Alba Zurita Sánchez

EXTRACTION OF PROTEINS FROM BUFFALO WITH COMMERCIAL IONIC LIQUIDS IN AQUEOUS TWO-PHASE SYSTEMS

MASTER'S DEGREE THESIS

Supervised by Dr Christophe José Bengoa

MASTER'S DEGREE IN NANOSCIENCE, MATERIALS AND PROCESSES

UNIVERSITAT ROVIRA i VIRGILI

Tarragona

2022

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Alba Zurita Sánchez
Master Program in Nanoscience, Materials and Processes, 2021-2022
e-mail: alba.zurita@estudiants.urv.cat

Supervisor: Dr. Christophe José Bengoa
Department of Chemical Engineering. Universitat Rovira i Virgili
Campus Sescelades, Avinguda dels Països Catalans, 26. Tarragona, 43007, Spain.

Keywords: Insects; Ingredients; Ionic liquids; Aqueous two-phase systems; Protein extraction and purification

Abstract
The need for more sustainable protein sources to feed a growing World population has put insects under the spotlight, and greener approaches for the extraction of their proteins are under development. The present work studied the extraction of proteins from Buffalo, the larvae form of the beetle *Alphitobius diaperinus*, with a protein content of 68.10%. This was done in aqueous two-phase systems (ATPS) constructed using commercial ionic liquids (ILs) together with a salt solution. Different extraction aspects were evaluated, such as: the biomass addition procedure (solution or mass of Buffalo powder + mass of water); the effect of the ionic liquid ([bmim][Cl] or [bmim][Br]); the extraction point (3 mmol of IL + 1 mL of 50% w/v K_2HPO_4 or 11.45 mmol of IL + 3.815 mL of 50% w/v K_2HPO_4); or the effect of the volume or equivalent volume of insect solution (from 1 mL to 3 mL). In none of the studied ATPS setups 10% of protein recovery was reached, which was because of the poor solubilisation of the insect powder. This starkly contrasted with the recoveries that were obtained when testing ATPS with bovine serum albumin (BSA), as the systems constructed with [bmim][Cl] presented recoveries ranging from 62 to 92%, and from 66 to 85% when working with [bmim][Br]. Then, the purification of the protein extracts of the upper phase of the ATPS was conducted via dialysis, but protein losses occurred during the process, and the complete removal of the IL was not achieved in any of the dialysed samples. Because of this, new ways of incorporating the Buffalo powder and the design of novel ILs are needed to enhance protein recovery in ATPS. Additionally, more efficient purification methods for protein separation from the IL need to be developed.

1. Introduction

The rapid increase human population has experienced in the last 100 years has turned the obtention of sustainable protein sources into a major challenge to be addressed, both for human and animal consumption (Henchion et al., 2017). This is since protein consumption per capita is growing worldwide, especially in emerging economies (Ritchie and Roser, 2017).

With a view on satisfying this growing protein demand without harming the environment, and offering an affordable and nutritious alternative, insects have been suggested as potential protein sources (FAO and Wageningen UR, 2013; Riddick, 2013; Pal and Roy, 2014; Sánchez-Muros et al., 2016; van Huis, 2017; FAO, 2020; Gravel and Doyen, 2020; Moruzzo et al., 2021). There are more than 1,900 known species of edible insects (Pal and Roy, 2014), and they show similar protein content and amino acid profile to conventional meat products. For example, some flies have a similar nutritional profile to that of a fishmeal, thus being affordable protein sources for feeding poultry, pigs and a wide range of fish and shrimp (Riddick, 2013; Sánchez-Muros et al., 2016). Moreover, insects have the following advantages when compared to conventional livestock: (i) less need for land (FAO and Wageningen UR, 2013; van Huis, 2017) and water; (ii) less greenhouse gas (GHG) emissions: pigs produce 10 to 100 times more GHG per kg of weight than mealworms; (iii) higher feed conversion efficiency: cattle require (on average) four times more feed than insects for the same body weight gain; (iv) insects can be fed on bio-waste, stimulating circularity (FAO, 2020).

There is a plethora of protein extraction protocols, but the general scheme involves a first step of breakage of interprotein interactions with sodium hydroxide, and then a step of pH-dependent protein precipitation with the addition of an acid or a base. The selection of a suitable acid or base for this last step depends on the isoelectric point of the protein, which is the pH value in which it has minimal solubility and no net charge (Huet et al., 2020; Mohan et al., 2020; Zeng et al., 2021). Current industrial processes are based on these approaches, which involve the use of these volatile and aggressive compounds. These compounds are not only detrimental for the environment, but can ultimately damage the extracted proteins, highlighting the need for a transition to greener processes (Caligiani et al., 2018; Tan et al., 2021).

"Green chemistry" seeks to develop safer processes and products in a cost-effective way, and it does so by assessing their impact on human health and the environment throughout their life cycle (Roosen et al., 2008; Mishra et al., 2015; Paike and Sun, 2015;

Shamshina et al., 2018). It is within this approach that ionic liquids (ILs) have caught the attention of the scientific community (Roosen et al., 2008; Shamshina et al., 2018; Kaur et al., 2022). The origin of this interest lies in their tuneable and extraordinary properties, which make them potential solvents for the extraction of value-added compounds from biomass: they are able to extract and keep the stability of proteins, enzymes and amino acids (van Rantwijk and Sheldon, 2007; Roosen et al., 2008; Lee et al., 2017). Moreover, they show better efficiency, performance, and are easier to recover than the volatile organic compounds (VOCs) that are normally used for these purposes (Shamshina et al., 2018; Kaur et al., 2022). It is this evidence what makes ILs promising tools for the extraction and isolation of proteins from insects (Zeng et al., 2021).

