33 (2013) 111–117, <http://dx.doi.org/10.1016/j.foodhyd.2013.03.002>.
- [46] M.R. Brown, The amino-acid and sugar composition of 16 species of microalgae used in mariculture, 145 (1991) 79–99.