Fractionation of Squid Pens with Ionic Liquids – An upgraded β-Chitin and Shellfish Protein Production

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Abstract:

In this work, the foundation of a biobased ionic liquid utilization of squid waste has been scrutinized. An ionic liquid screening showed that the biocompatible ionic liquid choline acetate, [Ch][OAc], can extract more than 80 wt% of the protein and precipitate it upon solvent swing between ethanol and water. Process parameter optimization was carried out with factorial design of experiments to yield 75% of recovered protein with an estimated 90% purity and a highly acetylated, crystalline β-chitin with up to 95% purity. Physico-chemical analyses of these two streams confirmed the efficiency of ionic liquid separation and the amino acid profiling of the protein isolate revealed the presence of three major essential amino acids, histidine, leucine and valine that could be a valuable alternative protein source for feed in aquaculture. Molecular dynamic simulations show that the affinity of $[OAc]^-$ to protein surfaces is greater than that of alternative anions, facilitating protein solubilization. However, its combination with $[Ch]^+$ is optimal because the interactions of this cation with the protein surface are relatively weak, allowing the protein to be recovered from the IL. A material mass balance of the process has shown the solvent usage is high, which ultimately impacts on high energetic requirements. Techno-economic confirmed that solvent usage makes up to nearly 65% of the minimum selling price of the protein, which reaches up to 9 \$/kg, but decreases with co-production of β-chitin down to 0.6 $\$\cdot kg^{-1}$ for each 1 $\$\cdot kg^{-1}$ of chitin. Solvent usage also impacts negatively on CO_2 emissions with up to 4.27 kg $CO_2\cdot kg^{-1}$ of product. These emissions are split into 61% for the protein and 39% for the β-chitin production.

Keywords: Process design, Squid waste, techno-economic analysis, molecular dynamics, alternative protein.

Seafood is a highly traded global food commodity, and it encompasses a wide range of species, including freshwater and marine finfish, bivalves, decapods, cephalopods, algae, and cyanobacteria[1]. In 2020, global seafood production reached 214 million tonnes, with the squid fishery representing about 4.3 percent of all marine catches by volume and about 7% by value worldwide[1,2]. The Food and Agricultural Organization of the United Nations estimates the seafood trade to be worth USD 151 billion, with a projected 13% growth in production value by 2030. Seafood categories are generally considered to have better environmental performance compared to other protein-rich foods, particularly in terms of sustainability[3]. However, the seafood supply faces various challenges, including increasing demand due to overfishing, species depletion, environmental pollution, global warming, and biodiversity changes that ultimately have impacted the squid population over the last decades[4].

Interest in the bio-refining of seafood waste through environmentally friendly processing is a result of a growing understanding of how traditional fishing processes affect the environment and the stringent pollution control requirements imposed by regulatory bodies[3,5]. Squids differ from fish by their high growth rate, short life span, and high feeding behaviour. These characteristics end up exerting a high predation pressure on zooplankton, fish, and other squid prey, as in the case of the invasive Humboldt squid in the eastern North Pacific[4,6]. There is then a necessity for population control and, of course, the introduction of biorefinery strategies for completely sustainable consumption and utilization of squid waste.

Large amounts of squid waste are produced from wild-caught fisheries. It is estimated that more than 40% of the total body weight of squid ends up as processing byproducts, including the viscera, pens, and skins[7]. These byproducts are routinely dumped in landfills or released into bodies of water. Incineration, composting, and anaerobic digestion could be an alternative, but only in more developed countries such as the UK and Australia, with disposal costs up to 150 US$ per tonne[8]. This introduces demand for the implementation of efficient and cutting-edge treatment and utilization methods to minimize any adverse environmental effects on aquatic ecosystems or land and to encourage more productive practices that minimize biomass, energy, or nutrient losses[1].

The squid pen, or gladius, is an internalized shell that acts as both a point of attachment for significant muscle groups and a barrier to protect the visceral organs[9]. The pen's distinctive protein and β-chitin composition is what gives it its strength and flexibility. The weight distribution of the pens from different species of squid is 25–49% β-chitin, 43–75% protein, and relatively low ash content[9]. A protein layer surrounds the β-chitin nanofibrils, which are arranged parallel to the pen's long axis by α-helical protein coils[9,10].

Squid pens valorization has been focused on obtaining protein hydrolysates with antioxidant properties or as fermentation media for microorganisms[7,11]. These methods, however, do not entail the isolation of the squid protein to obtain a high-purity protein isolate, as the protein recovery methods such as dialysis or membrane separation, would be costly[12]. Squid pen utilization for β-chitosan production usually employs harsh chemicals such as hydrochloric acid and sodium hydroxide[13–15] and generate high volumes of wastewater, which negatively impacts the water footprint of the process[16].

Ionic liquids (ILs) are liquid salts at room temperature that present interesting physicochemical properties such as high vapor pressure, conductivity, and high thermal stability. They can be tailored for different purposes by choosing different cation and anion combinations[17]. There has been a debate on the 'green-ness' of ILs due principally to their eco-toxicity[18], but this depends on the IL and process configuration. If the IL is fully recovered and the processing water streams are also recycled, then, a greener process can be ensured. Past literature studies entailed solubilizing chitin and chitosan with ILs for energy, pharmaceutical, and medical applications but not the fractionation of shellfish waste. A study by Shamshina and Abidi (2022)[19] has shown that acidic ionic liquids such as 1-butyl-3-methylimidazolium hydrogen sulfate, [BMIM][HSO_4], can efficiently fractionate shrimp shells to produce high-purity chitin. However, there are no studies on holistic biorefinery utilization of squid waste with ILs.

In this study, we aim to explore the use of ILs in the fractionation of squid pens to produce high-purity β-chitin and protein as two solid streams. We investigated the influence of the structure of the anion and cation of the IL on protein extraction in an IL screening. Once the IL was chosen, optimization of the fractionation parameters determined the best conditions to ensure high protein yield. Then, recycling of the IL under a limited number of batches was probed to ensure the IL performance was retained along the cycles. Characterization of the produced β-chitin and protein confirmed the efficiency of fractionation. A mass balance of the fractionation process, together with a techno-economic analysis, indicated the process's feasibility. Lastly, molecular dynamic simulation helped us to theorize the explanation of the choline acetate selectivity towards protein extraction and further isolation.

2. Materials and methods

2.1 Feedstock and reagents

All experiments used European-caught (*Loligo sp.*) squid pens sourced from a fish market in Spain and stored at -80°C. Before fractionation, the squid pens were defrosted, washed in deionized (DI) water, rinsed with ethanol, and dried at 40°C overnight. Then, they were blended into a powder and sieved to a particle size between 180 and 850 µm. Chemicals were sourced from Sigma-Aldrich, UK and included: monoethanolamine (MEA, ≥ 98%), methane sulfonic acid ($MeSO_3H$, ≥ 99%), glacial acetic acid (HOAc, 100%), choline bicarbonate ([Ch][HCO_3], 80 wt%) and sulphuric acid (H_2SO_4, 66.3 wt%).

