Universidade de Aveiro Departamento de Química

2018

Filipa Alexandra Lima Moreira

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ii

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iv

The important thing is to not stop questioning. Curiosity has its own reason for existing.

Albert Einstein

o júri

presidente

Prof. Doutora Maria Inês Purcell de Portugal Branco
Professora Auxiliar do Departamento de Química da Universidade de Aveiro

Doutora Sónia Patrícia Marques Ventura
Equiparada a Investigadora Auxiliar do Departamento de Química da Universidade de Aveiro

Prof. Doutor Oscar Rodriguez Figueiras
Investigador "Ramón y Cajal" Departamento de Engenharia Química, Escola Técnica Superior de Engenharia (ETSE), da Universidade de Santiago de Compostela

agradecimentos

Antes de mais, gostava de começar por agradecer ao meu co-orientador, Pedro Carvalho, pela imensa paciência e disponibilidade que teve sempre para comigo. Ao Pedro um enorme obrigada! À minha orientadora, Drª Sónia Ventura também um obrigada pela disponibilidade e atenção que teve para comigo. Também quero agradecer aos membros PATh por todo o carinho e ajuda que sempre me demonstraram. Foram muito mais que um grupo de colegas de trabalho, uma segunda família que sempre soube apoiar. Um especial obrigada à Liliana por todos os desabafos, ajudas e troca de gargalhadas. Por último, mas mais importante, um enorme obrigada à minha mãe pelo apoio incansável que teve comigo sempre neste percurso. Sem ela nada teria sido possível! Obrigada pelo incentivo, pela força, e pelos inúmeros conselhos que sempre fizeram que não perdesse o rumo. Um grande obrigada aos avós e namorado pelo constante apoio. Todos vocês são a minha única família e que fizeram de mim a pessoa que eu sou hoje e, portanto, as minhas vitórias são as vossas vitórias também! Uma coisa vos garanto, a pessoa que me tornei hoje deve-se a cada um de vocês. Obrigada mais uma vez por acreditarem sempre em mim, por terem feito parte deste percurso, e claro, por nunca terem desistido de mim. Obrigada por me mostrarem que sem empenho e sem trabalho não se vai a lado nenhum, e que para tudo na vida é preciso ter força e dedicação. Sinceramente, acho que nunca vos conseguirei agradecer o suficiente!

x

Palavras-chave

Gracilaria sp, R-ficoeritrina, purificação, sistemas micelares de duas fases aquosas, processo de purificação em regime contínuo, surfatantes, líquidos iónicos tensioativos, co-surfatantes

Resumo

Atualmente, as macroalgas têm sido amplamente estudadas como nova matéria-prima na indústria. Este elevado interesse deve-se ao facto de as macroalgas terem condições de produção aliciantes, para além de apresentarem na sua constituição compostos de alto valor acrescentado, como é o caso das ficobiliproteínas. Ficobiliproteínas, em particular a R-ficoeritrina, são proteínas que atuam como pigmentos captadores de luz natural, as quais possuem propriedades biológicas bastante atrativas, especialmente propriedades antioxidantes, anticancerígenas e anti-inflamatórias. Assim sendo, dadas as características interessantes desta proteína, muitas empresas têm vindo a desenvolver um elevado interesse por estas macromoléculas, considerando nomeadamente o elevado potencial destas nos setores farmacêutico, cosmético e energético. Posto isto, é necessário estabelecer métodos de extração e purificação eficientes que permitam a utilização da R-ficoeritrina. Relativamente aos métodos já existentes, estes envolvem múltiplas etapas, as quais representam normalmente processos de elevada complexidade e/ou que requerem um elevado consumo energético o que implica o encarecimento do produto final. Neste sentido, este trabalho terá como objetivo o desenvolvimento e otimização de um processo de purificação da R-ficoeritrina em regime contínuo, utilizando sistemas micelares de duas fases aquosas (AMTPS). Estes sistemas apresentam-se como mais seletivos e mais biocompatíveis, por não interferirem com as biomoléculas. Neste trabalho, será pela primeira vez projetada uma unidade de separação com controlo de temperatura para a aplicação de AMTPS em regime de fluxo contínuo.

Abstract

Currently, macroalgae have been widely studied as a new raw material in the industry. This high interest is due to the fact that macroalgae have attractive production conditions, as well as having high added value compounds, such as phycobiliproteins. Phycobiliproteins, in particular R-phycoerythrin, are proteins that act as natural light-picking pigments, which have very attractive biological properties, especially antioxidant, anticancer and anti-inflammatory properties. Therefore, given the interesting characteristics of this protein, many companies have developed a high interest in these macromolecules, considering in particular the high potential of these in the pharmaceutical, cosmetic and energy sectors. Having said that, it is necessary to establish efficient extraction and purification methods that allow the use of R-phycoerythrin. In relation to the existing methods, these involve multiple steps, which normally represent processes of high complexity and/or which require a high energy consumption which implies the enhancement of the final product. In this sense, this work will aim to develop and optimize a continuous purification process of R-phycoerythrin using two-phase aqueous micellar systems (AMTPS). These systems appear to be more selective and more biocompatible because they do not interfere with biomolecules. In this work, a separation unit with temperature control will be designed for the first time for the application of AMTPS in continuous flow regime.

CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF ABREVIATIONS

AMTPS – aqueous micellar two-phase systems

ATPS – aqueous two-phase systems

CMC – critical micelle concentration

ILs – ionic liquids

LLE – liquid-liquid extraction

[N$_{1,1,1,12}$]Br – benzyldodecyldimethylammonium bromide

[N$_{4,4,4,4}$]Cl – tetrabutylammonium chloride

[N$_{4,4,4,4}$]DBS – tetrabutylammonium dodecylbenzenesulfonate

[N$_{4,4,4,4}$]DS – tetrabutylphosphonium dodecylsulphate

[P$_{4,4,4,4}$]DS - tetrabutylphosphonium chloride

[P$_{4,4,4,4}$]DS – tetrabutylphosphonium dodecylsulphate

R-PE – R-phycoerythrin

SAILs – surface-active ionic liquids

1. INTRODUCTION

The red algae are one of the most abundant classes of macroalgae spread all over the world. There are more than 8000 known species, most of which grow in the sea. These are found in the intertidal and subtidal zones to depths that can vary from 40 to 250 meters.[1] Red macroalgae are considered the most important source of numerous biologically active metabolites in comparison with green and brown macroalgae.[1] The walls are made of cellulose, agars and carrageenans, however the biochemical composition varies with the species and, potentially, with location, seasonality and growth conditions.[1][2]

