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Fractionation of *Isochrysis galbana* Proteins, Arabinans, and Glucans Using Ionic-Liquid-Based Aqueous Biphasic Systems

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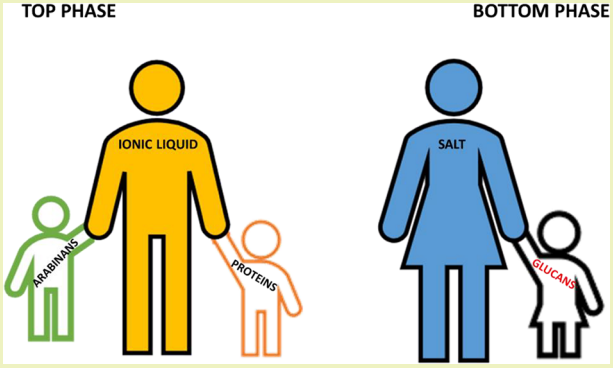
Supporting Information

ABSTRACT: *Isochrysis galbana* (*I. galbana*) is a microalgae species rich in biomolecules of high commercial value and industrial relevance. Despite the numerous studies done on the extraction of different compounds from microalgae, the complete valorization of the biomass under the biorefinery concept is scarce or even inexistent. The mixture of different compounds found in the different extracts obtained from the microalgae biomass is one of the most important drawbacks in the field. Despite the large interest of academia and industry in the different classes of bioactive compounds composing microalgae, these are still poorly explored due to the low efficiency of the downstream processes used up to date. In this context, this work proposes the development of an efficient purification process by applying ionic liquid (IL)-based aqueous biphasic systems (ABS) to separate proteins from arabinose- and glucose-rich polysaccharides. For this purpose, the nature of the inorganic salt, the IL structural features (anion and alkyl chain length) and the extraction point (using different inorganic salt concentrations) were the conditions optimized. After the proper selection of the most performant IL-ABS (%EE_{carb} = 71.21 ± 5.21% to the bottom phase and %EE_{prot} = 100% to the top phase), a complete downstream process was developed and implemented, in which the isolation of the target biomolecules (proteins, arabinose- and glucose-rich polysaccharides) and the reuse of the phase formers and main solvents applied was considered. In summary, the approach proposed using IL-ABS appears as a simple and efficient method of purification easily integrated into continuous flow processes, thus demonstrating its industrial potential.



KEYWORDS: *Isochrysis galbana, Arabinose- and glucose-rich polysaccharides, Proteins, Aqueous biphasic systems, Ionic liquids, Purification*

INTRODUCTION

Microalgal biomass has recently gained a relevant role as an alternative source of high-value bioactive compounds.[1,2] Examples of these biomolecules are the pigments, proteins, lipids, carbohydrates, and vitamins, with applications in cosmetic, nutritional, and food industries.[2−4] Due to their high commercial and industrial interest, the complete processing of this biomass is in huge demand. It is in this context that the concept of blue biorefinery applied to microalgae has gained industrial receptiveness as an emerging integrative concept to obtain the maximum product output and profit from a single raw material source while maintaining the minimal waste formation.[1,5,6] The microalgae biorefinery exploits microalgal biomass for the production of a vast range of bioproducts with commercial value by combining upstream and downstream processing as the main steps under the Blue Biorefinery concept.[7] *Isochrysis galbana* (*I. galbana*) is industrially produced for feedstock in aquaculture for the production of mollusks, fish, and crustaceans.[8] It is a very good example of marine biomass rich in water-soluble polysaccharides, which exhibit interesting bioactivities, as antioxidant,[9−11] antimicrobial,[9,12] and anti-inflammatory,[9,13] also presenting considerable amounts of proteins.

Traditionally, the downstream processes are those steps used to extract and purify the different classes of biomolecules composing any biomass.[14] Since these steps correspond to the most complex task on the production of each bioproduct, their economic impact is actually the most relevant on the entire process.[15] Moreover, the costs of the downstream process could increase even more when the objective is to separate a specific biomolecule from a very complex mixture if compounds with similar chemical structures and properties are present.[16]

The carbohydrates mainly composing microalgae are starch, cellulose, and different types of polysaccharides. These polysaccharides are recognized by their biological functions

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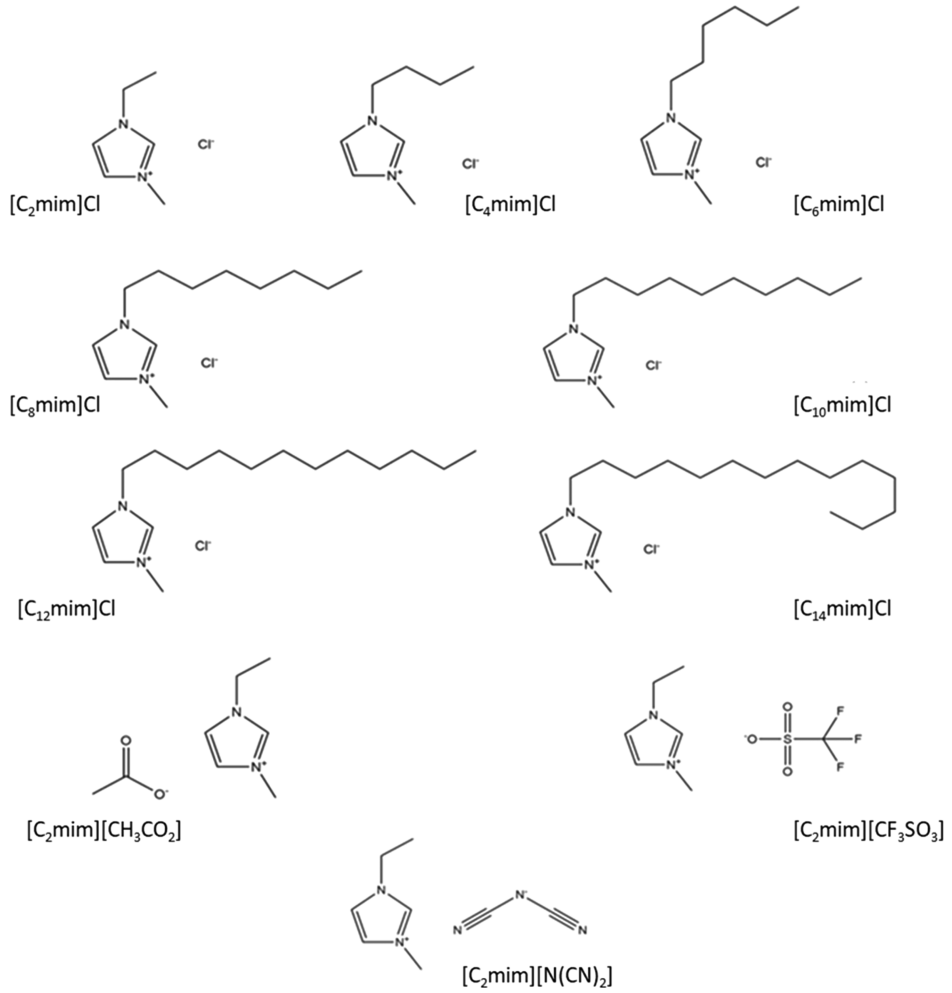

Figure 1. Chemical structures of the imidazolium-based ILs under study: $[C_2mim]Cl$; $[C_4mim]Cl$; $[C_6mim]Cl$; $[C_8mim]Cl$; $[C_{10}mim]Cl$; $[C_{12}mim]Cl$; $[C_{14}mim]Cl$; $[C_2mim][CH_3CO_2]$; $[C_2mim][CF_3SO_3]$; $[C_2mim][N(CN)_2]$.