2.2 Squid pen chemical characterization

Squid pens were characterized in a similar procedure by Chaussard and Domard (2004)[20]. Briefly, the extractive content of squid pens was determined by consecutive 12 h Soxhlet extraction with ethanol and cyclohexane. Then, the squid pens were extracted with 1 mol·L^{-1} at 80 ˚C for 3 h for quantitative protein removal; the dry β-chitin mass was considered protein-free after CHN analysis established its nitrogen content was 6.90%. The ash content of the squid pen samples was determined after ashing the dry squid pens in a muffle oven for 6 h at 600˚C.

2.2 IL synthesis and characterization

Six ILs were synthesized by dropwise addition of equimolar amounts of the acids – acetic, methane sulfonic, or sulfuric – to the base – choline bicarbonate or monoethanolamine under stirring and cooling with an ice bath (0˚C). The six ILs were: choline hydrogen sulfate, [Ch][HSO_4], choline methane sulfonate, [Ch][$MeSO_3$], choline acetate, [Ch][OAc], monoethanolammonium hydrogen sulfate, [MEA][HSO_4], monoethanolammonium methane sulfonate, [MEA][$MeSO_3$] and monoethanolammonium acetate, [MEA][OAc]. Once the addition finished, the synthesized IL was allowed to stir for 1 h and had its acid-base ratio and water content adjusted to 1:1 and 20 wt%, respectively. IL water content was measured using a V20 Volumetric Karl-Fischer Titrator (Mettler-Toledo). Acid-base ratios of [Ch][HSO_4] and [MEA][HSO_4] were determined by titration with 0.1M NaOH, using a G20S Compact Titrator (Mettler-Toledo, Columbus, USA), using potassium hydrogen phthalate (Sigma Aldrich, UK) as a primary standard.

2.3 IL screening assays

Briefly, 9.0 g of the IL was added to 1.0 g of squid pen powder in a flat-bottomed glass vial. The vials were then heated to the target temperature in a hot plate with constant stirring at 300 RPM for 3 h in a similar procedure to Polesca et al. (2023)[21]. The vials were then allowed to cool to room temperature, and then 25 mL of ethanol was added. The slurry was filtered, and the filtered solid β-chitin was washed three times with 5 mL ethanol portions. The filtrate had its ethanol content evaporated in a rotary evaporator. Approximately 10 g of DI water was added to precipitate the protein from the IL, the mixture was left sitting for 30 min, and it was centrifuged at 13.3 × G for 10 minutes. The protein was washed thrice with 10 mL DI water and freeze-dried (FreeZone 6, Labconco, MO, USA) to yield a white powder (ESI). β-chitin and protein purity were estimated by CHN analysis (Medac Ltd., UK), the calculations were detailed in the Calculations section 3.

2.4 Optimization of protein extraction for [Ch][OAc]

Once [Ch][OAc] was chosen as the best IL for protein extraction, the optimization of the extraction parameters consisted of three series of experiments: 1) design of experiments (DoE): a 2^3+3 (center points) factorial design; 2) single variable experiments for water content and extraction time. The main goal of these experiments was to attain high yield and purity of the squid protein while also producing a high purity β-chitin. A complete description of the experiments can be found in the ESI (Section X).

2.5 Recycling of [Ch][OAc]

Once protein extraction had been optimized, an IL recycling experiment was performed. The protein extraction was performed at 100 ˚C, 10 wt% solids loading, 20 wt% water content, and 2 h. The main difference from the previous experiments was that the wash water fractions from the β-chitin and protein were used to precipitate the protein and then added back to the aqueous IL respectively (Figure 1). The IL underwent recycling for five iterations, totalling six cycles in all.

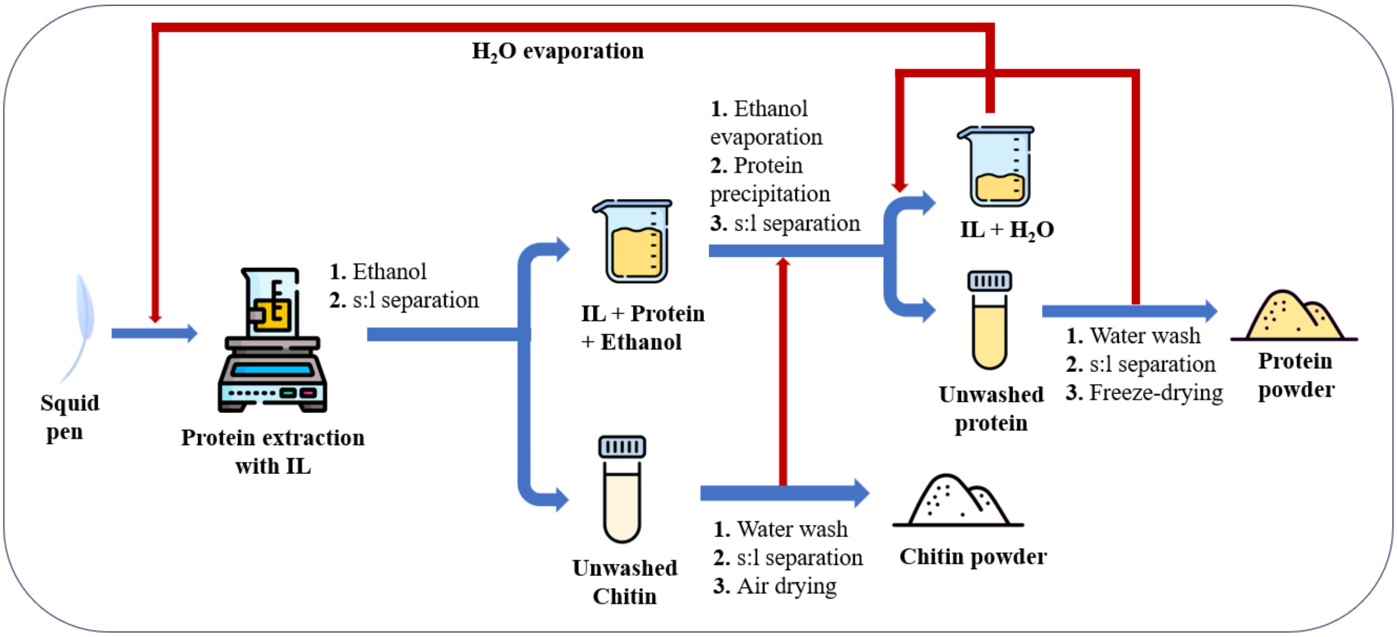

Figure 1. Recycling of [Ch][OAc] used for squid pen protein extraction. The red arrows correspond to the recycling changes in the IL and wash water fractions.

2.6 Material characterization - Analytical Methods

Once the β-chitin and protein were obtained under the optimized conditions with [Ch][OAc] — 100 ˚C, 2 h, 5 wt% solids loading and 20 wt% water — a comprehensive material characterization was performed to better understand the two streams that were produced. Details regarding the statistical analyses and analytical procedures for the characterization techniques ^{1}H-NMR, FT-IR, TGA, SEM, XRD, SDS-PAGE, CHN analysis, amino acid profiling can be found in the ESI (section X).

2.7 MD simulations

Equilibrium molecular dynamics simulations were conducted utilizing GROMACS 2018.3 CUDA[26,27] to explore the enhanced efficiency of protein extraction from squid pen by ILs based on acetate and choline. The model protein used was ubiquitin. Initial systems were prepared with 3.0 mol·L^{-1} IL aqueous solution boxes generated via Packmol[28,29]. These systems comprised ILs formed by the combination of choline ([Ch]$^+$), monoethanolamonium ([MEA]$^+$), acetate ([OAc]$^-$), and methanesulfonate ([MeSO$_3$]$^-$) ions. Additional information regarding the simulations can be found in the ESI (section X).