Phycobiliproteins are a group of coloured proteins (pigments) commonly present in cyanobacteria and red algae, possessing a large spectrum of applications.[3] Phycobiliproteins, also known as chromoproteins, are classified into four groups based on the presence of different chromophores, namely the Phycoerythrin (PE, purple), Phycocyanin (PC, blue), Phycoerythrocyanin (PEC, orange) and Allophycocyanin (APC, bluish), and present a UV-VIS spectra with characteristics absorbance wave lengths of 565-575 nm, 615-640 nm, 575 nm and 650-655 mm, respectively.[3][4] PE is widely abundant in a large range of natural sources, like red algae, cyanobacteria and cryptomonads[5][6]; however it is in red algae where phycoerythrin is most abundant[6], with protein contents higher than 20% (dry weight).[7] PE can be divided into four types: R-phycoerythrin (R-PE), B-phycoerythrin, C-phycoerythrin and b-phycoerythrin, a classification attributed depending on their source/origin. Actually, B-phycoerythrin and R-PE are the most abundant phycobiliproteins in red algae, with a molecular weight varying between 240 and 260 kDa.[6].

Phycoerythrin is widely used in the biochemistry and clinical area for medical diagnostics.[8] Moreover, PE has interesting biological properties for health care, such as anticancer,[4] antioxidant,[9][10] antidiabetic,[10] immunosuppressive and antihypertensive properties.[11] Furthermore, phycoerythrin is also used in the food industry, as an alternative coloring agent to artificial dyes, with antioxidant properties and protection character towards food's lipid oxidation.[12]

Phycoerythrin has gained a special importance due to its particular characteristics and properties, such as its nature as an optically active center, translated into high

absorption coefficients and emission quantum yields.[13][14] This characteristic behavior foster the interest of this fluorescent protein in new fields, like its inclusion as optically active centers in luminescent solar concentrators, specific devices being created to improve the absorption efficiency of photovoltaic panels.[15][16] More recently, other applications of phycoerythrin have been envisioned, namely its use as a fluorescent probe in flow cytometry and immunofluorescence microscopy[17], and as a probe in proximity or interaction assays between two molecules by fluorescence resonance energy transfer, as a protein marker in electrophoretic techniques and size-gel exclusion chromatography, as tracers in fluorescent immunoassays, immunophenotyping, just to mentioned a few.[18][4] Taking into account all the possible applications for PE it becomes clear its high potential commercial value[8], and thus, how important is the demand for sustainable and efficient downstream processes to recover phycoerythrin from red macroalgae.[19]

Over time different downstream processes have been proposed.[8][20] However, the processes envisioned are normally characterized by their low selectivity and purification performances, and low yields processed, which implies quite complex and time consuming extraction and purification processes.[21] Thus, the development of new, efficient, economically and technically viable downstream processes to recover pure R-PE from marine biomass is a crucial demand. Regarding the R-PE extraction processes, methods involving osmotic shock and cell wall breakage, are widely reported.[8] However, this type of procedure presents important disadvantages, namely the fact that it implies degradation of R-PE by proteases.[8] Furthermore, the use of liquid nitrogen, despite facilitating the destruction of the cell wall, imposes high costs at any scale.[8] Recently, Dumay and co-workers[22] proposed an enzymatic process based on xylanases for the *Palmaria palmata* cell wall disruption as a new extraction method, with yields 62 times greater than those obtained without the enzymatic treatment.[8][6] However, this methodology is limited by the availability of specific enzymes adapted to attack the cell wall and also the specificity of the enzymes for each type of biomass.[7]

Phycoerythrin is typically purified by the combination of several techniques, such as ammonium sulphate precipitation, ion-exchange chromatography, gel filtration and

hydrophobic interaction, chromatography on hydroxylapatite, among others.[23][18][20] Ammonium sulphate precipitation is commonly used as a first purification step, where different saturation percentages of salt are used depending on the species.[24] The solubility of proteins varies in accordance with the ionic strength of the solution, which is affected by the salt concentration. *Salting-out* is a very valuable phenomenon to facilitate the purification of a given protein, since proteins differ evidently in their solubility in water at high ionic strength.[6] Nevertheless, the chromatographic methods, such as ion-exchange, gel filtration, affinity chromatography, and hydrophobic interaction chromatography representing the technique with highest application on the proteins purification. The most popular is the ion-exchange chromatography, in which the separation of ions and polar molecules based on their net charge is assessed. Ion-exchange chromatography retains molecules on the column based on ionic interactions.[6][25] The gel filtration chromatography process describes the protein purification based on the molecule size.[6] The affinity chromatography takes advantage of the high affinity of many proteins for specific chemical groups present in the chromatographer matrix. The hydrophobic interaction type (HIC) uses the properties of hydrophobicity to separate proteins. The separation is based on the reversible interaction between a protein and the hydrophobic ligand bound to the chromatographer matrix.[6] The downstream processes available are somehow complex, since all these methods comprise a combination of sequential steps of purification as summarized in **Table 1**.

Nowadays, both the extraction and purification of PEs are being investigated but the methodologies employed remain expensive and time-consuming, as they involve multiple steps.[26] Therefore, it is important to work to improve the performance of these methods.[8]

In this sense, liquid-liquid extraction (LLE) appears as an alternative procedure allowing the elimination of the traditional multiple steps and reducing costs.[27] Liquid-liquid extraction consists in the selective transfer of molecules from one phase to another when immiscible or partially soluble liquid phases are brought into contact with each other.[27][28] This extraction technique becomes advantageous in the sense that it is represented by good extraction capacity, high selectivity, and high

yields, while providing low costs and fast processing.[27][29] However, standard LLE methods rely on the use of volatile organic solvents, known to lead to denaturation/chemical degradation of the proteins.[27][30]

Table 1. List of methods described in literature for the R-PE purification. The parameters described are the extraction yields and the purity indexes.