A way to employ ILs for this purpose can be by using them in aqueous two-phase systems (ATPS), also known as aqueous biphasic systems (ABS). These are greener and more protein-benign alternatives to the conventional aqueous/organic solvent liquid-liquid extraction systems (Yang, 2012; Nunes et al., 2021). This protein-benignity stems from the fact that if the system is properly designed, mild operating conditions can be achieved, thus creating a biocompatible environment that enables protein extraction with great efficiency (75-100% recovery in one single step), selectivity and short equilibration time (Nunes et al., 2021). There are multiple ways through which these systems can be constructed, for example, by adding IL aqueous solutions either salts, polymers, carbohydrates, or amino acids (Yang, 2012; Nunes et al., 2021). When working with salts, the structure-making ions (PO_4^{3-}, HPO_4^{2}…) define the formation of the two phases, as ions with higher valences are better salting-out agents because of their ability to hydrate more water molecules (Pei et al., 2009).

Although there are some works about the extraction of proteins in ATPS from commercial proteins (Lin et al., 2013; Pereira et al., 2015; Belchior et al., 2020), microalgae (Suarez Ruiz et al., 2020), urine (Du et al., 2007) or plasma (Vicente et al., 2019), no articles to date report the use of ATPS for the extraction of proteins from insects, proving the novelty of the present work.

2. Materials and procedures

2.1. Materials

Buffalo powder was supplied by Kreca Ento-Foods. 1-butyl-3-methylimidazolium bromide (ref. 95137), 1-butyl-3-methylimidazolium chloride (ref. 94128), Bradford reagent (ref. B6916), bovine serum albumin (ref. A9647), copper sulphate pentahydrate (ref. 209198), D-+-glucose monohydrate ≥ 99.5% (ref. G8270), deuterium oxide (ref. 151882), dipotassium hydrogenphosphate > 98% (ref. P3786), Folin-Ciocalteu's phenol reagent (ref. F9252), and sodium tartrate dibasic dihydrate (ref. S4797) were supplied by Sigma Aldrich. Hexane (ref. 34859), sodium hydroxide (ref. 30620) and sulfuric acid 95-97% (ref. 32051) were supplied by Honeywell Fluka. Phenol 99.9% (ref. 134852.1209) and sodium carbonate (ref. 131648.1210) were supplied by Panreac. Hydrochloric acid 50% v/v (ref. 11333717) and silver nitrate 0.1 M (ref. 15615020) were supplied by Fischer Scientific.

2.2. Buffalo characterisation

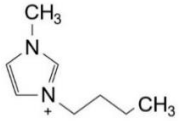
Buffalo (the larvae form of *Alphitobius diaperinus*, a beetle species) powder was characterized with a view on determining its humidity, ash, carbohydrate, lipid and protein content. Total solids (TS) and humidity, and total volatile solids (TVS) and ashes were determined following the standard methods 2540B and 2540E, respectively (Rice et al., 2012). Carbohydrates were determined following the Dubois method (Dubois et al., 2002). Lipid determination was conducted via conventional Soxhlet extraction, as defined in the standard method 5520E (Rice et al., 2012). Finally, proteins were determined with the Lowry method (Lowry et al., 1951).

2.3. Protein extraction in ATPS

The protein extraction procedure in ATPS consisted of six steps: (i) selection of IL-salt; (ii) construction of the binodal curves; (iii) construction of the ATPS; (iv) selection of the extraction points; (v) extraction of BSA; (vi) extraction of Buffalo proteins.

The selection of the ILs and salts was based on previous works (Pei et al., 2007; Lin et al., 2013). Imidazolium-based ILs ([bmim][Cl] and [bmim][Br]) with various phosphate salts were used to extract pure proteins in ATPS. The cation and anions of the ILs used in the ATPS are shown in Table 1.

Table 1. Cation and anions of the ILs used in the ATPS.

Cation	Anions
 (1-butyl-3-methylimidazolium)	Cl^- (Chloride) Br^- (Bromide)

Binodal curves were constructed for each of the studied IL–salt combinations following the cloud point method (Tanimura et al., 2019). First, 0.50 g of IL were added into a Falcon recipient together with 0.50 g of distilled water. Then, drops of a salt solution of a known concentration were added until two phases were observed. At this point, the mass of the system was annotated, and distilled water was added until the system looked turbid again. Mass was annotated again, and more salt solution drops were added until the system newly split into two phases. This process of adding drops of distilled water or salt solution was repeated until no more points could be obtained.

When constructing ATPS, the role of the IL–salt concentration must be considered. This is because if the point corresponding to the IL–salt combination is

placed below the binodal curve, no phase separation will occur, while if it is above, phase separation is ensured. Despite studying different extraction points, the way of constructing the ATPS remained the same: once the desired quantities of ionic liquid and salt solution were added into the Falcon tube, the system was vortexed to enhance phase contact for the formation of the ATPS. When the ATPS was visible, the desired biomass sample was added to study its behaviour, and after this, the Falcon was newly vortexed and then left to settle inside the fridge for at least 24 h. As a final step to ensure good phase distribution, samples were exposed to ultrasounds under stable temperature conditions (20-22ºC) for 52 minutes. To guarantee homogeneous ultrasound exposure, Falcon tubes were placed inside a beaker filled with water and then immersed into the water bath of the ultrasound system.

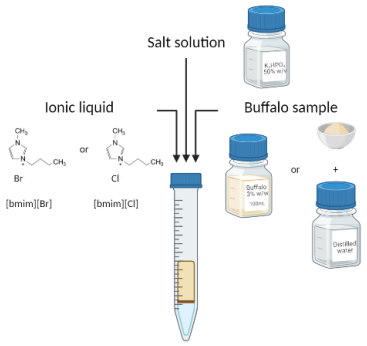


Figure 1. Setups of ATPS constructed with Buffalo. Created with BioRender.com.