2.8 Techno economic analysis

Aspen Plus v11 was employed for process simulation to estimate economic costs and the environmental impact of removing ethanol and water from the IL for its reuse. This was done following similar methodologies reported previously[21,37]. Additional information regarding the simulations can be found in the ESI (section X).

3. Results and Discussion

3.1 Chemical composition of squid pen

The chemical composition of the squid pens on a dry basis is: 30 ± 1.3 wt% chitin, 68.6 ± 2.6 wt% protein, 0.5 ± 0.02 wt% ash and 1.1 ± 0.26 wt% lipids. These values are quite similar to those obtained for the composition of *Ilex argentinus'* squid pen by Cortizo et al. (2008)[43] with 31 wt% of chitin, 64 wt% of protein, 1.0 wt% ash and 2.3 wt% lipids. Additionally, Kurita et al. (1993)[44] also found similar values for the squid pens of *Ommastrephes bartrami*, 35–40 wt% chitin and 58 wt% of protein. Squid pens are a relatively simple shellfish waste due to its composition, consisting mainly of proteins and chitin.

3.2 IL screening for protein extraction

Acid-base ratio characterisation of the ILs employed in the screening is depicted in Table S15 in the ESI. The ILs present acid-base ratios close to 1.00:1.00, from which, most present acid-base ratios slightly lower than 1, from 0.95 to 0.99, except [Ch][MeSO$_3$] with 1.07:1.00. Some interesting trends related with the relationship between the pH of the ILs and protein removal can be found by inspecting Fig. 2. From the three acidic ILs — [MEA][HSO_4], [Ch][HSO_4] and [Ch][$MeSO_3$] — only the [HSO_4] ILs were able to remove the protein from the biomass. The explanation relies on the amount of acidic protons available in the medium. Acidic ILs with [HSO_4]$^-$ anions present a high pool of protons available for exchange. In a study by Firth et al. (2024)[45], they showed through DFT (Density Function Theory) calculations that spontaneous proton dissociation from [HSO_4]$^-$ to water occurred for ammonium-based protic ILs, preceded by the formation of an anion trimer structure. Whereas with [Ch][$MeSO_3$], the slight excess of methane sulfonic acid did not provide sufficient acidity to the system. Although the proteins were removed from the squid pen, the protein yield was near zero because the proteins most likely were hydrolysed in the acidic media. The neutral IL, [MEA][$MeSO_3$], was also inefficient in promoting any protein solubilisation. And lastly, the alkaline ILs [Ch][OAc] and [MEA][OAc] were able to extract a considerable amount of protein. However, upon anti-solvent addition to the protein-[Ch][OAc] mixture, instantaneous protein precipitation occurred (Video S1 on the ESI), which confirms the superiority of this IL towards protein extraction and recovery. Of course, the pH trend does not solely explain the protein solubility and precipitation; other phenomena related to ion hydrophilicity, intermolecular-interactions and effect of cosolvent which will ultimately impact on the salting-in and out effects of the IL will be covered in section 3.5 with the MD simulations.

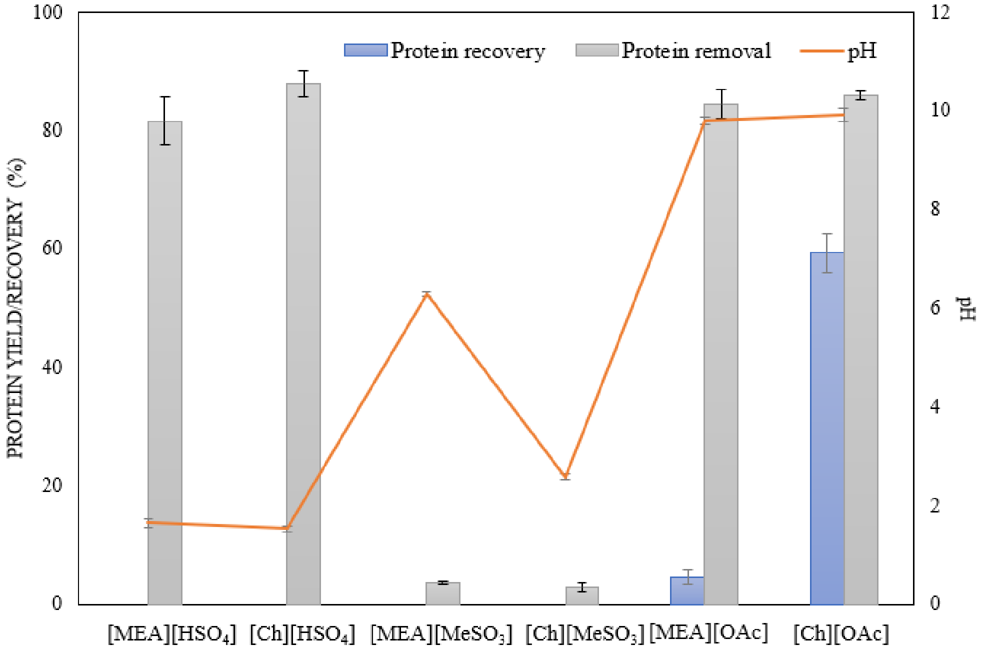

Figure 2. IL screening of squid protein extraction: protein removal, recovery, and pH of the IL solution.

3.3 Optimisation of protein extraction for [Ch][OAc]

Once [Ch][OAc] was chosen, optimisation of the protein extraction parameters was performed. Extraction time, solids loading, and temperature were chosen as optimisation variables, and two main response variables were assessed: protein removal and protein yield. Protein extraction experiments performed under 100 ˚C did not yield any measurable amount of recovered protein. It can be argued that high temperatures are necessary to disrupt both the hydrogen bonding and covalent bonds between protein and chitin. In fact, past studies employed harsh conditions with 1M NaOH under high temperatures (< 80 ˚C) at prolonged times to fully remove proteins from squid pens[9,10,14]. Sodium hydroxide is a much stronger alkali than [Ch][OAc] due to the presence of hydroxide anions in solution. Therefore, protein-chitin bond disruption can occur from 80 ˚C.

A new 2^2 design (with only time and solids loading as parameters) was then extracted from the previous 2^3 DoE, with three extra centre point triplicates being added, 10wt% solids loading and 3 h extraction time. The DoE analysis (ESI) showed that all parameters are significant, including time and temperature interaction. The statistical significance of the model term coefficients, ANOVA, and Pareto chart for both protein recovery and removal were shown in the ESI. The analysis showed that all of the model terms are statistically significant ($p < 0.05$). The first-order model fit to the experimental data predicts the response using the following equation:

Protein removal (%) = 79.73 + 19.02T – 28.63S + 14.32 TS (3)

Protein recovery (%) = 44.80 – 28.09T – 33.13S + 33.8TS (4)

Where T and S correspond to the time and solids loading factors (dummy coded, i.e., from -1 to +1), and the R^2 values for the protein removal and recovery were 0.99 and 0.70, respectively, indicating that the efficiency of protein removal is better predicted than how much protein is precipitated. Logically, protein removal is a simpler outcome variable as it only depends on the mass of the samples before and after protein extraction. On the other hand, protein recovery is more complex because it involves both protein removal and their salting out via anti-solvent addition. The surface response plots were shown in Fig. S3 (ESI). Protein removal was favoured by long extraction times and low solids loadings.