Purification procedure steps	Extraction Yield (mg/g DW)	Purity Index	References
$(NH_4)_2SO_4 \rightarrow GF \rightarrow IE \rightarrow UF + GF$	0.117	4.70	[5]
$(NH_4)_2SO_4 \rightarrow IE \rightarrow UF$	0.038	2.10	[31]
$(NH_4)_2SO_4 \rightarrow GF$		4.50	[32]
Preparative electrophoresis	0.562	3.20	[18]
$HIC \rightarrow IE$	0.152	3.60	[33]
Hydroxyapatite + GF	0.600	6.67	[34]
$HIC \rightarrow IE$	0.825	4.60	[35]
$(NH_4)_2SO_4 \rightarrow IE$	0.841	5.59	[36]
$HIC \rightarrow$ hydroxyapatite	0.425	3.90	[26]
$HIC \rightarrow IE$	0.500	3.26	[26]
$2GF \rightarrow IE$	0.940	4,89	[37]
$(NH_4)_2SO_4 \rightarrow$ hydroxyapatite	0.540	3,20	[38]
$(NH_4)_2SO_4 \rightarrow IE$	0.810	5,20	[25]

GF: gel filtration; IE: ion exchange; UF: ultrafiltration; HIC: hydrophobic chromatography

To overcome these limitations aqueous two-phase systems (ATPS) have been proposed as an alternative to apply on the LLE extraction processing. ATPS are characterized by the formation of two aqueous-rich phases rich in two structurally distinct compounds, which are immiscible at given conditions.[39] Typically, ATPS have been reported as systems composed by the combination of two polymers,[30] two salts,[30] a salt and a polymer,[40][41] or surfactants.[42] For this reason ATPS are considered as a flexible system capable of obtaining high selectivity and yields

when applied on the development of alternative downstream process.[27][39][43] However, some limitations associated with the most traditional ATPS have been identified, such as the high cost of some polymers allied to the high viscosities of the phases limiting the scale-up and increasing the corrosion inherent to the high content of inorganic salts, leading to regular and costly equipment maintenance and wastewater treatment.[39] Ionic liquids (ILs) have been studied as alternative solvents on the formation of ATPS, due to their unique properties, such as their high solvation capability for a wide range of compounds, and the possibility of changing their properties to be applied in a specific application, only by the different cation and anion combinations.[44] Moreover, when these are applied as phase formers, they input a lowest viscosity to the system, fast phase separation, and high extraction efficiencies for most of the biomolecules.[44][39] The first study on the application of an imidazolium based IL with an inorganic salt was reported by Rogers *et al*, in 2003.[45] Since then, several studies have been reported with the IL-based ATPS going, currently, beyond the simple use of salt + IL combinations, to combinations with amino acids, carbohydrates, polymers and others.[46][47]

Aqueous micellar two-phase systems (AMTPS) are a particular class of ATPS that use surfactants as solvents and appear as a promising technique for bioseparation processes.[29] AMTPS present many advantages, such as the simplicity, the rapid mass transfer due to low interfacial tension, a higher selectivity performance while maintaining the native conformation structure of biomolecules.[48] When surfactant molecules are dissolved in water at concentrations above their critical micelle concentration (CMC), they form aggregates known as micelles.[39][42] Micelles are structures formed by the non-covalent aggregation of surfactant monomers, which can assume a spherical, cylindrical or planar shape.[42][49] The micellization process results from a subtle balance of intermolecular forces, including hydrophobic, steric, electrostatic, hydrogen bonding, and van der Waals interactions. However, the main attractive force outcomes from the hydrophobic effect associated with the nonpolar surfactant tails, and the main opposing repulsive force results from steric interactions and electrostatic interactions between the surfactant polar heads. At certain surfactant concentrations and temperatures, micellar solutions can macroscopically

phase separate into a surfactant-rich phase and a surfactant-poor phase. These systems feature a single phase below a certain temperature, known as cloud point temperature, T_{cloud}.[39] Thus, as illustrated in **Figure 1**, below the T_{cloud} occurs the phase separation.[39]
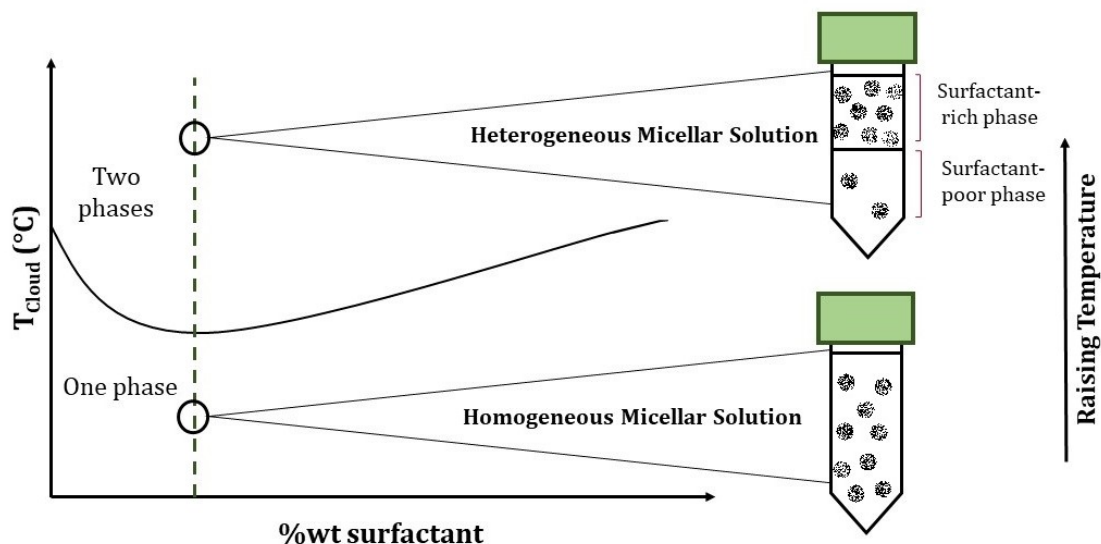
Figure 1. Representation of a typical phase diagram representing a conventional AMTPS, in which the spontaneous phase separation occurs when increasing the system temperature. The curve corresponds to the binodal curve, the boundary between the monophasic and biphasic regions, as indicated in the graphical representation.

Surfactants can be either anionic or cationic, when the charge is on the head; dipolar (zwitterionic); or nonionic, when they have no charge.[42][29][48] Recently, the addition of additives, such as electrolytes and polar organic solutes, is being studied since it was proved that depending on their physico-chemical properties, these can cause significant changes in the T_{cloud} of the main surfactant, thus increasing it or decreasing it.[43][29][50] Actually, in the last years, a new proposal of AMTPS involving the use of ILs as co-surfactants, has been investigated.[29][51] In a study developed by Bowers *et al.*[51] the authors described the behavior of ILs that self-aggregate due to the presence of sufficiently long alkyl chains, with at least eight carbons, turning them amphiphilic molecules.[51] It was proved that the use of ILs as co-surfactants may increase or decrease the CMC and the aggregation number, depending on the chemical structure of the IL (cation, anion, alkyl chain length). The