as storage, protection and structural molecules.[4,17] Some of these polysaccharides could be responsible for the modulation of the immune system and inflammatory reactions, making them highly interesting as cosmetic additives, food ingredients, and natural therapeutic agents.[18,19] Proteins are also an important part of the main constituents of microalgae, comprising 50−70% of the microalgae composition, which has a relevant role in human and animal nutrition.[20] As previously discussed, the commercial impact of some specific polysaccharides is huge due to their main properties and potential applications. In this context, there is a huge demand for the development of efficient processes of purification of these biomolecules. One of the major associated drawbacks is the concomitant extraction of polysaccharides and proteins. Commonly, the conventional methodologies to obtain the polysaccharides involve acetone extraction and/or ethanol precipitation to remove lipids and pigments,[21,22] followed by different purification steps, namely, those based in filtration-based[18] and chromatographic-based methods, like ion-exchange chromatography (IEC)[21,22] and size-exclusion chromatography (SEC).[22] Most of the recovery methods employed to purify the polysaccharides are based in multiple purification steps.[23−25] Moreover, the biomass treatment with expensive proteolytic enzymes is being widely adopted to remove proteins from polysaccharide-rich extracts, avoiding their recovery and subsequent application.[18] More recently, some other approaches were reported,[26] namely, those using a sequential extraction by applying as solvents (i) supercritical carbon dioxide ($ScCO_2$), (ii) gas-expanded liquid ($SsCO_2$ and ethanol), (iii) pure ethanol, and (iv) pure water. However, despite the good effort on the development of a microalgae-based biorefinery, these processes are not very selective. If the authors were able to successfully obtain different bioproducts from this biomass, then these were always composed of at least two classes of compounds and sometimes more, e.g., carotenoids+nonpolar lipids; carotenoids+chlorophylls+middle- and highly polar lipids; and proteins+polysaccharides.[26] However, some of the most interesting and commercially viable applications of polysaccharides require pure extracts, thus imposing the elimination of the proteins. Proteins are traditionally being extracted from microalgae by solvent extraction,[27,28] filtration,[29] and again, supercritical carbon dioxide extraction.[30] Despite these efforts, the isolation of these biomolecules is still a challenge regarding their contamination with polysaccharides or even pigments.[29] Moreover, in addition to their low selectivity, the large-scale application of these methods is also limited by their complex operations, low yields, and high operational costs.[31,32] Additionally, the same problem of selectivity is found when the objective is the purification of a specific polysaccharide from microalgae, which has not been much explored up to now. Thus, the lack of profitable and scalable downstream

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processes for polysaccharides is currently the greatest obstacle to their industrial application as nutraceuticals and food additives,[33] and consequently in their commercialization.[34]

Aqueous biphasic systems (ABS) are one of the alternatives with high potential to be applied on the development of an efficient downstream process to separate polysaccharides from proteins and to isolate specific polysaccharides from a complex mixture.[35] ABS based in hydrophilic ionic liquids (ILs) as phase formers are a particular type of ABS, first developed by Rogers and co-workers.[36] Due to their high solvation capability, large hydrophilicity/hydrophobicity range, and "designer solvent" nature, ILs-based ABS are nowadays recognized as a very interesting tool for the fractionation of biomolecules. In fact, IL-based ABS possess multiple advantages, such as the absence of volatile organic solvents, negligible emulsion formation, minimal viscosity, fast phase separation, and mild and biocompatible environment (since some are mainly composed of water).[36−38] It was already demonstrated that IL-ABS can be used to separate proteins and carbohydrates. Pei et al.[39] were pioneers in the application of IL-ABS for the selective separation of proteins from a mixture contaminated with polysaccharides. They found that 82.7−100% of the protein, bovine serum albumin (BSA), migrated to the top phase, while the carbohydrates were preferentially concentrated in the bottom phase. These results demonstrated that IL-ABS were effective in the selective separation of proteins and carbohydrates,[39] and in this sense, further studies were carried out.[40−42] Nonetheless, to the best of our knowledge, the fractionation of polysaccharides and proteins and the isolation of specific polysaccharide structures have never been reported in a single step and using IL-based ABS.

In the present study, the application of IL-ABS, based on the methylimidazolium family ([$C_n$mim][X]) and inorganic salts for the development of a single-step fractionation process was performed. In this sense, two main objectives were pursued, namely, the (i) separation of polysaccharides from proteins, and the (ii) simultaneous fractionation of two distinct polysaccharides, the arabinan- and glucan-based. After process optimization and extract characterization, a simple process to fractionate proteins from arabinose- and glucose-rich polysaccharides was designed, in which the isolation of the bioactive compounds and the reuse of the main phase formers were envisioned.

MATERIALS AND METHODS

Materials. The lyophilized sample of *I. galbana* was provided by Necton S.A., packaged and transported under vacuum and darkness conditions.

For the degreasing process, the following reagents were used: chloroform ($CHCl_3$, purity 99.0%); methanol (CH_3OH, purity of 99.9%); ethanol (C_2H_5OH purchased from Merck) and acetone (C_3H_6O, purity of 99.9%). All reagents, except ethanol, were purchased at VWR Chemicals.

The inorganic salts used in the ABS formation were tripotassium phosphate (K_3PO_4, 98% of purity), dipotassium hydrogen phosphate (K_2HPO_4, ≥98% of purity), and the potassium phosphate buffer, consisting of a mixture of K_2HPO_4 and potassium dihydrogen phosphate, KH_2PO_4 (99.5% of purity). All salts were purchased from Sigma-Aldrich. The ILs used (Figure 1) were 1-ethyl-3-methylimidazolium chloride, [C_2mim]Cl (purity = 98%); 1-ethyl-3-methylimidazolium acetate, [C_2mim][CH_3CO_2] (purity = 98%); 1-ethyl-3-methylimidazolium dicyanamide, [C_2mim][$N(CN)_2$] (purity = 99%); 1-ethyl-3-methylimidazolium triflate, [C_2mim][CF_3SO_3] (purity = 99%); 1-butyl-3-methylimidazolium chloride, [C_4mim]Cl (purity = 99%); 1-hexyl-3-methylimidazolium chloride, [C_6mim]Cl (purity = 99%); 1-methyl-3-octylimidazolium chloride, [C_8mim]Cl (purity = 99%); 1-decyl-3-methylimidazolium chloride, [C_{10}mim]Cl (purity = 98%); 1-dodecyl-3-methylimidazolium chloride, [C_{12}mim]Cl (purity ≥ 98%); 1-tetradecyl-3-methylimidazolium chloride, [C_{14}mim]Cl (purity = 98%). All ILs were purchased from Iolitec.

The reagents required for the preparation of the Bradford reagent were the Coomassie brilliant blue G-250 (proteome purity acquired in Amresco); absolute ethanol (C_2H_6O, purity of 99.9%, Fisher Scientific); phosphoric acid (H_3PO_4, purity of 85%, Panreac); and bovine serum albumin (BSA) (purity ≥ 99.0%, Acros Organic). The reagents required for the phenol-sulfuric acid method include the phenol (C_6H_6O, purity ≥ 96%, Sigma-Aldrich) and sulfuric acid (H_2SO_4, purity > 95%, Fisher Chemical). Moreover, other reagents were used specifically for the quantification of the neutral sugars, namely the 2-deoxyglucose ($C_6H_{12}O_5$, purity ≥ 99%, Sigma-Aldrich); sodium borohydride ($NaBH_4$, purity of 98%, Riedel-de-Haën); acetic anhydride ($C_4H_6O_3$, purity ≥ 99%, Fluka); 1-methylimidazole ($C_4H_6N_2$, purity ≥ 99%, Sigma-Aldrich); and dichloromethane (CH_2Cl_2, purity of 99.9%, Sigma-Aldrich). The Bicinchoninic Acid Protein Assay (BCA) Kit was acquired at Sigma-Aldrich.