Alternatively, protein recovery was favoured by low solids and low extraction times. Long extraction times promote protein breakdown into oligopeptides, peptides and amino acids, which may have remained in the IL even upon anti-solvent addition. A compromise between high protein removal and protein recovery must be achieved. A time course experiment (Fig. S6 ESI) showed 2 h of extraction was sufficient to ensure high protein yields (> 60%) . Therefore, an extraction time of 2 h and 5 wt% solids loading was chosen as the optimised condition. However, one last parameter needed assessment — the water content of the IL. The protein recovery as a function of the water content can be seen in Fig. S7 (ESI). A water content of lower than 5% was difficult to achieve by rotary evaporation due to the high hydrophilicity of [Ch][OAc], thus the initial water content was 10 wt%. Protein recovery remained high under a low water content, then decreased upon the addition of water to 60% protein recovery with a 40% water content. A higher water content promotes the salting out of the protein and therefore decreases the ionic liquid/protein interactions. As there were no significant differences (section 4.2 from ESI) between a 10 and 20% water content, 20% was chosen as optimal for the IL recycling experiments. A higher water content in the IL decrease the cost related to solvent removal, once [Ch][OAc] is synthesised via diluted aqueous solutions of either [Ch][OH] (46 wt%) or [Ch][HCO_3] (80 wt%).

3.4 IL recycling – protein build-up

Recycling of [Ch][OAc] was carried out for three samples along five cycles, and the protein recovery yields are reported in Figure 3. It is noticeable that the protein recovery drops after the first cycle to nearly 50% for sample 1 on the third cycle, showing a possible decrease in efficiency. However, the yields sharply increase on the subsequent cycles up to 160%, which suggests that the proteins accumulate in [Ch][OAc] and after a certain threshold, they precipitate back. Sample 1 spiked earlier than the other two samples possibly due to a lower protein recovery on cycles 1-3, which resulted in a higher amount of protein accumulating.

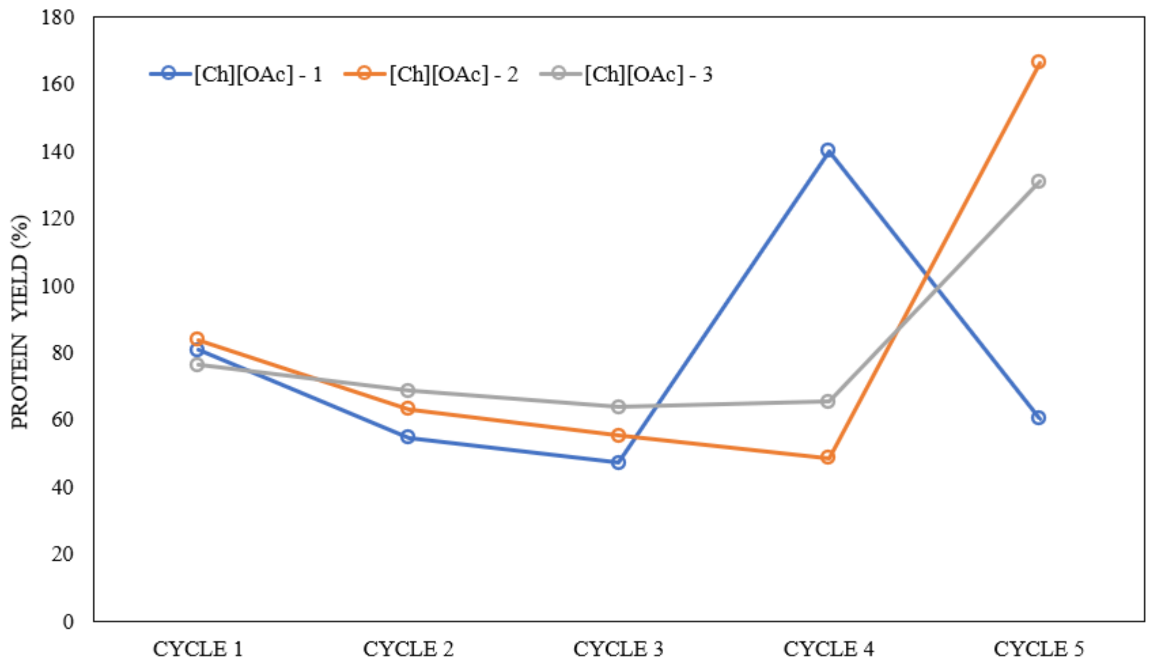

Figure 3. Protein recovery as a function of [Ch][OAc] recycling.

Although there has been a significant number of studies on ionic liquid or deep eutectic solvent (choline-chloride based) extraction of proteins[46,47], there is still a great gap in the investigation of their recovery and reuse, which poses difficulty to establish comparisons with other approaches. However, similar behavior can be observed in the lignin accumulation during the recycling of trialkyl ammonium hydrogen sulfate IL fractionation of lignocellulosic biomass, as observed by Brandt et al. (2017)[48] and Abouelela et al. (2021)[37]. Lignin can be salted out from the IL after fractionation upon the addition of excess water, in a similar method to squid proteins obtained in this work. The phenomenon underlying the lignin precipitation can be attributed to lignin cross-condensation along multiple pretreatment cycles[48] which does not reflect what happened to the protein. However, the addition of water triggers changes in both ionic strength and pH of the medium, two parameters known to impact the solubility of proteins and facilitate the precipitation of amorphous protein aggregates[49]. Lower ionic strength in aqueous choline-based IL systems is known to increase the likelihood of protein aggregation [50]. A continuous process would yield a different protein accumulation pattern in the IL with a steady state being reached, minimizing the likelihood of sudden spikes in protein precipitation.

The IL recovery is also shown in Table S13 (see ESI). On average, most of the [Ch][OAc] was recovered with the washing steps of both chitin and protein. The slight losses along the recovery cycles are most likely due to the protein-bound IL via hydrogen bonding and/or electrostatic interactions. Wahlstrom at al. (2017)[51] have shown that residual [Ch][Cl], up to 2.9 wt%, could be still found on the protein concentrate after dialysis on the extraction of Brewer's spent grain protein with the deep eutectic solvent [Ch][Cl]/urea. Polesca et al. (2023)[21] employed [Ch][OAc] to extract keratin from chicken feathers and managed to recycle the IL for four cycles without major losses in keratin extraction and IL recovery yields above 90 wt%. Nevertheless, they did not observe keratin accumulation on IL along the cycles, which may be related to differences in protein polarity. Once keratin is known to be hydrophobic and more insoluble in solvents due to the high amount of disulfide bonds, salting out with ethanol/water mixtures promoted their full precipitation from the bulk of the IL[52]. The [Ch][OAc] samples along the cycles have shown no signs of contamination (Figure S8 from ESI) on their ^{1}H-NMR spectra, which shows that no major by-products were produced upon the extraction.