Two extraction points (EP) were considered. EP 1 was based on the work of Lin et al. (Lin et al., 2013). 3 mmol of [bmim][Cl] or [bmim][Br] were added into a Falcon tube together with 1.00 mL of a 50% w/v K_2HPO_4 solution (36.76% in mass). EP 2 was selected to study the effect of the scaling up of the system. In this case, 11.45 mmol of IL were added into a Falcon tube as well as 3.815 mL of a 50% w/v K_2HPO_4 solution. Both systems presented basic pH, which is ideal for the purification of Buffalo proteins. This is since the isoelectric point of Buffalo proteins was determined by adding some drops of 40% NaOH solution and some of 50% HCl. The solution turned transparent when adding NaOH, and remained turbid when adding HCl. The isoelectric point of Buffalo proteins was at pH 10.

Extraction with BSA was first assessed in each IL-salt combination because of the heterogeneous nature and poor solubility of Buffalo powder. This was since BSA is more homogeneous and soluble. BSA standards of different concentrations (0.2, 0.4, 0.6, 0.8, 0.9, 1.0, 1.2 and 1.4 mg/mL) were prepared. 1.00 mL of BSA standard was added into a Falcon tube constructed in EP 1 using both ILs. Once the ATPS were ready, proteins were quantified using the Bradford method (Bradford, 1976). After knowing the behaviour of BSA in the ATPS, it was determined if that IL–salt combination would be suitable for working with Buffalo.

Buffalo protein extraction was conducted in EP 1 with [bmim][Cl] and [bmim][Br]. Insect powder was first sieved to have a diameter ≤ 850 µm, and a 3% w/w solution was prepared. To enhance homogenisation and extraction, an ultrasonic pre-treatment was applied before stirring. This was done using Hielscher UP200S ultrasonic processor. The employed setup was 50% of amplitude and cycles of 0.5 s using Sonotrode S14 (tip 14) for 25 minutes. The sample was placed inside an ice bath to avoid the heating and subsequent damage of the proteins during its sonication. After this, the sample remained under stirring for 1 hour, and then 1.00 mL was added into the ATPS. To verify that the desired mass of Buffalo was really added, procedure was modified to add 0.03 g of Buffalo first, and then 0.97 g of distilled water. On the other hand, with a view on optimizing the system, the role of the volume of the insect solution was studied in the two EPs. In EP 1, using [bmim][Cl], the following volumes of 3% w/w Buffalo solution were studied: 1.0, 1.5, 2.0, 2.5 and 3.0 mL. In EP 2, the same volumes of 3% w/w Buffalo solution were used with both ILs.

2.4. Separation and concentration of extracts

A series of ATPS that had exhibited good protein recovery in their upper phase were selected to conduct the separation and later concentration of their proteins. ATPS with protein concentration below the limit of detection of the Bradford method were also selected to test the protein recovery efficiency of the process.

The separation of protein from the IL was performed by two methods: protein concentrators and dialysis. The purification efficiency of both processes was qualitatively assessed by adding 1 dialysate drop into an Eppendorf containing 500 µL of a 0.1 M $AgNO_3$ solution to detect the presence of chlorine. Pierce™ Protein Concentrator PES with a molecular cut-off weight of 10 kDa and a capacity of 2-6 mL was tested with the selected samples. Dialysis was conducted using Viskase MD10 14 x 100 CLR membranes with a cut-off of 14 kDa. Dialysis bags got both their ends sealed with Parafilm® and held with dialysis tubing closures from Sigma before being immersed in a beaker, which was filled with approximately 600 mL of distilled water and placed on a magnetic stirrer. To make the samples float, Slide-A-Lyzer® buoys from Thermofisher were used. On average, dialysis rounds lasted for 2 hours, although all samples went through an overnight one. When the dialysate did not turn turbid after two rounds when adding $AgNO_3$, it was the inside of the dialysis bags what was studied: if the inside looked turbid, two more rounds were conducted, otherwise the dialysis process was stopped. When the dialysis was finished, the content of the bags was carefully emptied on a beaker, and the inside of the bag was cleaned with MiliQ water to ensure that no sample was lost on the walls. This cleaning water was also poured into the beaker. After this, volumes were

measured and spectrally screened from λ=1100 nm to λ=190 nm. This was done in the spectrophotometer UV-VIS 4600 from Dinko Instruments to see whether there was a signal at λ=280 nm, indicating the presence of proteins (Layne, 1957).

Samples were lyophilised to remove their water content. To do this, the water volumes recovered from the dialysis bags (except from 0.50 mL, which were kept from each sample for their later analysis) were divided into two Falcon recipients, which were carefully weighed before and after the addition of the samples. Once filled, Falcons were covered with a layer of needle-punched Parafilm®, and were placed tilted on a laboratory rack. Then, they were kept in a freezer at -4ºC before being lyophilised in a LABCONCO FreeZone benchtop freeze dryer at 4 mbar and -51ºC. The process lasted for 4 days because of the big volumes of the samples. After lyophilisation, Falcons were newly carefully weighed to determine the mass of the recovered protein.

Desiccation under vacuum and temperature was used to remove the residual humidity of the samples. Falcon tubes were covered with a needle-punched aluminium foil layer, which was subjected with Parafilm®. Then, they were placed inside a desiccator P SELECTA VACUO-TEMP at 40ºC and -76 cmHg for 24 h. This temperature was selected since BSA denatures at 60ºC (Matsarskaia et al., 2020). This was repeated until the mass of the Falcon exhibited minimal or no variation.

2.5. Analytical procedures for extracts and ILs

Lowry method (Lowry et al., 1951) was used for the quantification of proteins after extraction.

Bradford method (Bradford, 1976) was conducted using the protocol from Sigma Aldrich.

Protein absorbance at λ=280 nm was used to detect and/or measure protein content in samples (Layne, 1957). Because of the 50% w/v K_2HPO_4 solution exhibiting low absorbance at this wavelength, it was decided to use this method as a qualitative approach for the detection of proteins when dealing with ATPS extracts.