8

use of salts, whether simply salts or buffer solutions, has the effect of a typical additive, *i.e.*, it always has the same effect on T_{cloud}, since they are not part of the formation of micelles or aggregates. However, the addition of tensioactive ILs (or surface-active ILs) with long alkyl chains imply different effects in the formulation of AMTPS and in the T_{cloud}, as recently discussed.[52] In general, the AMTPS based only in the common surfactant are referred as the conventional AMTPS and the systems based in the common surfactant-based and IL as co-surfactant are referred as mixed AMTPS. For conventional AMTPS, where no additives are used, the operating temperature is controlled only by the surfactant structure, while mixed systems, which use additives (ILs), shows that the T_{cloud} is significantly influenced (*i.e.* it can be increased or decreased) by the incorporation of IL, even at very low concentrations of the co-surfactant (*e.g.* 0.3 wt%).[52]

SAILs have been the subject of several studies, because they exhibit physical and chemical properties and they can self-aggregate to form micelles in aqueous solutions, which makes them potentially attractive for many applications, such as separation, condensation, and extraction.[53][54] Not only studies of SAILs based on imidazolium have been reported, but also SAILs based on ammonium, phosphonium and pyridinium structures.[52][53] Whereas the SAILs based on imidazolium, ammonium (with only one long alkyl chain) and pyridinium induce the formation of smaller micelles and, as such, promote water-head group interactions and the cloud points increase; for the most hydrophobic phosphonium and ammonium-based SAILs (with 2 long alkyl chains), an increase in the micelles occurs and thus, the clouds points decrease.[52] These behaviors are also concentration-dependent as recently analyzed.[52]

Thus, AMTPS stand out as an advantageous and interesting biphasic system able to be applied on the development of alternative downstream processes, raising the attention of many researchers.[29][52] Moreover, the physical and chemical properties of each of the compounds added to the systems allied to the operational conditions will affect the stability of the molecules of interest to be extracted.[39] Regarding the application of the SAILs in AMTPS, there is not literature yet that

discusses these systems, and therefore, in this work it is also proposed to make a study about the viability of SAILs in AMTPS.

Envisioning the industrial potential of the use of AMTPS as biphasic systems to develop more efficient liquid-liquid extraction systems, the continuous purification regime should be developed.

Scope and Objectives

R-PE has been focus of strong research because of its main properties, allowing it to have a wide range of applications, ranging from the cosmetic, food, and pharmaceutical sectors to the energy sector. Most extraction and purification processes require multiple steps imposing thus, high operating costs, high complexity, and sometimes, lower efficiencies. In addition, unfortunately, the target proteins are always present in low concentrations, and therefore the downstream processing of proteins is difficult due to the requirement of achieving a high protein yield with high purity level. Since the PE purity level is normally defined considering the different applications, nowadays, there is still a huge demand for task-specific downstream processes, able to recover the protein with the purity levels required. Due to the lack of industrial knowledge in what concerns the development of downstream processes, several aquaculture companies have shown a strong interest in the development of processing technology able to allow the continuous extraction and purification of R-PE with high efficiencies, but without involving high costs, long and complex steps.

In this work, it is intended to develop an efficient, selective, sustainable and continuous downstream process to recover and purify R-PE from the red macroalga *Gracilaria sp*. To accomplish this objective, several AMTPS were applied. Briefly, a conventional AMTPS based in Tergitol 15-S-7 as the surfactant and different mixed AMTPS, in which it was used the Tergitol 15-S-7 as the main surfactant and benzyldodecyldimethylammonium bromide, $[N_{1,1,1,12}]$Br, as the co-surfactant were investigated. Two different families of SAILs (ammonium and phosphonium) were studied considering the preparation of the respective AMTPS and their application on

the R-PE purification. After defined and characterized the most performant AMTPS, an apparatus was designed and optimized aiming the application of a process of purification based in AMTPS in continuous regime and easy to scale-up to an industrial process.