Included in the reagents required for the quantification of the uronic acids are the sodium borate ($Na_2B_4O_7$, 99% of purity, Riedel-de-Haën), m-phenylphenol ($C_{12}H_{10}O$, 85% of purity, Sigma-Aldrich), sodium hydroxide (NaOH, 98.8% of purity, Labchem), and galacturonic acid ($C_6H_{10}O_7$, purity ≥ 99%, Sigma-Aldrich). The reagents required for the quantification of sulfate groups were the barium chloride ($BaCl_2$, purity of 98%, Carlo Erba); trichloroacetic acid (CCl_3CO_2H, purity ≥ 99%, Sigma-Aldrich); and potassium sulfate (K_2SO_4, ≥ 99% of purity, Carlo Erba).

The reagents applied on the determination of the protein amino acid composition were the sodium phosphate buffer consisting of a mixture of disodium hydrogen phosphate (Na_2HPO_4) and sodium phosphate monobasic (NaH_2PO_4), both purchased from Sigma-Aldrich (99.5% of purity). Pronase lyophilized powder from *Streptomyces griseus* (activity ≥6,000 $U.g^{-1}$ lyophilizate) manufactured by Roche, purchased from Sigma-Aldrich, prolidase lyophilized powder from porcine kidney (activity ≥100 $U.mg^{-1}$ protein) L-norleucine (purity ≥ 98%), and pyridine (purity ≥ 99%), respectively purchased from Sigma-Aldrich, ethylchloroformate (purity ≥ 98%) purchased from Fluka, and anhydrous sodium sulfate (purity 99.2%) purchased from Labsolve.

Degreasing and Aqueous Extraction of Carbohydrates from *I. galbana*. The lyophilized *I. galbana* sample was treated with a chloroform:methanol (2:1) (v/v) mixture in the solid−liquid ratio of 1:4 (w/v), being homogenized using an Ultraturrax (IKA T25 basic with a dispersion tool S25N-18G) at 13500 rpm for 15 min.[22] Posteriorly, the mixture was stirred for 1 h, and placed on ice during 30 min. Then, it was centrifuged (Heraus Multifuge from Thermo scientific) at 15000 rpm, 0 °C for 30 min and the supernatant removed. The residue was treated again with the same solvent at the same ratio, stirred for 1 h and centrifuged under the conditions previously described. This step was repeated six times until no color was observed in the supernatant. Then, ethanol was added to the residue in a solid−liquid ratio of 1:2 (w/v), centrifuged at 15000 rpm, 0 °C for 30 min and the supernatant removed. Finally, acetone was used as the extraction solvent in the same conditions of ethanol. The defatted sample was air-dried, placed overnight in a vacuum oven with phosphorus pentoxide, and subsequently used for the aqueous extraction.

For the aqueous extraction of polysaccharides and proteins, water was added to the degreased sample in a solid−liquid ratio of 1:10 (w/v) and the mixture was then placed in a water bath at 65 °C for 3 h, according to the methodology described by Balavigneswaran et al.[10] Then, the mixture was cooled down, centrifuged at 15000 rpm, at 2 °C for 25 min and the supernatant obtained (aqueous extract rich in polysaccharides and proteins) used in the purification fractionation step.

Fractionation of Proteins and Different Polysaccharides by Applying IL-Based ABS. *Optimization Study.* ILs-based ABS were

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prepared gravimetrically ($\pm 10^{-4}$ g) by adding a specific amount of IL, inorganic salt and aqueous extract of *I. galbana* (obtained as detailed in section *Degreasing and aqueous extraction of carbohydrates from I. galbana*). The extraction mixture points adopted for each system analyzed, as well as the respective phase diagrams[35,43−46] used are shown in Table 1. In the study of the different conditions, and after

Table 1. IL-Based ABS Applied in the Optimization Studies Carried for the Purification of Polysaccharides Obtained from *I. galbana*[a]

Effects analyzed	Extraction point	Ref
(i) inorganic salt nature	IL: 15 wt %	43
(ii) IL′ anion	inorganic salt: 22 wt %	35
(iii) IL′ alkyl chain length	aqueous extract of *I. galbana*: 63 wt %	44, 45, and 46
(iv) mixture point (concentration of K_3PO_4) (for ABS based in [C_8mim]Cl + K_3PO_4)	IL: 15 wt %	46
	inorganic salt: 20, 22, 25, and 27.5 wt %	
	aqueous extract of *I. galbana*: 57.5, 60, 63, and 65 wt %	45

[a]Respective references presenting the phase diagrams used in this work are also presented, as well as the main effects evaluated.

the mixture of all components, each system was agitated to allow the contact between the phase formers and the aqueous extract of *I. galbana*. To reach the thermodynamic equilibrium, each system was then centrifuged at 10,000 rpm for 10 min in a VWR Microstar 17 centrifuge. Then, the phases were separated carefully using syringes. For all systems tested, the bottom phase corresponded to the inorganic salt-rich phase, while the IL-rich layer was defined as being the top one. The total protein and the total carbohydrate concentrations were determined by the Bradford[47] and phenol-sulfuric acid[48] methods, respectively.

The evaluation of the protein and carbohydrates partition for the top and bottom phases was carried by the extraction efficiency (%EE) parameter determined for proteins (eq 1) and carbohydrates (eq 2), respectively.

$$EE_{prot}\ (\%) = \frac{[prot]_{top} \times V_{top}}{[prot]_{extr} \times V_{extr}} \quad (1)$$

$$EE_{carb}\ (\%) = \frac{[carb]_{bot} \times V_{bot}}{[carb]_{extr} \times V_{extr}} \quad (2)$$

where $[prot]_{top}$ and $[carb]_{bot}$ correspond to the total protein and polysaccharides concentration in top and bottom-phases, respectively. $[prot]_{extr}$ and $[carb]_{extr}$ represent, respectively, the total protein and carbohydrates amount present in the initial aqueous extract. The volume of both top and bottom phases is represented as V_{top} and V_{bot}, and the volume of the initial aqueous extract corresponds to V_{extr}.

The mass balances for all the ABS evaluated in this work were also calculated for proteins and carbohydrates, through the following:

$$MB_{prot}(\%) = \frac{([prot]_{top} \times V_{top}) + ([prot]_{bot} \times V_{bot})}{[prot]_{extr} \times V_{extr}} \quad (3)$$

$$MB_{carb}(\%) = \frac{([carb]_{top} \times V_{top}) + ([carb]_{bot} \times V_{bot})}{[carb]_{extr} \times V_{extr}} \quad (4)$$

Isolation of Proteins and Polysaccharides. To further characterize the polysaccharides and protein content after purification, the IL-ABS composed of 15 wt % [C_6mim]Cl + 20 wt % K_3PO_4 + 65 wt % H_2O and 15 wt % [C_8mim]Cl + 20 wt % K_3PO_4 + 65 wt % H_2O were used. After the purification by applying IL-based ABS, both top and bottom phases were carefully separated and the pH of each adjusted to 6.5 with acetic acid to precipitate the inorganic salt in the form of potassium acetate. Both fractions were individually dialyzed (cutoff 12−14 kDa) against distilled water aiming at to remove the inorganic salt, IL and other low molecular weight molecules. After dialysis, the material with a molecular weight higher than 12−14 kDa (called high molecular weight material) retained into the dialysis membrane for both top and bottom phase was lyophilized. Absolute ethanol was added to the Top-phase in a concentration of 30 wt % to separate proteins from arabinose-rich polysaccharides, through selective protein precipitation. The mixture was centrifuged at 3500 rpm, 20 min and the Top-phase Supernatant (Sn Top-phase) and the Top-phase Precipitate (Pp Top-phase) were separated. The ethanol was evaporated and subsequently, both fractions were dialyzed and lyophilized as previously described.