SimpliNano procedure was used when it was expected not to have salts in samples. 5 μL of samples were pipetted in the SimpliNano spectrometer from Biochrom. This device enabled the determination of the protein concentration of the samples using a program based on BSA absorbance at λ = 280 nm, and it also provided a graph plotting the absorbance of the sample between λ = 250 nm and λ = 350 nm. Centrifugation was necessary, and it was conducted for 2 min at 4200 rpm and room temperature conditions in Ortoalresa unicen20 centrifuge. After this, 200 μL of the liquid phase of the Falcon were pipetted into an Eppendorf, and since the two replicas of 0.030 g of Buffalo powder + 0.970 g of water extracted in EP 2 were still very turbid, they were added 400 μL of distilled water.

Determination of the N content of the samples served two different purposes: the quantification of ionic liquid and the quantification of proteins by nitrogen correlation (Boulos et al., 2020). A TOC analyser (Shimadzu total organic carbon analyser TOC-L and total nitrogen unit TNM-L ROHS) was used.

FTIR was used to evaluate more precisely the quality of the dialysis process. The first step was to compare the infrared spectra obtained for samples before and after dialysis. Buffalo powder in solid and diluted form (3% w/w filtered solution ≤ 20 μm), [bmim][Cl] in solid and diluted form (7.4% w/w solution), a 50% w/v K_2HPO_4 solution and a 2 mg/mL BSA solution were analysed to locate their peaks. All FTIR analysis were conducted using the infrared spectrophotometer Jasco FT/IR-6700 from the Scientific and Technical Resources Service of URV.

NMR allowed the detection of the organic part of the ionic liquid (1-butyl-3-methylimidazolium cation). A Bruker Avance Neo 400 Mhz NMR system from the Scientific and Technical Resources Service of URV was used. 0.10 mL of deuterated water were added to 0.40 mL of all samples. The peaks corresponding to a [bmim][Cl] solution (7.4% w/w) were used to locate them.

3. Results and discussion

3.1. Buffalo characterisation

The results of the characterisation of Buffalo powder are presented in Table 2.

Table 2. Characterisation of Buffalo powder.

Parameter	Average content (%)
Moisture	0.61
Ash	4.85
Carbohydrates	15.35
Proteins	68.10
Lipids	11.24
Total	100.15

As it can be seen in the table, results proved how the high protein content of Buffalo, > 68%, makes it an attractive source of proteins, since it accounts for more than half of its composition. Lipids and carbohydrates were present in quite similar quantities. These results are aligned with those from (Kurečka et al. 2021), who obtained a 63.5 ± 1.5% of protein.

3.2. Construction of the binodal curves

Figure 2 presents the ternary diagrams obtained for both ionic liquids, [bmim][Cl] and [bmim][Br], with K_2HPO_4 salt. Both diagrams are similar to those obtained by (Pei et al., 2007) and (Bai et al., 2013).

When constructing ATPS, the addition of the salt into the ionic liquid solution led to a salting-out effect, which formed an ionic liquid-rich upper phase, and a salt-rich lower phase. This was since K_2HPO_4 is a kosmotropic (i.e. water-structuring) salt, so it increased the dielectric constant of the aqueous phase, making the [bmim] cation migrate towards the upper phase with its corresponding anions (Gutowski et al., 2003).

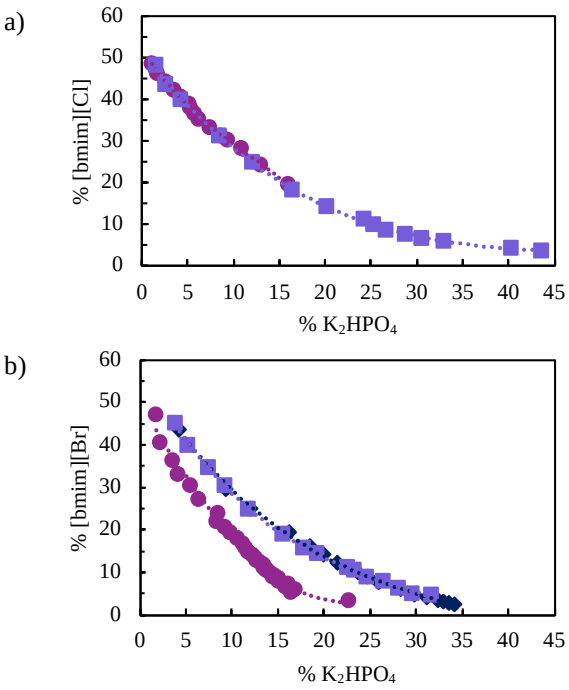


Figure 2. Binodal curves for a) [bmim][Cl] and b) [bmim][Br] ionic liquid-salt equilibria. (●) represents experimental data, (■) data from (Bai et al., 2013) and (♦) data from (Pei et al., 2007).

3.3. Extraction of BSA

The protein extraction efficiency of each ionic liquid-salt system was first studied with BSA. This way, it would be seen if this extraction approach would work. All ATPS were constructed adding 1.00 mL of BSA standard to an ATPS constructed in EP 1.

Table 3. BSA recovery in the upper phase for the [bmim][Cl] and [bmim][Br]−salt ATPS.