3.5 Mass Balance

When developing a new process, it is essential to track mass flows for better understanding of critical quantities such as biomass fraction yields and solvent usage. The mass balance for the optimised squid pen extraction with [Ch][OAc] is depicted in Figure 4. The β-chitin yield was 0.34 $kg\cdot kg^{-1}$ of squid pen, which is consistent with values obtained by Chaussard and Domard (2004) — between 30 and 35 wt% — on the conventional extraction of squid protein from squid pens[20]. Cuong et al. (2016)[14] also managed to obtain 35.8 wt% of chitin from *Loligo chenisis* pens via conventional alkaline extraction. McReynolds et al. (2022) and [53] Lv et al. (2023) [54] obtained nearly 30 wt% of β-chitin by using the alkaline deep eutectic solvent K_2CO_3/glycerol. Sulthan et al. (2023)[55] employed the deep eutectic solvent ChCl/malonic acid to obtain nearly 43 wt% of β-chitin. Approximately, 0.53 kg of protein can be produced per kg of dry squid pen, which is a valuable yet multifunctional stream, due to its several amino acids constituents and it can be obtained in a solid form, facilitating its preservation and storage. In terms of solvent consumption, nearly 40 L of water and 23 L of ethanol were employed per kg of dry squid pens, which is still a considerable amount. However, all the solvent streams can be recycled, which does not generate wastewater.

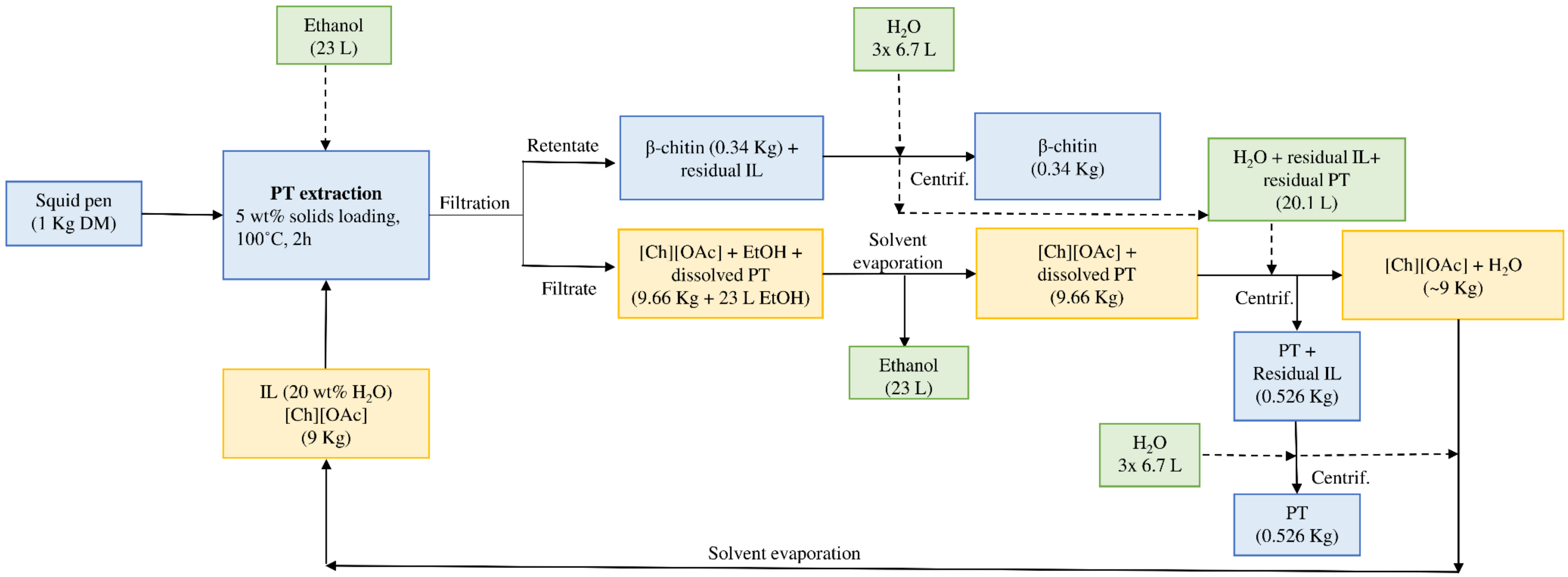

Figure 4. Mass balance for the optimised extraction of squid pen protein (PT) with [Ch][OAc]. The blue boxes represent the feedstock and fractions streams. The yellow boxes represent the IL streams and the green boxes represent the co-solvent streams. The dashed lines represent the flow of co-solvents.

3.6 Materials characterisation summary — β-chitin and protein

The extracted β-chitin presents a high degree of acetylation as shown by ^{1}H-NMR estimation, 94%. This suggests that, despite the use of an alkaline IL, there was insufficient basicity to promote deacetylation of the N-acetyl groups. FT-IR analysis showed the main differences across the samples were related to the relative intensity of the bands. It was noted that the protein and the squid pen (which is mostly composed of proteins) present strong absorptions at 1630 and 1511 cm^{-1} due to the amine bands. Whereas with the β-chitin, even though present—chitin contains N-acetyl groups, which are amides — such bands are lower in intensity. The TGA revealed a trend in which, similarly to the FT-IR spectra, the squid pen had intermediary behavior between the two components (chitin and protein) where T_{onset} for the squid pen was 318 °C, for the β-chitin, 326 °C, and for the squid protein, 276 °C. Additionally, the TGA pattern for the squid pens resembled more of the protein since it is the primary component. The XRD analysis revealed that the CrI of the β-chitin from [Ch][OAc] extraction was higher (82%) than those measured from the conventional extraction method with NaOH (50-70%). The electrophoretic profile of some of the squid proteins obtained in this study showed that the proteins presented similar electrophoretic patterns with two distinct bands, at 10 kDa and between 15 and 25 kDa. Such bands are likely to be related to muscular proteins from squid and which are typically present at low molecular weight[56]. The elemental analysis of the protein showed a nitrogen content of 14.4 ± 0.1%, which corresponds to a protein purity of 90 ± 0.6%, respectively. These values place the [Ch][OAc] fractionation as one of the best to produce high-purity streams from squid waste. The squid protein concentrate obtained in this work had an overall protein content of 43%, excluding tryptophan, which increases to 56% when cysteine and tyrosine are also included. The primary essential amino acids detected were histidine, leucine, and valine, whereas tyrosine, proline, and alanine made up the majority of non-essential amino acids. The biocompatible [Ch][OAc] is a suitable IL choice as the protein isolate can be used for high-protein content feed in aquaculture due to its biocompatibility [1,57,58]

The squid pen surface is slightly rough and is present in layers as visible in Figure 5a. These layers form as a result of the squid's secretion during their formation[9]. Removal of these superficial layers through treatment with [Ch][OAc] reveals a smooth and groovy surface on the β-chitin, as observed in previous studies[9,59,60]. The freeze-dried squid pen protein is highly porous with nano-sized pores distributed across its surface (Figure 5c-d). These pores were most likely were formed during the freeze-drying process and resemble the formation of hydrogels made of animal protein[61], soy protein[62] and other biopolymers such as agar[62], glucomannan[63]and modified cellulose[64].