Polysaccharides Analysis. *Total Carbohydrate Quantification by the Phenol−Sulfuric Acid Method.* The total carbohydrate content was accomplished through the phenol-sulfuric acid method.[48] It was started by pipetting 80 μL of each phase (top and bottom), 150 μL of 5% (w/v) of an aqueous phenol solution and 1 mL of concentrated sulfuric acid into a long test tube and then stirred. The test tubes were placed for 10 min in a water bath at 100 °C. The absorbance of the yellow-orange color was measured at 490 nm using the Biotek Eon spectrophotometer. A calibration curve (0.05−0.3 $mg.mL^{-1}$) was previously established using glucose as the predominant sugar in the microalgae under study.[49]

Determination of Neutral Sugars Composition. The neutral sugars were determined as alditol acetates by GC-FID.[50] Briefly, the samples were prehydrolyzed with 72 wt% of sulfuric acid (H_2SO_4) for 3 h at room temperature and, then, glycosidic linkages were hydrolyzed with sulfuric acid 1 M, at 100 °C during 2.5 h. The 2-deoxyglucose was used as the internal standard for quantification. The monosaccharides obtained after hydrolysis were reduced with sodium borohydride (15% (w/v) in NH_3 3M) at 30 °C during 1 h and acetylated with acetic anhydride and 1-methylimidazole, during 30 min at 30 °C. The alditol acetates prepared were injected into a PerkinElmer-Claurus 400 GC-FID, equipped with a DB-225 column (30 m longer, 0.25 mm diameter, and 0.5 μm of thickness). The temperature program used was the following: initial temperature of 200 °C, increasing at 40 $°C.min^{-1}$ to 220 °C, holding for 7 min. Then, the temperature rises 20 $°C.min^{-1}$ to 230 °C, holding for 1 min. The temperatures of the injector and detector were 220 and 230 °C, respectively. The carrier gas used was H_2 at a flow rate of 1.7 $mL.min^{-1}$.

Determination of Uronic Acids Content. The uronic acids content was determined by the modified colorimetric method described by Blumenkrantz et al.[51] and Coimbra et al.[52] Briefly, the samples were prepared by prehydrolysis in 72 wt% of H_2SO_4 for 3 h at room temperature, followed by a 1 h hydrolysis with an H_2SO_4 solution 1 M at 100 °C. After hydrolysis, 3 mL of 50 mM boric acid (prepared in concentrated H_2SO_4) was added to three tubes with the hydrolyzed sample, stirred and placed for 10 min in a water bath at 100 °C. Thereafter, upon cooling, 0.1 mL of 3-phenylphenol (0.15% w/v in 0.5% w/v NaOH) was added to two of the three test tubes, stirred and left in the absence of light for 30 min. Finally, the absorbance was measured at 520 nm using the Biotek Eon spectrophotometer and the uronic acid estimation was made using a D-galacturonic acid calibration curve (4−40 $mg.mL^{-1}$).

Determination of Sulfate Content. The sulfate content of the fractions was determined by turbidimetry. This experiment was carried out according to the method described by Oliveira et al.[53] The ester sulfate groups were hydrolyzed with 1 M of HCl, at 110 °C for 5 h. Then, 200 μL of hydrolysate was added to 3.8 mL of 3% (w/v) of CCl_3CO_2H and 1 mL of $BaCl_2$-gelatin reagent (0.5 g of $BaCl_2$ in 100 mL of gelatin solution). After reacting for 15 min, the $BaCl_2$ suspension absorbance was measured at 360 nm using the Biotek Eon spectrophotometer. Determination of sulfate was achieved using a calibration curve (20−300 $mg.mL^{-1}$) of K_2SO_4 standard solutions. Three replicates of each concentration and each sample were carried out, except for the Bottom-phase fraction, due to limitations on the amount of sample.

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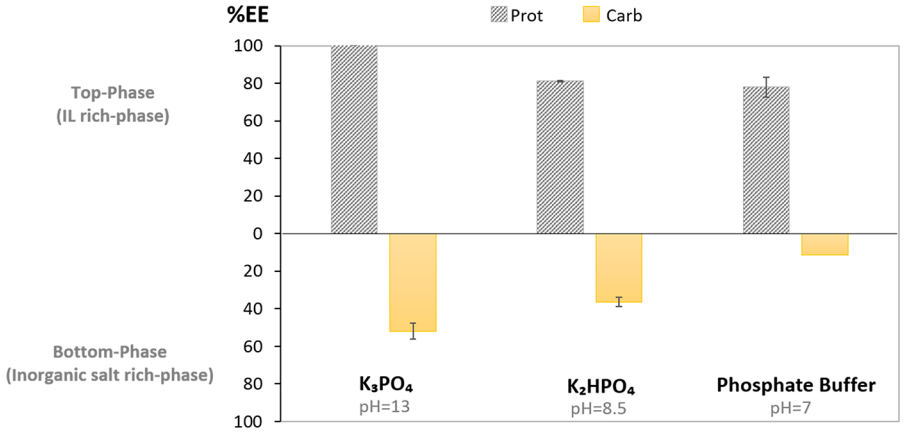

Figure 2. Effect of the inorganic salt on the extraction efficiency (%EE) of polysaccharides (yellow bars) and proteins (gray bars). The IL-ABS composed of 15 wt % $[C_2mim]Cl$ + 22 wt % inorganic salt + 63 wt % aqueous extract of *I. galbana* at room temperature was applied. The experimental results of pH measured for each system are also shown.

Protein Analysis. *Total Protein Quantification by Bradford Method.* The total protein content was determined for the top and bottom phases by the Bradford method.[47] To prepare the Bradford reagent, 0.1 g of Coomassie brilliant blue G-250 was dissolved in 50 mL of 95% (v/v) of ethanol, 100 mL of 85% (w/v) of phosphoric acid and 850 mL of distilled water. Briefly, 100 μL of each aqueous phase were pipetted and 5 mL of Bradford's reagent were added. After vortexing, the absorbance at 595 nm was measured using the SinergyHT spectrophotometer. A calibration curve (0.1−1 $mg.mL^{-1}$) was previously established using the protein bovine serum albumin (BSA) as standard. Three replicates of each concentration and each sample were carried. For the longest alkyl chain ILs ($[C_{10}mim]Cl$-based ABS), the top phases were diluted to eliminate quantification errors promoted by the interference of the IL presence.

Total Protein Quantification by Bicinchoninic Acid Method. The total protein quantification was based on the bicinchoninic acid (BCA) method using bovine serum albumin (BSA) as standard, and the Bicinchoninic Acid Protein Assay Kit. The Sn Top-phase and Pp Top-phase fractions (the only described as having protein) were incubated at 37 °C for 30 min. The absorbance was measured at 562 nm using the Biotek Eon spectrophotometer. Three replicates of each concentration and each sample were done.