[BSA] (mg/mL)	Recovery (%)	
	[bmim][Cl]–salt	[bmim][Br]–salt
0.4	62.0	65.9
0.6	79.6	73.6
0.8	78.0	78.5
0.9	81.1	68.6
1.0	76.7	83.2
1.2	92.0	85.0
1.4	87.5	82.6

The Bradford method was used to quantify proteins in the upper and the bottom phase of ATPS. All separations exhibited almost identical phase volumes, which in the case of the [bmim][Cl] were 0.7 mL in the bottom phase and 1.4 mL at the upper one, while with [bmim][Br], those volumes were 0.9 mL in the bottom phase and 1.5 mL in the upper one. Absorbance values of the bottom phase fell below the limit of detection of the method, proving that proteins showed no affinity towards that phase. In contrast, protein recovery was achieved with very good results in the upper phase, with recoveries ranging approximately between 62 and 92% when working with [bmim][Cl], and 66 and 85% when doing so with [bmim][Br]. Values corresponding to 0.2 mg/mL of BSA were discarded since they were below the limit of detection. These recoveries are aligned with those obtained by (Pei et al., 2009) and (Lin et al., 2013) when working in similar extraction points. The detailed results of the extraction of BSA in the upper phase are presented in Table 3.

Protein transfer towards the ionic liquid-rich phase was driven by the combination of two phenomena. The kosmotropic nature of K_2HPO_4 and subsequent salting-out effect helped protein migration, and so did the long alkyl chain of the imidazolium cation. The kosmotropicity of this chain stimulated the ion pairing with the kosmotropic carboxylic groups of the protein surface, leading to protein displacement (Yang, 2012). Additionally, the absorbance values that were measured not only exhibited a linear behaviour, but also a very clear partitioning relation when compared to those same BSA solutions without being extracted in ATPS. In the case of the [bmim][Cl]–K_2HPO_4, this value was 1.687, while it was 1.806 for [bmim][Br]–K_2HPO_4.

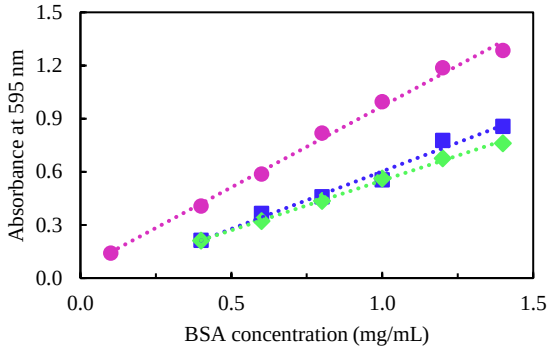


Figure 3. Absorbances obtained with the Bradford method for conventional BSA solutions (●) and extracted BSA in EP 1 with [bmim][Cl] (■), and [bmim][Br] (♦). Absorbance values of the 0.9 mg/mL solution were discarded.

3.4. Extraction of Buffalo proteins

When working with Buffalo, the recovery capacity of the ATPS was dramatically lower than that obtained with BSA. The cause is mainly the poor solubilization of the Buffalo powder in the ionic liquid.

Study of the effect of the IL and the biomass addition procedure on protein recovery

When emulating the extraction conditions for BSA with the insect solution (3 mmol of IL, 1.00 mL of 50% w/v K_2HPO_4, 1.00 mL of biomass sample) it was observed how protein recovery was significantly lower. The highest protein recovery using the sonicated 3% w/w Buffalo solution was achieved when working with [bmim][Br], obtaining an average recovery of 6.0%, which slightly dropped to 4.3% when working with [bmim][Cl]. This difference in protein recovery was caused by the difference in the hydrophobicity of the studied anions: as Br^- is more hydrophobic than Cl^- (Pei et al., 2007), it showed enhanced protein migration.

However, volumes were found to be almost identical in the ATPS of both ionic liquids: 0.6 mL in the bottom phase, 0.4 mL in the interphase and 1.4 mL in the upper phase.

No substantial improvement was observed in terms of protein recovery when adding masses of Buffalo and distilled water equivalent to those of the 3% w/w Buffalo solution (0.030 g of Buffalo + 0.970 g of water), since with [bmim][Br] a 7.0% recovery was achieved, which decreased to 4.0% with [bmim][Cl]. Despite this, phase distribution was found to be better in this case, since the volume of the interphase experienced a significant decrease. The volume of the phases in both types of ATPS were: 0.8 mL in the bottom phase, 0.1 mL in the interphase and 1.5 mL in the upper phase. Therefore, it was determined that to minimize potential inhomogeneities that may arise from the presence of solids in the interphase, it was better to add the equivalent masses of insect and distilled water.

Table 4. Results of protein extraction in EP 1, in which the effect of the ionic liquid and the biomass addition procedure were studied.

IL	Biomass addition procedure	Volume (mL)	Average phase volume (mL)			Average protein recovery (%)
			Bottom phase	Interphase	Upper phase	
[bmim][Cl]	Sonicated 3% w/w Buffalo solution	1.0	0.6	0.4	1.4	4.3
	0.030 g_{Powder} + 0.970 g_{H_2O}	1.0	0.6	0.4	1.4	7.0
[bmim][Br]	Sonicated 3% w/w Buffalo solution	1.0	0.8	0.1	1.5	6.0
	0.030 g_{Powder} + 0.970 g_{H_2O}	1.0	0.8	0.1	1.5	4.0

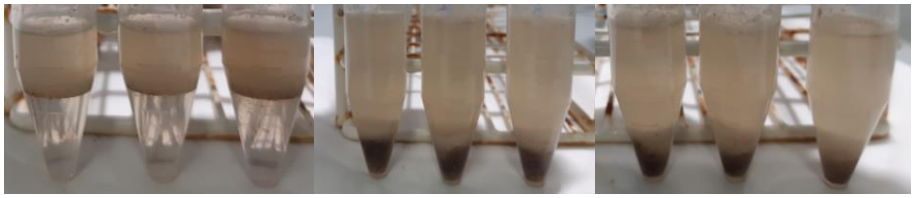


Figure 4. Appearance of the ATPS constructed in extraction point 1 using [bmim][Cl] when adding the following volumes (mass of Buffalo powder + mass of water): 1.00 mL (left), 2.00 mL (centre), and 3.00 mL (right).