2. EXPERIMENTAL SECTION

2.1. Materials
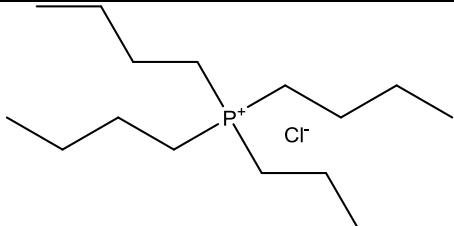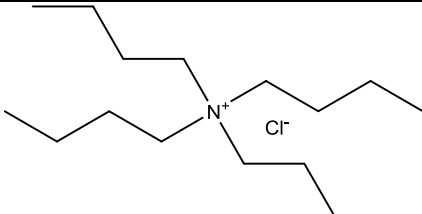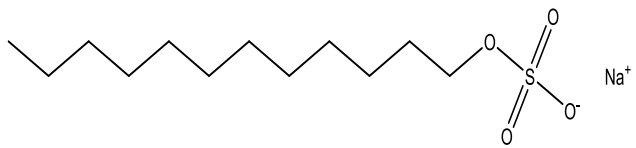
Tetrabutylphosphonium chloride, $[P_{4,4,4,4}]Cl$, with purity ≥ 95% acquired from Iolitec, tetrabutylammonium chloride, $[N_{4,4,4,4}]Cl$, with a purity ≥ 97% acquired from Sigma-Aldrich, sodium dodecylsulfate, SDS, with purity ≥ 99%, acquired from Acros Organics, and sodium dodecylbenzenesulfonate, SDBS, acquired from Aldrich with technical grade, were used as precursors for the synthesis of tetrabutylphosphonium dodecylsulfate, $[P_{4,4,4,4}]DS$, tetrabutylammonium dodecyl sulphate, $[N_{4,4,4,4}]DS$, and tetrabutylammonium dodecylbenzenesulfonate, $[N_{4,4,4,4}]DBS$. Dodecyltrimethylammonium bromide, $[N_{1,1,1,12}]Br$, with purity ≥ 99%, was acquired from Sigma-Aldrich. The tetrabutylammonium dodecyl sulphate, the tetrabutylphosphonium dodecylsulfate and the tetrabutylammonium dodecylbenzenesulfonate, were synthesized in house following the synthesis procedure proposed by Özdil *et al.*[55] and described in the following section.
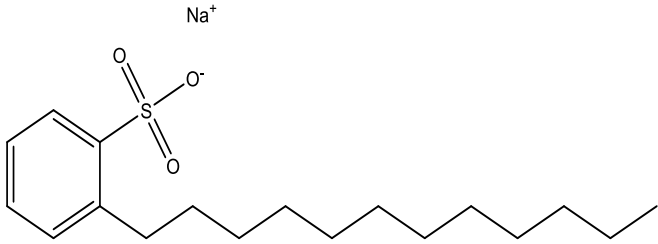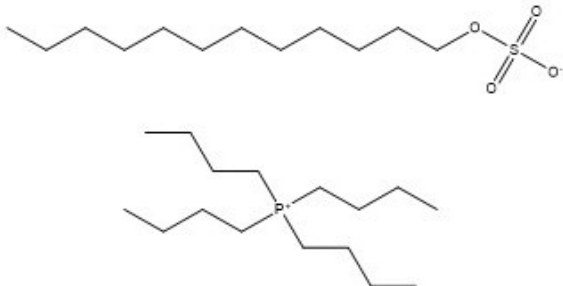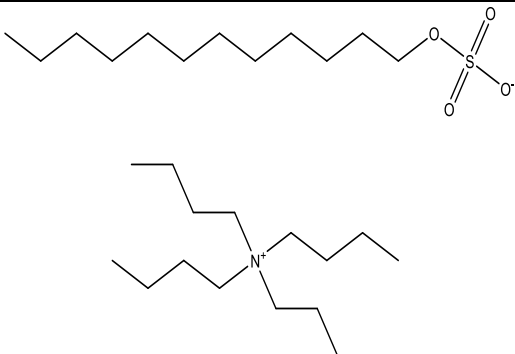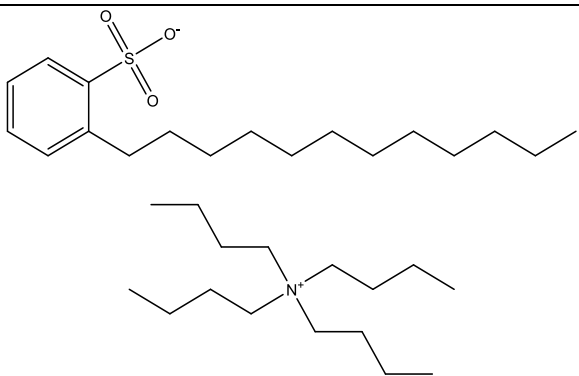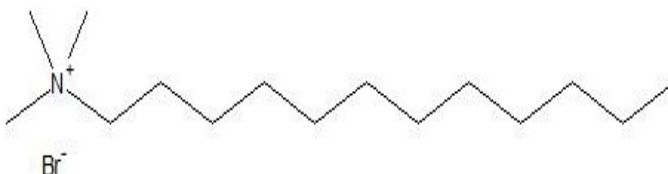
The non-ionic surfactant Tergitol 15-S-7 was purchase from Sigma-Aldrich with a mass fraction purity of ≥ 99% and used as received. The McIlvaine buffers of different pH, see **Table 2**, were prepared in the laboratory using di-sodium hydrogen phosphate anhydrous, N_2HPO_4, with purity ≥ 99%, and citric acid anhydrous, $C_6H_8O_7$, with purity 99.5%, acquired at Fisher Chemical and Synth, respectively. The compounds name, abbreviation, chemical structures, mass fraction purity, supplier and molecular weight of the chemicals are reported in **Table 3**.
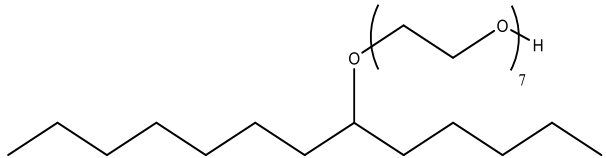
Table 2. McIlvaine buffer composition for a final volume of 1L.[56]

pH	V (mL) of 0.2M Na_2HPO_4 ± 0.5	V (mL) of 0.1M citric acid ± 0.5
3.0	205.5	794.5
4.0	385.5	614.5
5.0	515.0	485.0
6.0	631.5	368.5
7.0	823.5	176.5
8.0	972.5	27.5

Table 3. Compounds name, abbreviation, chemical structures, mass fraction purity (%), supplier and molecular weight of the chemicals used.

Compound	Structure
Tetrabutylphosphonium chloride [P$_{4,4,4,4}$]Cl Purity: ≥ 95% Supplier: Iolitec M_w: 294.89 g.mol^{-1}	
Tetrabutylammonium chloride [N$_{4,4,4,4}$]Cl Purity: ≥ 97% Supplier: Sigma-Aldrich M_w: 277.92 g.mol^{-1}	
Sodium dodecylsulfate SDS Purity: ≥ 99% Supplier: Acros Organics M_w: 288.38 g.mol^{-1}	

Sodium dodecylbenzenesulfonate SBDS Purity: Tech. Grade Supplier: Aldrich M_w: 348.48 g.mol^{-1}	
Tetrabutylphosphonium dodecylsulphate [P$_{4,4,4,4}$]DS Purity: ≥ 90% M_w: 524.86 g.mol^{-1}	
Tetrabutylammonium dodecylsulphate [N$_{4,4,4,4}$]DS Purity: ≥ 95% M_w: 507.86 g.mol^{-1}	
Tetrabutylammonium dodecylbenzenesulfonate [N$_{4,4,4,4}$]DBS Purity: ≥ 95% M_w: 567.96 g.mol^{-1}	
Benzyldodecyldimethylammonium bromide [N$_{1,1,1,12}$]Br Purity: ≥ 99% Supplier: Sigma-Aldrich M_w: 384.45 g.mol^{-1}	

| Tergitol 15-S-7
$C_{13}H_{27}O(CH_2CCH_2O)_7H$
Purity: ≥ 99%
Supplier: Sigma-Aldrich
M_w: 515 g.mol^{-1} | |

2.1. Methods

2.1.1. Ionic liquids synthesis

The reagents, SDS and $[N_{4,4,4,4}]Cl$ or SDS and $[P_{4,4,4,4}]Cl$ or SDBS and $[N_{4,4,4,4}]Cl$, were dissolved in 50 mL of distilled water and allowed to react overnight. The double exchange reaction, *i.e.* the anion exchange, results on the formation of the compound of interest and sodium chloride, NaCl; the reactions of each compound are reported in *APPENDIX A – REACTIONS*. The water, present in the mixture, was removed by means of a rotary evaporator operating at 323.15 K and 102 mPa. To further remove traces of water, the mixture was further dried on a vacuum line operating at 1 Pa, 313.15 K and continuous stirring. The mixture was washed with absolute ethanol to precipitate the NaCl and placed in the refrigerator at 277.15 K overnight. It is important to note that ethanol with high purity, free of impurities, was used aiming at ensuring no secondary compounds formation. Subsequently, a filtration was performed to remove all the formed precipitate (NaCl), and the final mixture was taken to the rotary evaporator at moderate vacuum (175 mPa) to remove the ethanol. In the end, the traces of ethanol were removed from the mixture by drying on a vacuum line, operating at 1 Pa, 313.15 K and continuous stirring.[55] The SAILs structures and purities were verified by nuclear magnetic resonance, NMR, to the proton, H^1, carbon, C^{13}, and phosphorus, P^{31}, elements and found to be 95 % for $[N_{4,4,4,4}]DBS$ and $[N_{4,4,4,4}]DS$, and 90% for $[P_{4,4,4,4}]DS$.