Determination of Amino Acid Composition. The amino acid composition was determined solubilizing the samples in 1 mL of sodium phosphate buffer (50 mM and pH 7.0), containing 0.06% (w/v) of sodium azide and 10 μL of Pronase (10 $mg.mL^{-1}$) and incubated at 37 °C for 24 h with stirring.[54] After, 10 μL of Prolidase (1 $mg.mL^{-1}$) were added and incubated at 37 °C for 2 h. The amino acids obtained after enzymatic hydrolysis were subsequently derivatized according to the method described by Qiu et al.[55] and analyzed by GC-MS. Briefly, to 600 μL of amino acid solution, 800 μL of absolute ethanol and 150 μL of the internal standard L-norleucine (12 $\mu g.mL^{-1}$) were added, followed by the addition of 200 μL of pyridine and 100 μL of ethyl chloroformate. The mixture was sonicated for 60 s at 20 °C and after, 600 μL of chloroform was added followed by 200 μL of 7 M of NaOH and 100 μL of ethyl chloroformate. The phases were vortexed for 1 min and the phase separation was performed by centrifugation for 3 min at 3000 rpm. The aqueous phase was removed and the organic phase was dried by adding 100 mg of anhydrous sodium sulfate. Three replicates of each sample were performed. The organic phase was analyzed by GC-MS (Shimadzu QP-2010) using a nonpolar column DB1 (30 m length, 0.25 cm internal diameter and 0.25 μm of stationary phase thickness), by injection of 1 μL in split mode (split ratio 2.0) with the injector temperature of 260 °C. The initial column temperature was 70 °C increasing to 260 °C at 10 $°C.min^{-1}$ and then to 300 °C at a rate of 20 $°C.min^{-1}$. The carrier gas (helium) was maintained at a constant flow rate of 1.51 $mL.min^{-1}$. The transfer line temperature was 300 °C and the temperature of the ionization source was 260 °C. Mass spectra were acquired in the full-scan mode (45−550 m/z) after ionization by electron impact with 70 eV.

Elemental Analysis. The elemental analysis was made for the nitrogen atom quantification by thermal conductivity, using the Truspec 630−200−200 equipment. The nitrogen-protein conversion factor (4.59) calculated by Lourenço et al.[56] for *I. galbana* was used to convert the percentage of nitrogen atoms into protein percentage.

COSMO-RS Simulation Data. The energy value of hydrogen-bonding interaction energy, E_{HB} (kJ mol^{-1}) of the 1-ethyl-3-methylimidazolium-based $[C_2mim]$-based ILs was calculated using the COSMO thermodynamic model (COnductor-like Screening MOdel for Real Solvents).[57] The COSMO calculations were performed on an ideal conductor, which means that the molecules are assumed to be surrounded by a virtual conductor environment, and interactions are entirely made at the conductor interface, considering the electrostatic shielding and polarization of the solute molecule.

RESULTS AND DISCUSSION

Screening of IL-Based ABS on the Separation of Polysaccharides and Proteins. This work has as main aim the development of a fractionation process to separate efficiently proteins from the polysaccharides extracted from the *I. galbana*, by using ILs-salts-based ABS.[58] Thus, the purification optimization is needed aiming to select the ABS with the highest selectivity, i.e. the system allowing the (i) concentration of proteins and polysaccharides in opposite phases, and (ii) the efficient separation of different types of polysaccharides. The mass balances were determined for all the ABS studied for both proteins and carbohydrates (Table S1 from ESI).

Effect of Inorganic Salt Type. The effect of inorganic salt type in the partition of polysaccharides and proteins is an important parameter to take into consideration. All the inorganic salts tested (K_3PO_4, K_2HPO_4 and K_2HPO_4/KH_2PO_4 buffer) formed ABS with $[C_2mim]Cl$. It should be noted that the K_2HPO4/KH_2PO_4 buffer, originally prepared at pH 6.6 was not able to form ABS as previously shown,[41] so its adjustment at pH 7.0 was carried to guarantee the two-phase formation.

The effect of the inorganic salt nature on the carbohydrates and proteins partition is presented in Figure 2, considering the extraction efficiency parameters, $\%EE_{prot}$ and $\%EE_{carb}$, for the top (IL)-rich and bottom (salt)-rich aqueous phases, respectively. The results indicate an increase of $\%EE_{prot}$ and

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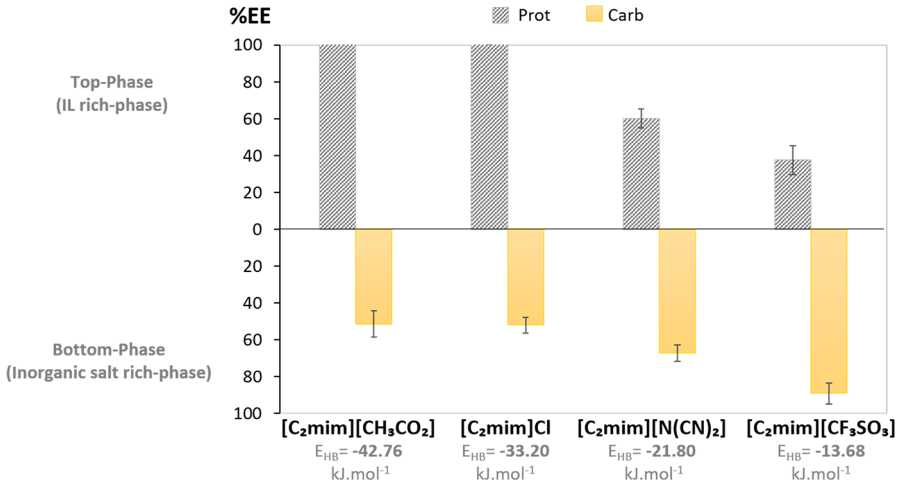

Figure 3. Effect of the IL anion on the extraction efficiency (%EE) of polysaccharides (yellow bars) and proteins (gray bars). The IL-ABS composed of 15 wt % $[C_2mim]X$ + 22 wt % K_3PO_4 + 63 wt % aqueous extract of *I. galbana* at room temperature was applied. The values of the E_{HB} parameter (kJ mol^{-1}) calculated by COSMOS-RS are also presented.

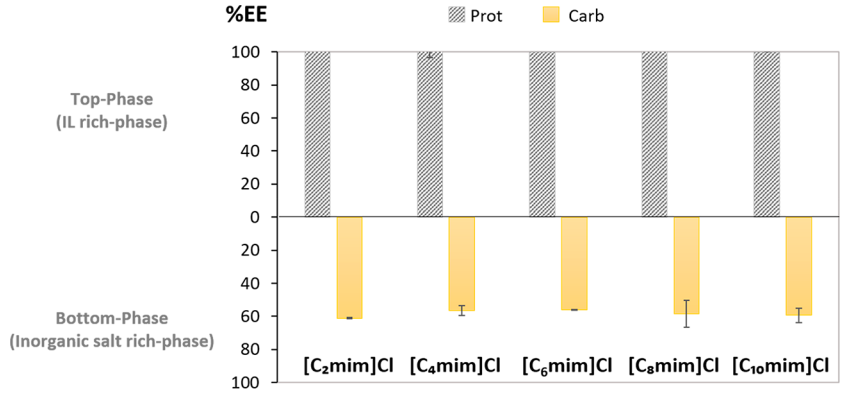

Figure 4. Effect of the alkyl side chain length of imidazolium cation on the extraction efficiency of polysaccharides (yellow bars) and proteins (gray bars). The IL-ABS composed of 15 wt % $[C_nmim]Cl$ + 22 wt % K_3PO_4 + 63 wt % aqueous extract of *I. galbana* at room temperature was applied.