Study of the scaling up and the role of the volume of a 3% w/w Buffalo solution in protein recovery

When increasing the volume of the 3% w/w Buffalo solution adding masses of Buffalo and distilled water equivalent to those of the 3% w/w Buffalo solution in extraction point 1 with [bmim][Cl], the points corresponding to 1.00 mL, 2.00 mL and 3.00 mL were first studied. However, the system only split into two phases in the case of 1.00 mL, as shown in the Figure 4. The reason why the ATPS was formed in the case of 1.00 mL only was because when adding the desired amounts of Buffalo powder and distilled water in the other experiments, the mass fractions of both the IL and the salt reached values below the binodal curve. Hence, no phase separation could happen.

As opposed to the previous extraction point, when scaling up the system and working in extraction point 2 (11.45 mmol of [bmim][Cl] and 3.815 mL of the 50% w/v K_2HPO_4 solution), the ATPS was formed in all the studied equivalent volumes, as all points were placed above the binodal curve.

Protein recovery was still low, but it was superior to that achieved in extraction point 1. However, no clear conclusions could be extracted from the impact of the increase of the added volumes, although the general trend indicated that there was a reduction in protein recovery when increasing the insect and distilled water quantities.

Additionally, protein quantification was conducted through the combustion of the sample for the quantification of N. This way, the results obtained with the Bradford method could be contrasted. However, no concluding results were obtained, since the N contribution of the ionic liquid masked the N coming from proteins.

Unlike the scaling up with [bmim][Cl], the one conducted with [bmim][Br] led to a decrease in protein recovery. These experiments were conducted starting from an equivalent volume of 1.00 mL and of 3.00 mL of 3% w/w Buffalo solution, and since the difference on the extraction efficiency was found to be just 0.15%, it was decided not to construct the other ATPS.

The scaling-up with [bmim][Cl] was repeated using a 3% w/w Buffalo solution which had not been sonicated. It was observed how there were greater variations between phase volumes despite pipetting while stirring the sample, and even protein recovery was lower. Moreover, in some cases a supernatant appeared.

3.5. Concentration and purification of the extracts

The selected samples for the purification and concentration of their extracts are indicated in Table 6. This table also indicates the number of purification steps and the maximum protein content to be recovered based on the dialysed volume and the sample volume that was used in lyophilisation. All contents of the dialysis bags exhibited absorbance at λ=280 nm, indicating the presence of proteins.

Table 5. Results of the scaling-up (EP 2), and effect of increasing volumes of 3% w/w Buffalo solution or equivalent solution (mass of Buffalo powder + mass of distilled water).

IL	Biomass addition procedure	Volume (mL)	Average phase volume (mL)				Average protein recovery (%)
			Bottom phase	Interphase	Upper phase	Supernatant	
[bmim][Cl]	0.030 g_{Powder} + 0.970 g_{H_2O}	1.0	2.6	0.2	3.7	-	6.8
	0.045 g_{Powder} + 1.455 g_{H_2O}	1.5	2.8	0.2	4.0	-	6.2
	0.060 g_{Powder} + 1.940 g_{H_2O}	2.0	3.0	0.4	4.1	-	6.0
	0.075 g_{Powder} + 2.425 g_{H_2O}	2.5	2.9	0.4	4.7	-	4.6
	0.090 g_{Powder} + 2.910 g_{H_2O}	3.0	3.0	0.5	5.0	-	5.4
	Non-sonicated 3% w/w Buffalo solution	1.0	2.5	0.3	3.7	-	4.5
		1.5	2.5	0.5	4.0	0.1	8.2
		2.0	2.5	0.3	4.7	-	3.7
		2.5	3.0	0.5	4.5	-	6.1
		3.0	3.0	0.5	5.0	-	3.9
[bmim][Br]	0.030 g_{Powder} + 0.970 g_{H_2O}	1.0	3.0	0.1	3.6	-	5.1
	0.090 g_{Powder} + 2.910 g_{H_2O}	3.0	3.5	0.5	4.6	-	5.3

Table 6. Conditions of the selected samples for protein concentration and purification as well as their number of purification rounds for the separation of [bmim][Cl]. The maximum protein content expected after lyophilisation is also indicated.

Sample name	Extraction point in ATPS with [bmim][Cl]	Biomass addition procedure	Number of protein concentrator and/or dialysis rounds	Maximum protein content to be recovered after lyophilisation based on the Bradford method and the volume used (mg)
1	1	1.00 mL 0.60 mg/mL BSA	6	0.17
2	1	1.00 mL 0.90 mg/mL BSA	6	0.25
3	2	0.005 g_{Powder} + 1.000 g_{H_2O}	6	$\leq$ 0.29
4	2		5	$\leq$ 0.29
5	2	0.030 g_{Powder} + 0.970 g_{H_2O}	5 concentrator 7 dialysis	1.04
6	2		11	0.99

Study of the content of the dialysis bag during the lyophilisation and desiccation

After lyophilising the content of the dialysis bags, the obtained Falcon tubes showed what was retained inside the dialysis membrane. In the case of BSA, all samples presented a cream/brownish coloration, while those containing Buffalo were greenish. Those colorations turned darker after the desiccation process, as shown in Figure 5.

Falcons were weighed after lyophilisation and each desiccation round to determine the recovered mass of protein through weight difference, and values were compared with the maximum theoretical recoveries. Despite seeing a significant weight variation thanks to the multiple desiccation steps, the recovered masses were still superior to those expected, indicating that apart from proteins, other compounds were recovered during the dialysis process.

Additionally, this weight difference approach presented the inherent difficulties of relying on something as sensitive to environmental variations as a scale can be, which could lead to the obtention of incoherent values. Because of this, all Falcons were added 0.50 mL of distilled water to solubilize their content. However, almost all samples exhibited poor solubilization.