2.1.2. Macroalgae conditioning

As mentioned, this work aims at extract and purify phycobiliproteins, more specifically R-PE, from the red macroalgae, *Gracilaria sp*. The macroalgae, collected in May (2018), was kindly offered by ALGAplus, an aquaculture company located in Ílhavo, Aveiro, Portugal. An image of the macroalgae collected is depicted in **Figure 2**. After received, the macroalgae samples were washed with distilled water, then dried with a chemical-free absorbent paper, weighted and stored in a freezer at 253.15 K.

Figure 2. Red mcroalgae sample of *Gracilaria sp.* obtained from ALGAplus.

2.1.2. Solid-liquid extraction

The macroalgae samples were defrosted and dried using a chemical-free absorbent paper. Then, the macroalgae was freezed with liquid nitrogen and grinded with a mortar. The macroalgae was further grinded to a powder using an electric mill, and finally suspended in distilled water in a proportion of 7.2 mL of distilled water *per* 5 g of fresh seaweed. Subsequently, the mixture was placed in a shaker incubator (IKA KS 4000 ic control) at 250 rpm and room temperature for 20 minutes. After, a filtration was carried out, and the resulting filtrate centrifuged (Thermo Scientific Heraeus Megafuge 16 R Centrifuge) at 5500 rpm, 277.15 K (to facilitate the cell debris sedimentation) and 30 minutes. This methodology was done following the extraction

procedure proposed by Martins *et al.*[57]. Finally, the extract was collected into a falcon tube and stored at 277.15 K and protected from the light; the precipitate formed was discarded.

Figure 3. Final macroalgae crude aqueous extract rich in phycobiliproteins used in the experimental work developed in this thesis.

In the determination of the AMTPS cloud point curves, different mixtures of known composition were prepared both for conventional systems as for systems composed of SAILs as the main surfactant. In both cases, the cloud point curves were determined using the cloud point method, using known compositions of each surfactant or SAIL.[39][46] The AMTPS cloud points were determined by visually identifying the phase transition, from a mono to a biphasic region (identifiable by the appearance of turbid systems), driven by the temperature increase. In other words, starting from a monophasic system and by raising the temperature a phase transition is promoted allowing the visual identification from a transparent to a cloudy state. The binodal curve of the nonionic surfactant, Tergitol 15-S-7, was experimentally assessed using a composition ranging from 0.5 wt% to 17.5 wt% both with the McIlvaine buffer at pH≈7.0 and distilled water. The temperature was controlled using

a water bath (ME-18V Visco-Thermostat, Julabo) able to control the temperature within 0.1 °C on a temperature ramp of 0.05 ºC/min. The experimental binodal curves were obtained by plotting the cloud point *versus* the surfactant concentration. The systems composed by SAILs ([P$_{4,4,4,4}$]DS, [N$_{4,4,4,4}$]DBS), were prepared with different concentrations: 5 wt%, 10 wt%, 15 wt%, 20 wt% and 30 wt% of SAIL, and McIlvaine buffer at pH≈7. In addition, systems composed of [N$_{4,4,4,4}$]DS were prepared with concentrations of 5 to 85 wt% in distilled water. All the systems were prepared with a volume of 10 mL on a 15 mL tube, minimizing thus, the loss of the volatile compounds to the vapor phase minimizing its impact on the initial mixture composition.

2.1.4. Liquid-liquid extractions – R-PE purification in batch regime

The R-PE purification on a liquid-liquid extraction system in a batch regime using AMTPS involved the preparation of various mixtures of known composition within the immiscibility region. Mixture points with the following compositions: 10 wt% surfactant + 10 wt% macroalgae extract + 80 wt% McIlvaine buffer at pH≈7, for the conventional AMTPS, and 10 wt% surfactant + 0.3 wt% of [N$_{1,1,1,12}$]Br + 10 wt% extract + 79.7 wt% McIlvaine buffer pH≈7, for the mixed AMTPS, were prepared. These were allowed to reach equilibrium at 313.15 K, in a temperature controlled water bath, protected from light, for periods never smaller than 4 hours for the conventional and mixed systems and 12 hours for the AMTPS based on both [N$_{4,4,4,4}$]DS and [N$_{4,4,4,4}$]DBS. The temperature of 313.15 K was selected after characterizing the liquid-liquid equilibrium cloud point curve as a function of temperature and composition. The time allowed for equilibration was stipulated taking into account the study reported by Cardoso *et al.*[19]*,* were the authors reported this time as enough for thermodynamic equilibrium to be reached, the physical separation of the two phases and migration of the protein of interest to the desired phase to occur. Two phases were formed, a top/surfactant-rich phase and a bottom/surfactant-poor phase.[29][41] Subsequently, both phases were separated, and their weights and volumes determined. Then, both phases were quantified in

terms of R-PE and total proteins concentration at wavelengths of, respectively, 565 nm and 280 nm by UV spectroscopy (Synergy| HT Microplate Reader, with a precision of 0.003 for R-PE and of 0.09 for total proteins in the bottom phase, and a precision of 0.003 for R-PE and 1.42 for total proteins in the top phase), which these correspond to their characteristic absorption peaks. The concentration of R-phycoerythrin and total proteins in the extracts was determined according to calibration curves, previously determined in the same UV-Vis equipment. The recovery (Rec) parameters of R-phycoerythrin and the total protein content towards the bottom (R_{bot}) and the top (R_{top}) phases were determined following **Eqs. 1** and **2**:

$$Rec_{bot} = \frac{[R-PE]_{bottom}}{[R-PE]_{top} + [R-PE]_{bottom}} \qquad (\textbf{Eq. 1})$$

$$Rec_{top} = \frac{[R-PE]_{top}}{[R-PE]_{top} + [R-PE]_{bottom}} \qquad (\textbf{Eq. 2})$$

The selectivity of the AMTPS herein developed was described as the ratio between the partition coefficient, K, values found for R-phycoerythrin and the total proteins, as indicated by **Eq. 3**:

$$S_{R-PE \,/\, Total\ proteins} = \frac{K_{R-PE}}{K_{Total\ proteins}} \qquad (\textbf{Eq. 3})$$

where the partition coefficients, K_{R-PE} and $K_{Total\ proteins}$, were determined by **Eqs. 4** and **5**:

$$K_{R-PE} = \frac{[R-PE]_{top}}{[R-PE]_{bottom}} \qquad (\textbf{Eq. 4})$$

$$K_{Total\ protein} = \frac{[Total\ proteins]_{top}}{[Total\ proteins]_{bottom}} \qquad (\textbf{Eq. 5})$$