$\%EE_{carb}$, for opposite phases, in the following crescent order: K_2HPO_4/KH_2PO_4 buffer < K_2HPO_4 < K_3PO_4. From the gathered data, the highest selectivity in the preferential separation of carbohydrates from proteins was found by using the IL-ABS composed of $[C_2mim]Cl$ + K_3PO_4 ($\%EE_{prot}$ = 100% and $\%EE_{carb}$ = 61.1 ± 0.4%, respectively, for the top and bottom phases). In these systems, not only the inorganic salt is different but also the pH associated with each system varies (Figure 2). The preferential migration of both classes of compounds is favored for opposite phases when systems with high pH values are applied. In alkaline media (pH 13) the intramolecular hydrogen bonds of the polysaccharides are partially destroyed so that they have free hydroxyl groups,[41] probably allowing preferential interactions between these free −OH groups and the more hydrophilic phase constituted by the aqueous inorganic salt.[59] For the remaining systems, as the pH is lower than 13, the polysaccharides are partitioned equally (K_2HPO_4, pH 9) or mostly for the top phase (K_2HPO_4/KH_2PO_4, pH 7).

Contrarily to what happens with the polysaccharides, the proteins are mainly migrating toward the IL-rich phase ($\%EE_{prot}$ = 81.16 ± 0.32% using K_3PO_4-based ABS), a common behavior found also for other systems.[60,61] Actually, this trend is normally justified by the salting-out nature[62] of the inorganic salt used and expelling the proteins for the opposite phase (rich in IL) or by the $\pi-\pi$ interactions between the IL cation aromatic ring and the aromatic amino acids of the proteins.[41]

Therefore, the ABS based in K_3PO_4 was selected as the most selective and thus applied for further optimizations.

Effect of IL Structural Features: Anion and Alkyl Chain Length. Despite the good selectivity results obtained by the ABS based in K_3PO_4, this parameter could be improved to achieve the maximum of recovering. Considering this, other conditions, such as ILs with different anions and alkyl chain lengths, were studied. Starting with the anion, the acetate $[CH_3O_2]^-$, chloride Cl^-, dicyanamide $[N(CN)_2]^-$ and triflate $[CF_3SO_3]^-$ were selected and their impact upon the partition of polysaccharides and proteins were evaluated (Figure 3). All ILs tested formed ABS with the inorganic salt K_3PO_4 at the extraction point studied. In general, the results of $\%EE_{prot}$ and $\%EE_{carb}$ depicted in Figure 3 indicate a dependency of the partition of both proteins and polysaccharides with the IL' anions tested. This behavior indicates that the hydrophobic/hydrophilic nature of the anions has an important role and that distinct interactions are taking place between the proteins, polysaccharides, and both phases, justifying the data. The hydrophilic/hydrophobic character of an IL can be analyzed by the hydrogen-bonding interaction energy (E_{HB}) parameter calculated by the COSMO-RS thermodynamic model.[62] In this sense, it seems that the anions with different E_{HB} values

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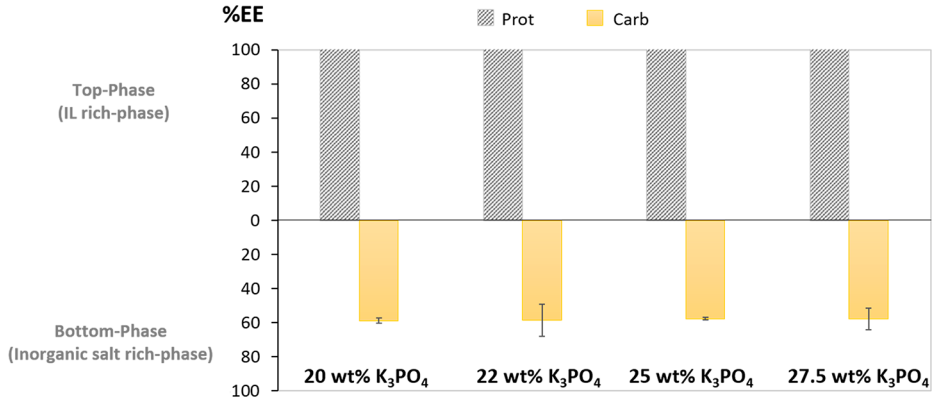

Figure 5. Effect of inorganic salt concentration to extraction efficiency of polysaccharides (yellow bars) and proteins (gray bars). IL-ABS composed of 15 wt % $[C_8mim]Cl$ + 20, 22, 25, and 27.5 wt % K_3PO_4 + 65, 63, 60, and 57.5 wt % aqueous extract of *I. galbana* at room temperature were applied.

(Figure 3) have different capacity to establish hydrogen bonds with the surrounding environment. Actually, the higher the E_{HB} the higher/strongest the IL cation−anion hydrogen bond interactions[63] and, consequently, the lower/weakest the IL cation−anion interactions with the surrounding media. In this sense, relatively low E_{HB} values (−42.76 and −33.20 kJ mol^{-1}), represent weaker IL cation−anion (hydrogen bond) interactions, which conjugated with the strong salting-out nature of the salt allow the complete partition of the proteins toward the top phase.

Regarding the polysaccharides partition profile, an opposite trend is observed. An increase in E_{HB} values leads to a higher partition of the carbohydrates toward the bottom phase.

Summing up, this study allowed the conclusion that the use of anions with a high E_{HB} ($[CH_3CO_2]^-$ and Cl^-) led to more selective systems in the protein/polysaccharides partition. Despite the same selectivity results were obtained for $[CH_3CO_2]^-$ and Cl^- anions, in the subsequent optimization studies, the Cl^- anion was chosen since it is the cheapest.

The alkyl chain length effect was also evaluated on the partition of proteins and polysaccharides (Figure 4). For this purpose, ABS based in the same extraction point, and composed of K_3PO_4 + $[C_nmim]Cl$ (with C_n = C_2, C_4, C_8, C_{10}, C_{12}, and C_{14}) were tested. All ILs tested, except $[C_{12}mim]Cl$ and $[C_{14}mim]Cl$, formed ABS with the inorganic salt. In fact, $[C_{12}mim]Cl$ and $[C_{14}mim]Cl$ ILs were not able to form ABS due to their high hydrophobic and tensioactive character that, helped by the salting-out effect of K_3PO_4, led to the ILs' precipitation.[64] The results indicate that the extraction efficiency of proteins ($\%EE_{prot}$ = 100%) and polysaccharides ($\%EE_{carb}$ around 60%) do not vary significantly with the increase of IL alkyl chain length.

Effect of the K_3PO_4 Concentration: The Extraction Point. The effect of K_3PO_4 concentration was analyzed in the most selective ABS platform ($[C_8mim]Cl$ + K_3PO_4) to guarantee the maximum polysaccharides partition toward the bottom phase, i.e. to increase even more the selectivity of the system. In this sense, the mixture points used to perform the extraction tests were different, as depicted in Figure S1, where the phase diagram and the respective extraction points and respective tie-lines are indicated. At all inorganic salt concentrations tested, namely 20%, 22%, 25% and 27.5 wt %, the formation of ABS was witnessed. By the analysis of Figure 5, both extraction efficiency parameters were not significantly affected by the alteration of the salting-out species concentration. In this sense, and considering the selectivity results, the lower concentration of salt was selected for further studies, considering the decrease in the overall cost of the process.