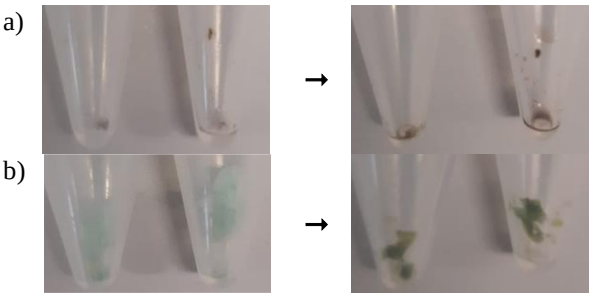


Figure 5. Content of the Falcon tubes after the lyophilisation process (left) and after the 3 rounds of desiccation at 40ºC and -76 cmHg for 24 h (right). a) corresponds to BSA (Sample 1), and b) to Buffalo (Sample 6).

When conducting quantification with the SimpliNano spectrometer, the obtained concentrations were very high in some cases. Since it was thought that that was caused by the turbidity originated from the bad

solubilization, samples were newly quantified with it after their centrifugation (and further dilution in the case of samples 5 and 6).
In this second quantification with SimpliNano, the concentration of two Falcons fell within the detection limit of the Bradford method, so they were quantified with it. However, as their values ended up being below the limit of detection, all the other samples were quantified. The only sample in which quantification with the Bradford method was possible was sample 6.

Table 7. Evolution in the mass recovery after the dialysis process for each sample after lyophilisation and desiccation at 40ºC and -76 cmHg, and mass of protein recovered after quantification with SimpliNano and the Bradford method.

	Mass recovered through weight difference (mg)				**Protein recovered (mg)**		
		Desiccation at 40ºC and -76 cmHg for 24 h			**SimpliNano**		
Sample	**Lyophilisation**	1st round	2nd round	3rd round	1st round	2nd round	**Bradford**
1	77.80	18.53	10.90	3.14	1.84	1.56	-
2	41.00	2.91	3.28	3.58	1.23	1.28	-
3	51.10	0.47	0.77	0.90	2.76	2.96	-
4	28.79	0.06	0.01	0.13	1.61	1.61	-
5	41.19	6.89	7.57	7.57	15.12	12.74	-
6	52.13	6.99	7.08	7.08	19.12	14.75	0.31

This impossibility to quantify all samples except from 6 with the Bradford method proved the potential presence of traces of [bmim][Cl] in the samples, which would lead to the distortion of the obtained absorbances in SimplyNano because of the proximity of its absorbance peaks to λ=280 nm.
This sample whose absorbance at λ=595 nm entered within the range of the Bradford calibration curve had a protein mass recovery of 0.31 mg, corresponding to just the 31% of the maximum one. Having seen this, it was concluded that there had been some protein losses during the dialysis process, or that the poor solubilization of the samples led to non-representative results.

Study of the purification efficiency of the dialysis process

Fourier transform infrared analysis (FTIR) of the individual compounds present in the extractions, showed that the BSA and the Buffalo solution exhibited identical peaks (3,273.57 cm^{-1}, 2,166.63 cm^{-1} and 1,635.34 cm^{-1}). [bmim][Cl] in solution format and the the K_2HPO_4 50% w/v solution presented them too, so [bmim][Cl] could only be distinguished from them because of 1 extra little peak it had at 1,169.2 cm^{-1}, and K_2HPO_4 50% w/v because of 2 different peaks: one at 1062.59 cm^{-1} and another at 985.447 cm^{-1}.

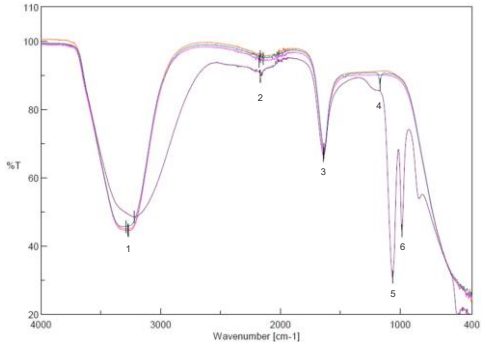


Figure 6. Plot of the FTIR spectra of diluted [bmim][Cl] (4 peaks, nº 1 to 4), 50% w/v K_2HPO_4 (5 peaks, nº 1 to 3, 5 and 6), and 2 mg/mL BSA and filtered 3% w/w Buffalo (3 peaks nº 1, 2 and 3).

Samples corresponding to the protein extractions before dialysis showed peaks indicating the clear presence of both [bmim][Cl] and K_2HPO_4, whereas those corresponding to the dialysed samples did not exhibit them, indicating their removal. However, this affirmation is not completely solid because of the shared peaks between substances. Spectra for samples containing BSA were almost identical, as was the case of those containing Buffalo.

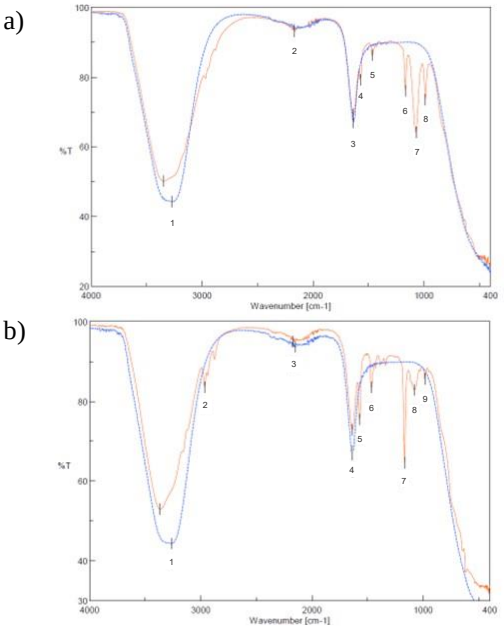


Figure 7. Comparison of the FTIR spectra obtained for samples before and after dialysis, where the orange lines correspond to the spectra of extracts and the blue ones to the ones of the dialysed samples. a) corresponds to sample 1 (containing BSA), while b) to sample 6 (containing Buffalo).