The mass balance was calculated by **Eq. 6**:

$$MB = \frac{[R-PE]_{extract}}{[R-PE]_{top} + [R-PE]_{bottom}} \qquad (\textbf{Eq. 6})$$

2.1.5. Liquid-liquid extraction – R-PE purification in continuous regime

The R-PE purification using a liquid-liquid extraction system in a continuous regime using AMTPS was performed using the apparatus depicted in **Figure 4**. This experimental setup represents one of the main objectives of this work. It is composed by a column, operating as separator, with a 90.2 cm length tube in spiral, with 22 curves, and with an inner diameter of 0.04 cm, followed by a separation unit with 16 cm length, an inner diameter of 3.9 cm and 110 cm^3 of volume with two exits, in which the poor surfactant phase is collected at the bottom and the surfactant rich phase in the top.
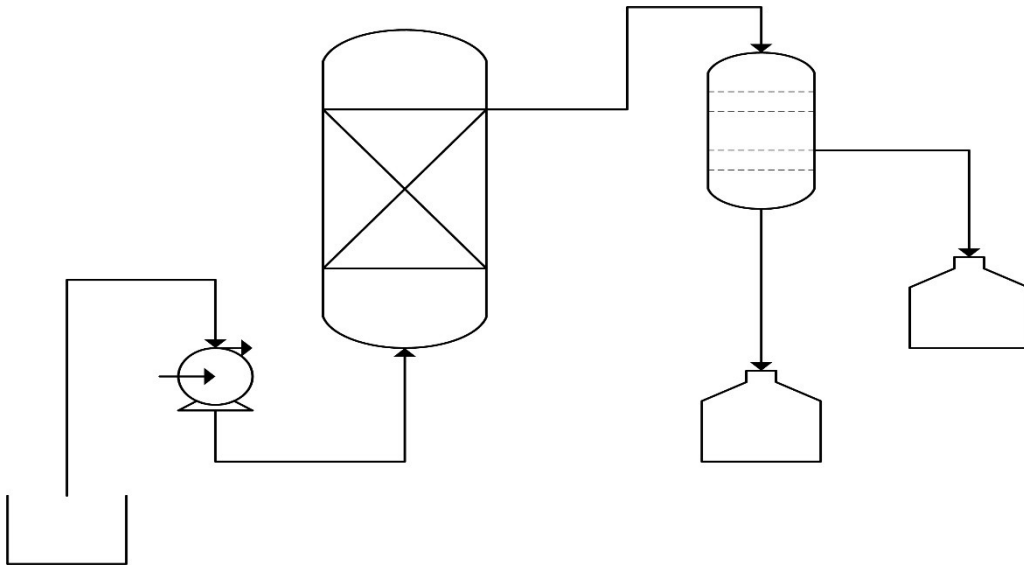
Figure 4. Schematic representation of the equipment designed to operate the P-PE purification is a continuous regime.

For the continuous liquid-liquid extraction, a stream composed of 10 wt% of surfactant, 80 wt% of McIlvaine buffer at pH≈7 and 10 wt% of crude aqueous extract, for a total volume of 300 mL, was used. The mixture was allowed to equilibrate for a minimum of 4 hours, protected from light. Then, this stream was pumped from a 24 mL syringe using a syringe pump (PERFUSOR Compact – B Braun Mcgaw) able to operate at a flow rate between 0.1 and 200 mL/h. The temperature of the separation units was maintained at 313.15 K through circulating water using a heater circulator able to maintain the temperature within ±0.5 K. The homogeneous mixture was pumped through the first column where it phases splits. To define flow rate, various flow rates were tested until the usage of the separation column could be maximized. At the end of this unit the complete phase separation is obtained allowing, with the second separation unit, decanter the two phases and collected them separately. Moreover, to verify that the equilibrium was reached, several aliquots of the two phases were taken during the experiment to evaluate the concentration of the R-PE and total proteins along the time and to verify the equilibrium condition.

3.   RESULTS and DISCUSSION

3.1. Characterization of the AMTPS cloud point curves

The first step of this work involved the characterization of the AMTPS cloud point curves, aiming at identifying the best conditions to operate the continuous separation unit to be developed. Thus, the cloud point curves for several AMTPS were determined [39][46] as described in Section 2.1.3.

The conventional AMTPS based in Tergitol 15-S-7 + water was reported by Li *et al.*[58] and, as depicted in **Figure 5**, small discrepancies are observed at surfactant concentrations below 1 wt%. Nonetheless, at higher concentrations the data measured here converges to that reported by the authors.[58] Although a minimum on the cloud point temperature, around 1 wt%, is perceived, the observed behavior is not so drastic as that reported by the authors. This difference, which occurs at low concentrations of surfactant can be justified by three factors: (i) the presence of impurities in the surfactant that can act as additives causing a change in the cloud point; (ii) because it is a visual method, and the cloud points may present discrepancies dependent on the operator; or (iii) insufficient time given to achieve the thermodynamic equilibrium. For some mixing points it was observed that after even though the system phase splits, if shaked and allowed to re-equilibrate, with the temperature constant, the mixture would become monophasic again. This behavior may lead to erroneous readings for a less critic operator or inadequate temperature ramps. Tergitol 15-S-7 presents a CMC value of around 35 ppm, or 35 mg/kg, which corresponds to ~0.35 wt% in aqueous solution. By a deep analysis of **Figure 5** a minimal cloud point is identified for a concentration similar/equivalent to the CMC of Tergitol 15-S-7. The results seem to indicate that two distinct behaviors should be defined before and after the surfactant CMC, which are probably evidencing the presence of different structures. In these systems, before the CMC the surfactant is distributed in the aqueous system as individual molecules and when the surfactant concentration is higher than its CMC, the presence of individual molecules is in equilibrium with the micelles being formed. The concentration of surfactant at the lowest cloud point is related to the surfactant CMC. Tergitol 15-S-7 presents a CMC value (35 ppm [59]) lower than the CMC for other nonionic surfactants, due to its

higher hydrophobicity, which leads to lower cloud points. Low CMC values indicate that less amounts of surfactant are needed to start the micelles formation by auto-aggregation of the tensioactive.[58]