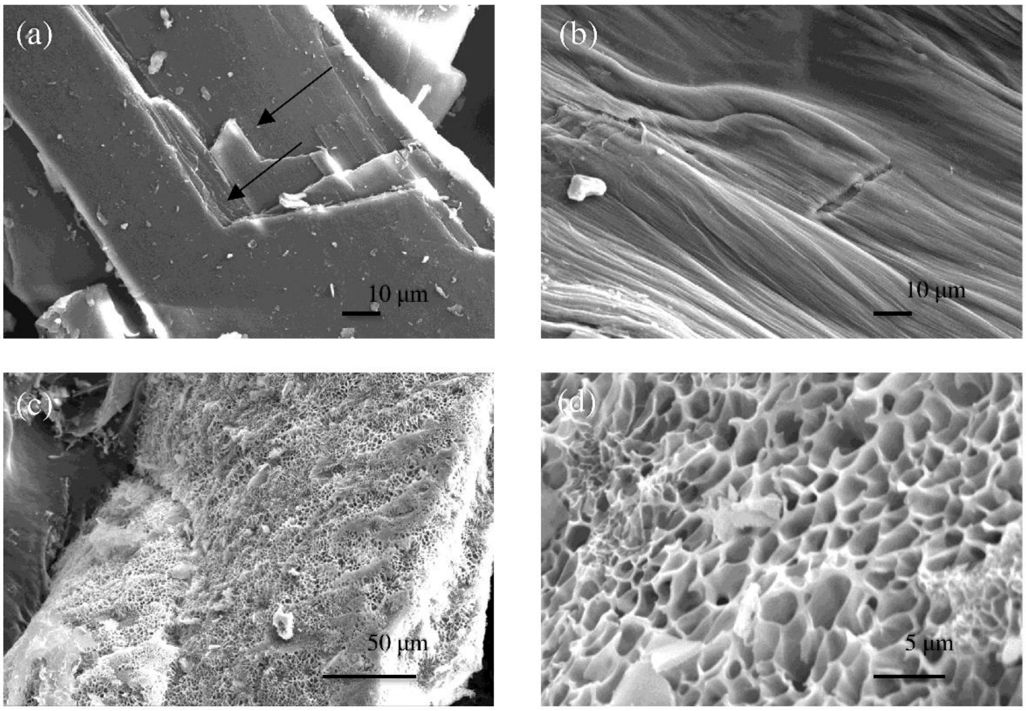

Figure 5. SEM surface image of (a) the squid pen; (b) β-chitin after protein extraction with [Ch][OAc]; (c) squid pen protein under low magnification; (d) squid pen protein under high magnification.

3.7 Molecular Dynamics (MD)

The phenomena behind the squid protein extraction with ILs at the molecular level can be quite complex due to the variety of inter- and intramolecular interactions involved. Nevertheless, past studies on the interaction of proteins with ILs have shown cooperative and competitive effects of IL solvation of proteins can be rationalized.[49,65,66]Squid proteins, especially muscular ones such as actin, myosin, and tropomyosin, are known to thermally denature from 40 °C to 80°C[9,67,68]. IL treatment at 100 °C most likely caused the squid pen proteins to denature. Bui-le et al.(2020)[66] have also shown that proteins such as the green fluorescent protein are more easily denatured in the presence of pyrrolidinium-based ILs, which may reinforce that protein denaturation may have happened even below 100 °C, which then caused their unfolding and exposure of the hydrophobic core. The acetate anion presents a high value of the Kamlet-Taft parameter β related to the hydrogen-bond basicity; it is known that high β values may lead to protein destabilization[49]. Once denatured, the protein remains soluble in the IL and upon the addition of ethanol as a co-solvent. After ethanol evaporation and the addition of excess of water, the protein was salted out from the IL due to the stronger IL-water interactions. The goal of these MD simulations was twofold: first, to understand why protein solubilization was more effective in ionic liquids with the [OAc] anion. Second, to rationalize why the combination of [OAc] with the [Ch] cation allows optimal recovery of the proteins.

3.7.1 Anion-protein interactions

Minimum distance distribution functions (MDDFs) for two distinct IL systems sharing the same cation are shown in Figure 6. The MDDFs peaks indicate the regions with the highest probability of locating the compound of interest in the solution relative to the protein surface. The MDDFs for the anions exhibit significant differences in the systems. Specifically, in the $[OAc]^-$ MDDF (Figure 6a), a sharp peak appears at ~1.9 Å, showing a high probability of acetate-protein hydrogen bonds.[65,69] Another important $[OAc]^-$ MDDF peak appears at ~2.9 Å and is a result of van der Waals interactions with the protein or interactions mediated by other ions or water molecules. The position of the cation peak, which intermediates the two anion peaks, suggests alternating charge solvation layers, thus a strong cation-anion cooperative solvation effect. The accumulation of $[MeSO_3]$ in $[Ch][MeSO_3]$ (Fig. 6b) is less pronounced than that of [OAc], being the second peak greater than the first, and both smaller than that of the cation. Clearly, [OAc] is a stronger binding to the protein surface than $[MeSO_3]$.