The characterization of the phases regarding the polysaccharides and proteins composition was performed for the best system composed of 15 wt % $[C_8mim]Cl$ + 20 wt % K_3PO_4 + 65 wt % *I. galbana* aqueous extract. For comparison purposes, the dialyzed initial *I. galbana* aqueous extract (AqExtract) was also included in this analysis.

The Top-phase and Bottom-phase fractions have polysaccharide contents of 16 and 45% and protein contents of 42 and 9%, respectively (Table 2). The Top-phase was further fractionated by ethanol precipitation, allowing to obtain one fraction soluble in ethanol (Sn Top-phase) and one insoluble (Pp Top-phase). Regarding the protein amino acid composition, the analysis showed that the protein in both fractions (Sn Top-phase and Pp Top-phase) have essentially the same composition, mainly tyrosine (35 and 44 mol %, respectively) and phenylalanine (27 and 18%, respectively). Other amino acids were also determined, such as leucine and isoleucine (13 and 11%, respectively), valine (7%), a mixture of glycine and alanine (5%), lysine (4%), and about 1% serine, aspartic acid, and tryptophan (Figure 6). This composition revealed that hydrophobic amino acids (i.e. phenylalanine, leucine, isoleucine, valine, glycine, and alanine) are half of the total amino acid content, in accordance with the composition reported in

Table 2. Polysaccharides, Proteins, and Sulfate Concentration Determined for Fractions Obtained from *I. galbana* Aqueous Extract by Applying the ABS Composed of 15 wt % $[C_2mim]Cl$ + 22 wt % Inorganic Salt + 63 wt % Aqueous Extract of *I. galbana* at Room Temperature

Fractions	Concentration (mg g^{-1} fraction)		
	Polysaccharides	Proteins	Sulfate[d]
AqExtract	415.2 ± 14.7	425.1 ± 1.9[a]	11.1 ± 1.9
Top-phase	164.9 ± 18.8	417.8[a,b]	9.7 ± 2.6
Bottom-phase	447.6 ± 34.0	91.7[a,b]	12.0[b]
Sn Top-phase	106.7 ± 0.7	442.6 ± 8.3[c]	
Pp Top-phase	786.6 ± 12.7	61.3 ± 1.5[c]	

[a]Determined by elemental analysis [b]Without replication by sample limitation. [c]Determined by BCA analysis. [d]Determined by turbidimetric analysis.

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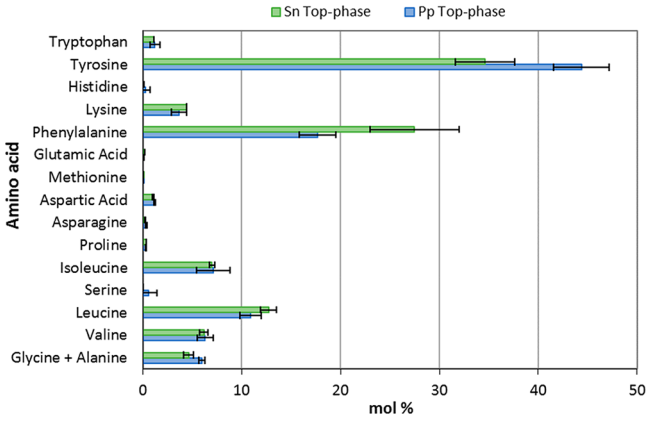

Figure 6. Protein amino acid composition of Sn Top-phase and Pp Top-phase fractions obtained from *I. galbana* aqueous extract using IL-ABS composed of 15 wt % $[C_8mim]Cl$ + 20 wt % K_3PO_4 + 65 wt % aqueous extract of *I. galbana*.

literature for *I. galbana* protein.[56] In addition, the amount of acidic amino acids is much lower than that reported in literature (14% glutamic acid and 10% aspartic acid).[56] The results show the predominance of amino acids with an aromatic ring, like tyrosine and phenylalanine in the salt-rich phase. In contrast, the proteins are preferentially concentrated in the top phase rich in IL. This phenomenon may be justified not only by the possible ($\pi-\pi$) interactions between the IL cation ring (methylimidazolium) and the aromatic amino-acids composing the proteins, but mainly by the strong salting-out nature of the salt (K_3PO_4) that expels the proteins to the opposite phase.

Concerning the polysaccharides composition (Figure 7), the Top-phase fraction is mainly composed of arabinose (45 mol %), whereas the Bottom-phase is constituted by glucose (56 mol %). These results permit us to infer that the application of IL-based ABS as purification platforms seems to allow the separation of proteins and polysaccharides, but they were also able to selectively fractionate different polysaccharides, which actually seems to justify the impossibility of obtaining $\%EE_{carb}$ higher than 60%. Relative to the negatively charged polysaccharides, due to the presence of uronic acids and sulfated residues, they were distributed equally by both fractions, uronic acids represent 39 and 63 mg g^{-1} in Top-phase and Bottom-phase, respectively, and sulfate content represents only 10 mg g^{-1} (Figure 7). Summing up, as these polysaccharides have a hydrophilic character, due to the presence of the negatively charged groups, it was expected their migration to the bottom phase. Meanwhile, the opposite migration of the arabinose-rich polysaccharides toward the Top-phase was achieved.

Because in Top-phase the coexistence of protein and arabinan prevailed, an attempt to separate these two biomolecules was made by ethanol precipitation. The analysis of total protein and polysaccharide contents on the supernatant (Sn Top-phase) and precipitate (Pp Top-phase) obtained showed that Sn Top-phase was mainly composed of protein (81%), while Pp Top-phase was constituted by 93% of polysaccharides (Table 2). The Pp Top-phase was composed mainly of arabinose (Figure 7), showing that the arabinan was precipitated with ethanol, while the protein remained in the supernatant. The polysaccharides recovered in the bottom phase consisted of 56 mol % glucose, 20 mol % mannose, and 12 mol % uronic acids as major sugars (Figure 7). The polysaccharides recovered in the top phase are mainly composed of arabinose (65 mol %), uronic acids (20 mol %), and xylose (16 mol %). This is consistent with the polysaccharide composition of AqExtract used for the ABS platform fractionation, which was mainly composed of a mixture of glucose (34 mol %) and arabinose (17%) (Figure 7). Although glucan has been previously reported as the main polysaccharide in *I. galbana*,[9,22,49] it is the first time that an arabinose-rich polysaccharide is reported.

In summary, using ABS based on ILs, it was possible to separate proteins from polysaccharides in an effective way to understand how to fractionate different classes of polysaccharides and to identify a new polysaccharide composing *I. galbana* not reported up to now.