Nuclear magnetic resonance (NMR) analysis of the inside of the dialysis bags confirmed the suspected presence of [bmim][Cl] in traces in the totality of the studied samples, as depicted in Figure 8. Therefore, it seems clear that the protein quantification problems that occurred when using SimpliNano spectrometer were caused by the presence of [bmim][Cl] in traces, which led to interferences in the measurement of absorbance at $\lambda = 280$ nm. Hence, it can be concluded that neither protein concentrators nor dialysis are efficient methods for the purification of the protein extracts.

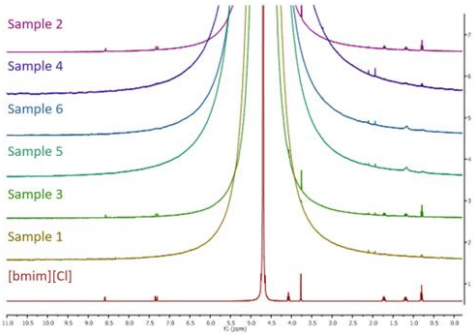


Figure 8. NMR spectra of all dialysed samples compared to the spectra of [bmim][Cl]. It can be clearly observed how all samples contain IL.

3.6. Recovery of the IL in the dialysates

The IL recovery during the dialysis process was studied in two of the dialysed samples: 2 (containing BSA) and 6 (containing Buffalo).

The amount of IL recovered in the dialysates was determined through the combustion of the samples for the determination of their N concentration, which was then converted into [bmim][Cl] mass considering the dialysate volume employed in each dialysis round.

In the case of sample 2, since it underwent a total of 6 dialysis rounds, all dialysates were studied, and so was the water in which the dialysis bag was kept hydrated during a weekend.

It must be considered that among N values obtained for this sample, the only one that fell within the range of the calibration curve of the system was the one of the first dialysis round. The rest were so diluted that were placed below the lowest point of the curve, corresponding to 5 mg/L of N. Considering that the maximum mass of [bmim][Cl] that could be recovered from this sample based on the volume of the dialysed extract was 0.186 g, it seems clear that most of the IL was removed after the first dialysis round, in which 0.177 g were removed. If all obtained masses of [bmim][Cl] were summed, the obtained value would be 0.193 g, which exceeds the maximum theoretical value. This may be since some values were placed below the minimum value of the calibration curve or because protein losses occurred through the dialysis membrane.

When studying dialysates of sample 6, since the employed [bmim][Cl] quantity was a lot higher than in the previous case, samples from the first three dialysis rounds were diluted 1/10, while those from rounds 10 and 11 were not diluted. Knowing that the maximum mass of IL that could be recovered in that case were 1.459 g and that in the first two dialysis rounds this value was exceeded (1.251 g in the first one and 0.323 g in the second one), and considering that these two concentrations fell within the range of the calibration curve, it seems possible that some proteins were lost during the dialysis process. This hypothesis would support the origin of the low protein recovery of this sample after its dialysis, which only accounted for the 31% of the maximum one.

4. Conclusions

The present work studied the viability of extracting proteins from Buffalo, a protein-rich insect, using ATPS formed by an ionic liquid ([bmim][Cl] or [bmim][Br]) and a salt solution (50% w/v K_2HPO_4).

ATPS showed very good protein recoveries in their ionic liquid-rich phase when working with BSA, with recoveries ranging between 62 and 92% in the case of [bmim][Cl], and between 66 and 85% in that of [bmim][Br].

However, the efficiency of those systems experienced a substantial drop when working with Buffalo powder: less than 10% of recovery. This was because of its poor solubilization in the media. In order to improve the recovery of proteins by increasing the homogeneity between phase volumes, it was decided to add the corresponding mass of powder first, and then the subsequent volume of distilled water instead of pipetting the insect solution. Still, ATPS were unable to extract proteins from the insect powder because the ionic liquid could not correctly solubilize it.

Regarding quantification methods, the Lowry method proved to be inviable because of the reaction of the ionic liquid with the Folin reagent, leading to the formation of a precipitate that absorbed the coloration of the sample.

On the other hand, the use of absorbance at $\lambda = 280$ nm resulted to be inviable for quantification too, since the proximity of the absorbance peaks of the ionic liquid with this wavelength led to distortions in the measurement, obtaining misleading results.

The same applied for the combustion of the samples for the determination of their N content, since in these two ionic liquids the N content was so high that it masked the N contribution of the extracted proteins.

At the end, the Bradford method was the only quantification method that provided reliable results.

The protein concentrators used to purify protein were found to be too slow to separate the ionic liquid from the protein extracts. Despite dialysis being apparently more effective, it turned out to be an unreliable approach as well, since ionic liquid traces were found in the totality of the dialysed samples. Additionally, protein losses occurred during the process. This was confirmed by the low recoveries quantified with the Bradford method, and also the high N concentrations obtained in the dialysates.

Therefore, it seems clear that further research is needed to find ways of enhancing Buffalo solubilization in the ionic liquid, and to design of novel ionic liquids to facilitate the recovery of its proteins. Apart from this, protein-benign, fast and reliable approaches for the total separation of the ionic liquid from the protein extracts need to be developed.

Acknowledgements
Alba Zurita would like to thank Dr. Katarzyna Glińska, Dr. Mabel Torréns, Dr. Mayreli Ortíz and Dr. Esther Torrens for their kind contribution. She also thanks the PI-SET Group for the hosting and supervision during her stay. The members of PI-SET are recognised by the Comissionat per a Universitats i Recerca del DIUE de la Generalitat de Catalunya (2017-SGR-396) and supported by the Universitat Rovira i Virgili (2017PFR-URV-B2-33).

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