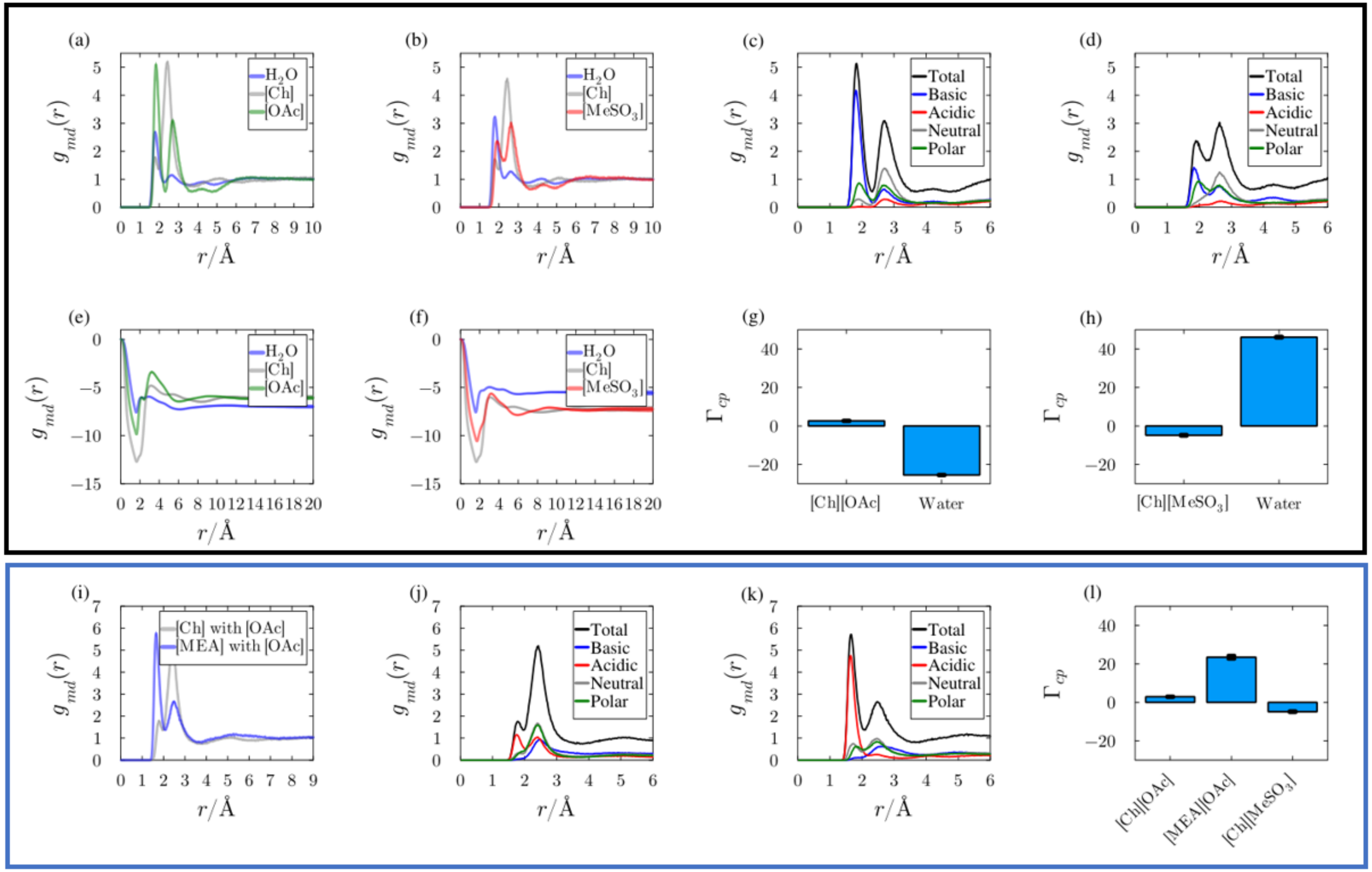

Figure 6. Protein solvation in IL systems using Minimum Distance Distribution Functions (MDDFs) and the Kirkwood-Buff (KB) theory of solvation. The figures enclosed in the black box show the distributions and KB-integrals of the anions $[OAc]^-$ and $[MeSO_3]^-$ in the presence of the same cation. The figures enclosed in the blue box refer to the distributions of the cations $[Ch]^+$ and $[MEA]^+$ in the presence of a shared anion. Panels (a) and (b) illustrate the MDDFs for of [Ch] and [OAc] and of [Ch] and $[MeSO_3]$, respectively. Panels (c) and (d) show a stronger association with basic residues, particularly for $[OAc]^-$. Panels (e) and (f) present the KB integrals for both IL systems, and panels (g) and (h) compare the preferential solvation parameters. Panels (i) to (k) offer a comparative analysis of protein solvation using the $[OAc]^-$ anion with $[Ch]^+$ and $[MEA]^+$ cations. Finally, Panel (l) contrasts the preferential solvation parameters between ILs with different cations, underscoring the variance in solvation preference and interaction strengths within these complex systems.

Protein residue-type contributions for the total MDDF distribution of ions are displayed in Figures 6c and 6d. In Figure 6a, the first peak of the MDDF of $[OAc]^-$ results from interactions with basic protein residues, mediated by interactions with the acetate oxygen (ESI, Figure S5). In contrast, $[MeSO_3]^-$ exhibits a smaller relative probability of interactions with basic residues than $[OAc]^-$. One can note, in Figure 6d the MDDF shows significant interactions with basic residues at hydrogen-bonding distances; however, such contribution to the total MDDF is also smaller than specific interactions displayed by the $[OAc]^-$ anion.

Kirkwood-Buff Integrals (KBIs) for the [Ch][OAc] and $[Ch][MeSO_3]$ IL systems are shown in Figure 6. In brief, when the water KBI exceeds that of the ions, it indicates a greater relative affinity of water for the protein surface. In the case of acetate, the ions' KBIs exhibit a stronger affinity for the protein surface, whereas for $[MeSO_3]^-$, the opposite trend is observed. This means that the IL with $[OAc]^-$ preferentially solvates the protein relative to water, while the protein is preferentially hydrated when the anion is $[MeSO_3]^-$, as shown in Figures 6c and 6d. The greater preferential solvation parameter implies that a greater number of IL ions is found around the protein for the system with [Ch][OAc] than with $[Ch][MeSO_3]$, for the same bulk concentration. In general, solutes that dehydrate proteins induce protein denaturation and may facilitate protein solubilization, as the protein tends expose its surface to the solution.

3.7.2 Cation-protein interactions

The distributions of components in solutions with different cations, but sharing the same anion are shown in Figure 6(i-k). The shared anion is $[OAc]^-$ and either $[MEA]^+$ or $[Ch]^+$ are the cations. The distributions of $[Ch]^+$ (in gray) and $[MEA]^+$ (in blue) display two primary peaks at approximately 1.8 Å, resulting of hydrogen bonds formed with the protein's surface. While both cations exhibit MDDFs with two peaks within 5 Å of the protein surface, the first peak is notably more pronounced for $[MEA]^+$. This suggests a higher likelihood of $[MEA]^+$ forming hydrogen bond interactions with the protein, particularly with acidic residues (Figure 6k). This significant contribution from acidic residues is not observed when the cation is $[Ch]^+$, as shown in Figure 6j. The inability of $[MEA]^+$ to induce protein precipitation, despite its solubilization capability, might be inferred from Figure 6l: Both ionic liquids

(ILs) with these cations preferentially solvate the protein, and thus interact favourably with the protein surface relative to water. Yet, the preferential solvation by $[MEA]^+$ is significantly stronger than for $[Ch]^+$. This potentially explains difficulty in precipitating the protein from the solutions with $[MEA]^+$. While the IL containing $[Ch]^+$ also preferentially solvates proteins when compared to water, its comparative affinity to the protein surface is much weaker than that of $[MEA]^+$, thereby facilitating protein recovery.

3.8 Techno-economic analysis

Techno-economic was conducted to evaluate the effects of the protein extraction process. These assessments are particularly crucial to minimise solvent consumption for environmental and economic issues, as ethanol and water are introduced at various stages of the process to facilitate the recovery of chitin and protein. Consequently, energy is required for the evaporation of these substances to achieve the appropriate composition for the recovery of ionic liquid (IL) for reuse. The IL recovery was set to 97%, following previous results shown in Table S13 (ESI). In addition, the experimental values reported in Figure 4 were considered for the estimation of heat consumption for ethanol and water evaporation.

The simulation takes into account heating the squid pen and IL mixture up to 100°C for protein extraction. Then, ethanol evaporation was simulated by a flash vessel at 0.1 bar, optimized by trial and error until no ethanol was detected in the bottom stream (IL + water). No IL was recovered in the top stream. 1.1 kg water·kg^{-1} of IL as low-pressure steam was necessary to provide the heat needed for the distillation. The top stream (ethanol) was condensed and then cooled to room temperature and recirculated, recovering the excess heat for its employment in the subsequent separations. Two-step multiple-effect evaporators were used for water removal and IL recovery. Pressure in both vessels (0.3 and 0.1 bar in the first and second vessel, respectively) was optimized by trial and error to minimize vacuum and energy consumption and ensure the recovered IL matched the required characteristics for its reuse. The excess heat from ethanol condensation was used in the first step, as well as an additional 1.78 kg water·kg^{-1} of IL as low-pressure steam for heating needs. Excess heat from water condensation in the top of the vessel was recovered and employed in the second step, with no additional heating needs. Water was cooled down to room temperature and recirculated. IL was then heated up again to 100°C and recirculated. From the simulation, annual costs were calculated as described in the methodology section; their percentual contribution to the minimum protein selling price is depicted in Fig. 7. Electricity contribution (due to pumping) is not shown as it accounted for less than 0.2%.