Purification Process Developed: *I. galbana* Biorefinery. The proposed purification approach appears as a promising, simple, and effective method to separate proteins from the polysaccharides extracts from *I. galbana*, simulta-

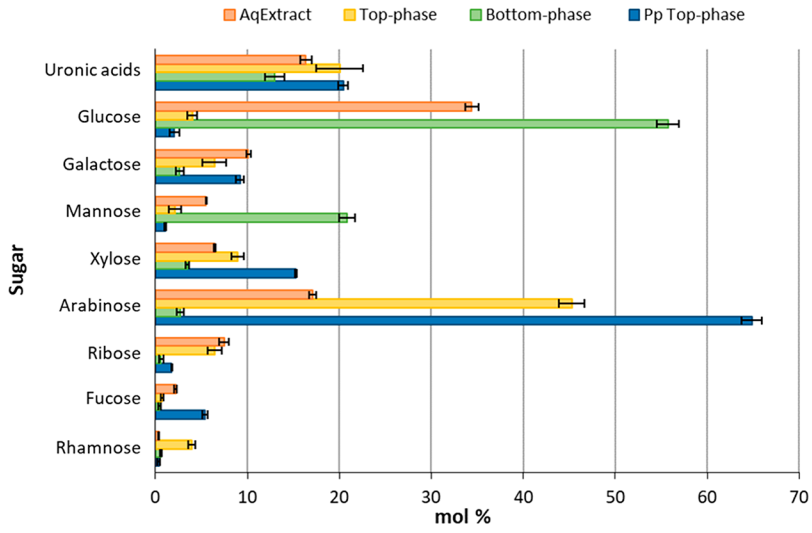

Figure 7. Polysaccharide composition of AqExtract, Top-phase, Bottom-phase, and Pp Top-phase fractions obtained from *I. galbana* aqueous extract using IL-ABS composed of 15 wt % $[C_8mim]Cl$ + 20 wt % K_3PO_4 + 65 wt % aqueous extract of *I. galbana*.

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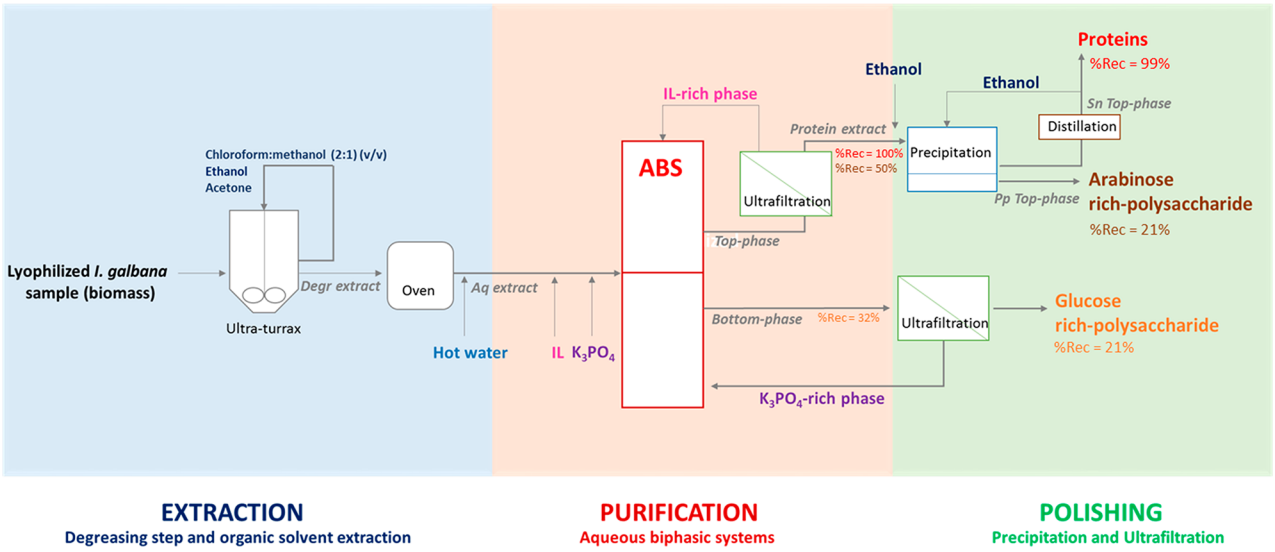

Figure 8. Diagram of the complete process developed to separate the proteins and arabinose- and glucose-rich polysaccharides in three consecutive steps: extraction of polysaccharides, their purification from proteins, and polishing. The isolation of the three classes of biomolecules from each aqueous/organic phase and the respective phase-forming components' reuse are also represented.

neously allowing the fractionation of distinct polysaccharides. Envisaging its industrial potential, the complete purification process was defined in Figure 8 for the fractionation of three types of bioactive products: two polysaccharide (arabinan and glucan) classes and proteins. This process comprises three main steps: (1) the extraction of polysaccharides, through the *I. galbana* biomass by defatting using an organic solvent extraction; (2) the separation of polysaccharides and proteins by applying the $[C_8mim]Cl + K_3PO_4$-based ABS; and (3) the polishing step to isolate (i) polysaccharides from the phase forming compounds and (ii) proteins from the arabinan-based polysaccharides. The reuse of the phase forming compounds (IL and inorganic salt) was effectively attained, to decrease the overall cost, while increasing the sustainability of the downstream process. To prove the complete recovery of the IL from the Top-phase of ABS and its reuse for a new cycle of purification, conductivity measurements were experimentally conducted. Moreover, the volatile organic compounds (VOC's) used in the process, mainly on the extraction of polysaccharides from the *I. galbana* biomass were reused as well. The overall mass balances obtained for polysaccharides and proteins are shown in Tables S1 and S2.

In the end, this work allowed us to prove that the use of IL-ABS is not only efficient on the separation of the different microalgae components but also a technology capable of overcoming some of the limitations of conventional up-scaling.[23−25] In fact, the results of purification obtained in the present work were better than those found in literature.[21,41,65] A special emphasis should be given to the higher recoveries representing the higher fractionation ability of the process developed to promote the polysaccharides decontamination caused by the presence of proteins.[41] Most important is the possibility of separating different classes of polysaccharides, which is normally obtained only with more complex steps including chromatography.[41,65,66]

Thus, the proposed approach appears as a promising, simple, and effective methodology of purification with industrial potential. Moreover, the use of IL-ABS can also be easily integrated into a fractionation process in continuous flow, through their posterior application on centrifugal partition chromatography[67] or continuous tubular separators.[68]

CONCLUSIONS

The use of IL-based ABS for the selective separation of proteins and polysaccharides seems to be a very promising approach. In fact, this technology may be an alternative to conventional polysaccharide purification platforms, which are expensive, complex, and present low yields. In this work, different conditions were optimized, namely, the nature of the inorganic salt, the IL anion and alkyl chain length, and the extraction point/tie-line length (by the use of different inorganic salt concentrations). After, the system based in 15 wt % $[C_8mim]Cl$ + 20 wt % K_3PO_4 was selected as the most performant ($\%EE_{carb}$ = 71.21 ± 5.21% to the bottom and $\%EE_{prot}$ = 100% to the top phase). It was the most efficient on the separation of polysaccharides from proteins, but it also allowed the fractionation of two distinct polysaccharides. Actually, a glucan and an arabinan (the latest identified in the *I. galbana* for the first time) were separated and concentrated in the IL- and salt-rich phases, respectively. Envisioning its industrial application, the complete process of purification to separate proteins, arabinose- and glucose-rich polysaccharides was developed and implemented, in which the isolation of the target biomolecules and the reuse of the phase formers and main solvents used was considered, by precipitation and ultrafiltration units, well-established technologies applicable at industrial scale.

The proposed approach using IL-ABS appears as a simple and efficient method of purification with industrial potential, which can also be easily integrated into continuous flow process, namely, using centrifugal partition chromatography or a continuous tubular separator.

ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acssuschemeng.8b02597.

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Phase diagrams and tie-lines (TLs) of the system composed of $[C_8mim]Cl$ + K_3PO_4, mass balance (MB) values for proteins and carbohydrates obtained for each ABS, Overall mass balance (OMB) values of proteins and carbohydrates taking into consideration the polishing steps (ultrafiltration and precipitation) for the optimized $[C_8mim]Cl$ + K_3PO_4-based ABS (PDF)

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Notes

The authors declare no competing financial interest.

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