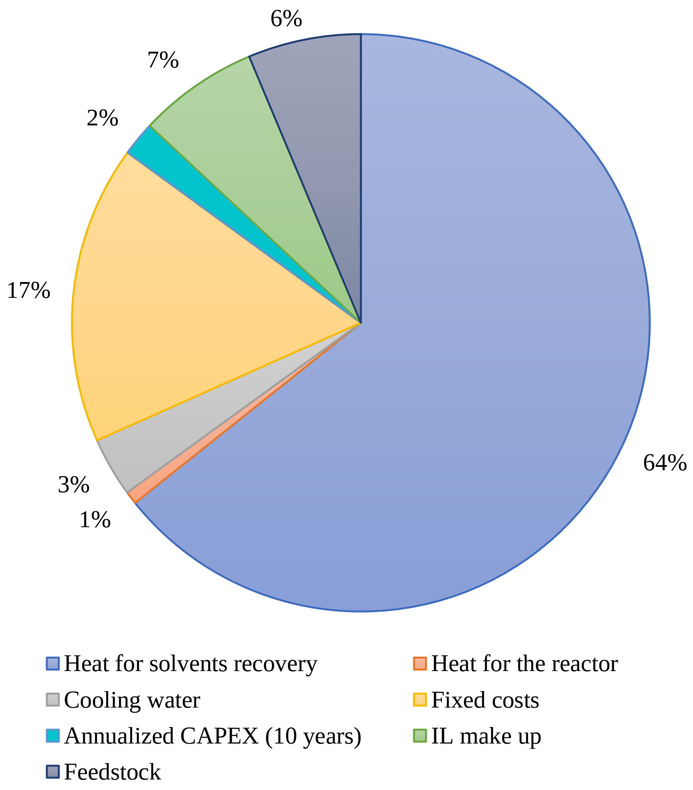

Figure 7. Percentual contributions to the minimum protein selling price.

As expected, the most significant contribution is the heat needed for solvent recovery due to the high volumes of ethanol and, mainly, water (85 wt% after mixing with the IL) employed in the process. This is especially important considering the heat of vaporization of water is 2.65 times higher than that for ethanol. For this, energy consumption in the multiple-effect evaporators for water evaporation is 1.6 times higher than for ethanol removal, accounting for 40% of the minimum protein selling price. Therefore, new methods for IL recovery are worth investigating to improve the energy efficiency of the process. IL makeup costs were calculated considering a 97% IL recovery from experimental results in laboratory, which may not completely translate to a larger scale[70]. The large volumes of solvents employed for relatively low productivity lead to high total field costs (due to equipment), which impacts the fixed costs, significantly contributing to the minimum selling price. The main contribution to these costs is the three evaporators employed in ethanol and water distillations, accounting for 15% of all fixed costs. Finally, it is important to note that feedstock costs are estimated using a squid pen powder price that may not be current, given the challenge of accessing updated pricing information. Hence, caution is advised in considering this aspect, as there is potential for cost reduction if the plant is integrated into the fish industry, where squid pens are commonly viewed as a waste material.

According to our simulation and considering all the costs explained in the methodology section, the minimum protein selling price obtained is 9 $\$\cdot kg^{-1}$, which was calculated considering a protein productivity of 416 ton/year, in accordance with the mass balance (Fig. 4) for simulated flow rates. However, it is worth mentioning that this price was calculated considering only protein and does not take into account the possible income from selling β-chitin as a by-product. Thus, based on chitin productivity and considering a price of 3.75 $\$\cdot kg^{-1}$[71], the protein selling price could be lowered to approximately 6.6 $\$\cdot kg^{-1}$. In terms of environmental impact, the process generates CO_2 emissions of 4.27 kg $CO_2\cdot kg^{-1}$ of products, factoring in the production of both protein and β-chitin, which account for 61% and 39% of CO_2 emissions, respectively. The predominant source of these CO_2 emissions is the significant energy requirements of the solvent recovery process, compounded by the limited availability of heat sources beyond steam. This underscores the critical need to explore alternative methods for recovering ILs from water streams, given their substantial impact on both economic and environmental considerations. A more flexible method for IL dehydration is pervaporation, a scalable membrane separation technique that can be selective toward the direct recovery of volatile solutes or solvents from non-volatile solvents in a quantitative manner[72].

4. Conclusions and perspectives

Designing ILs for protein extraction requires appropriate selection of cation and anion combination. Squid pens are an excellent model for a shellfish biorefinery because they present more simple composition when compared to crabs, prawns and lobsters. In this work, an ionic-liquid based process with [Ch][OAc] was designed and optimised that can generate two product streams of high purity, squid protein and β-chitin. Recycling of the IL showed that protein accumulates for a number of recycles and can eventually be recovered, without compromising the efficiency and purity of the IL. The mass balance of the process revealed that the solvent usage is high, however, process integration and IL recycling can ensure greener credentials for the process. Material characterisation of the two streams showed that the β-chitin presents a low protein content and is highly crystalline, whereas and the protein isolate has a high protein content and low molecular weight. Although it can be directed towards β-chitosan production — which would add an additional environmental burden due to its high waste generation[73] — β-chitin can be electrospun into scaffolds or mats for biomedical applications such as wound dressings, drug delivery agents or bone-growth scaffolds[74,75]. For instance, Gomes et al. (2020)[76] employed the same IL used in this study, [Ch][OAc], to produce biocompatible, highly porous, and interconnected sponges from crab shells α-chitin. Biocompatibility was probed by assessing the metabolic activity of L929 fibroblasts and seeding human adipose stem cells on the sponge surface. Despite a solvent intensive synthesis, chitin nano whiskers have been employed as reinforcing agents in elastomers synthesis, either in epoxidized natural rubber[77], in elastomeric composites with polycaprolactone[78] or modified polyurethanes[75].

Amino acid profiling of the protein isolate showed that the major essential amino acids were histidine, leucine and valine, which can be a source of valuable amino acids for aqua culture[57,58] and applications in human feed is also a potential avenue[56,79]. Nonetheless, caution needs to be taken regarding allergenicity triggered by tropomyosin[80]. MD simulations unveiled the reasoning behind the superiority of [Ch][OAc] to extract and precipitate protein from aqueous solutions, in comparison to other ILs screened with the same cation or anion, underpinning the role of the anion on the protein extraction and the role of the cation on the protein precipitation. Techno-economic analysis showed how energetic requirements for washing steps and IL recovery can heavily impact on the protein minimum selling price. The co-production of β-chitin decreases the minimum selling price of the protein. Solvent usage impacts negatively on CO_2 emissions and emphasizes that despite its promise, an IL-based refinery requires further design process integration and optimised heat recovery, as well as increased efficiency in separation processes. This work can be viewed as the foundation of an IL-based biorefinery for shellfish., where future works on carbonate-rich shellfish such as crabs, prawns and lobster can be beneficial for the holistic utilization of shellfish waste.

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