Given the cloud points of each of the systems it was found that for similar concentrations of surfactant - Tergitol 15-S-7 - and solvent, the values determined show significant differences when the solvent used was McIlvaine buffer at pH≈7 or distilled water. That is, for the system composed of Tergitol 15-S-7 and distilled water the temperatures representing the cloud points are higher compared to the system composed of Tergitol 15-S-7 and McIlvaine buffer of pH≈7 (**Figure 5**), since the presence of the salts composing the buffer are acting as additives [52].
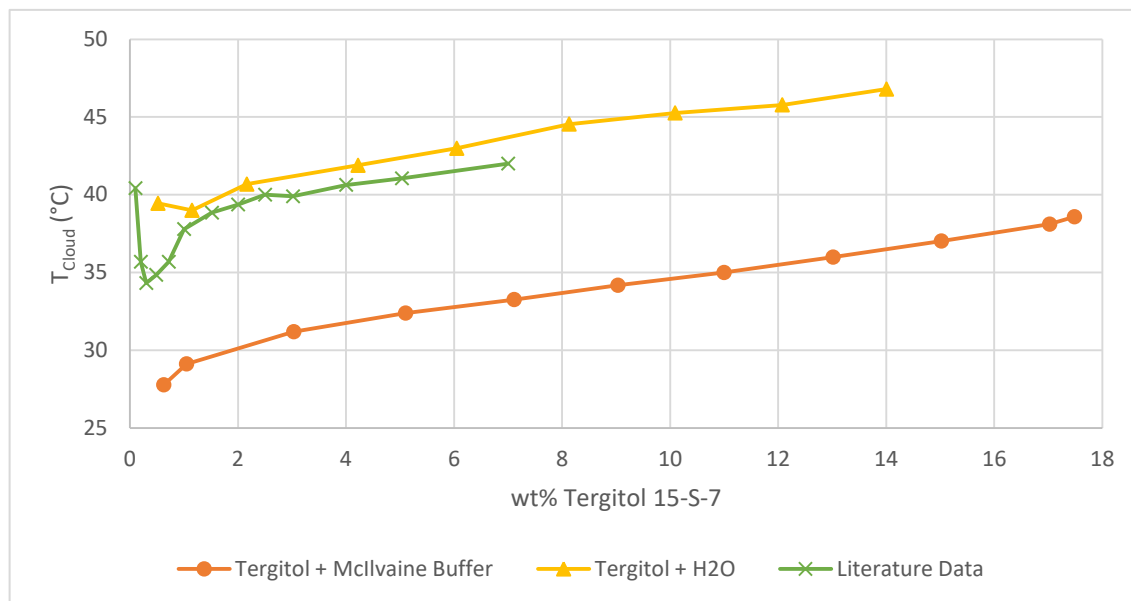
Figure 5. Binodal curves for the AMTPS based in Tergitol 15-S-7, where ▲ represents the system prepared with distilled water, ● corresponds to the system prepared with McIlvaine buffer (pH ≈ 7) and **x** represents the literature data for the AMTPS based in Tergitol 15-S-7 with distilled water.[58]

Cloud point represents a point of mixing of a homogeneous solution composed of amphiphilic substances, which is the case of surfactants, and which undergoes a clouding phenomenon that results in a phase separation: a surfactant-rich phase and a surfactant-poor phase.[60][58] This singularity is favored by changes in temperature, pressure or even by the addition of inorganic salts or organic compounds. Therefore, apparently both systems assume distinct behaviors, which is explained by the presence of a salt, when using the McIlvaine buffer at pH≈7. The presence of a salt imposes a greater affinity with the surfactant, establishing favorable interactions. In other words, the presence of a salt prevents the water from interacting with the monomer that is present in the system, allowing the micelles to cluster much more easily. Therefore, less energy is required to provide the system for the formation of micelles, and therefore, the temperature of the cloud point decreases. What is being described is a phenomenon called salting-out. If salts known to impose a salting-out effect are added, a decrease of the cloud point is observed;[47][61] salting-out salts are known as water-structure-making ions, with

29

strong electric fields at short distances and strong binding to water molecules. Consequently, since they have strong tendency to be hydrated by the water molecules, they compete for the solvent with the polyethylene oxide (PEO) chains composing the surfactant micelles. The resultant dehydration of the PEO chains leads to lower cloud point values.[61] Therefore, we can conclude that it would be much more advantageous to use this buffer solution, because, not only it has the ability to reduce the cloud point temperatures, allowing the design of less energy demanding separation units, but also it provides a more biocompatible environment to maintain the structural integrity of the fluorescent proteins, thus avoiding the potential degradation of the R-PE.

A second objective of this work was the identification of new AMTPS based in SAILs as the main surfactant in solution. So, two distinct families of SAILs were chosen, namely ammonium ($[N_{4,4,4,4}]$DS and $[N_{4,4,4,4}]$DBS) and phosphonium ($[P_{4,4,4,4}]$DS. The ammonium- and phosphonium-based ILs were not chosen randomly, these two families were selected due to their higher hydrophobic nature (which induce lower T_{cloud}) when compared to imidazolium-based ILs. These systems are not like those in which we have the IL acting as co-surfactant (mixed systems). In these systems, the ILs are acting as the main surfactant, with the tensioactive part in the anion and thus, representing anionic surfactants. With respect to $[N_{4,4,4,4}]$DS, the results obtained are shown in **Figure 6** for both the McIlvaine buffer of pH≈7 and the distilled water. As depicted, the same behavior obtained for the Tergitol 15-S-7 as main surfactant is observed, *i.e.,* the use of the McIlvaine buffer induces lower cloud points, as previously discussed.

As for the system composed of $[P_{4,4,4,4}]$DS, it presents a miscibility region at temperatures lower than those feasible to measure with the experimental setup. Therefore, this system cloud points were not possible to be measured. Comparing with the $[N_{4,4,4,4}]$DS, the only difference occurs in the central atom. Although phosphorus (atomic radius of 195 pm) is bigger than nitrogen (atomic radius of 160 pm), such difference is not so significant as to justify the observed behavior. Thus, this phenomenon seems to be in agreement with the work of Carvalho *et al.*[62]. In this work, the authors verified a difference of properties between ammonium *versus*

30

Figure 6. Cloud point curves for the AMTPS based in $[N_{4,4,4,4}]$DS, where ♦ represents the system prepared with distilled water and ● corresponds to the system prepared with McIlvaine buffer pH≈7.

phosphonium ILs related with the lower electronegative character of the ammonium cation, imposing a greater charge delocalization, and thus inducing a stronger cation-anion interaction and a more rigid structure. The phosphonium less rigid structure allows the anion to present more capacity to interact with different moieties of the cation (interacting either with the cation head group or with the cation's alkyl chain terminal hydrogens) and thus, allowing higher free-volume and polarizability.
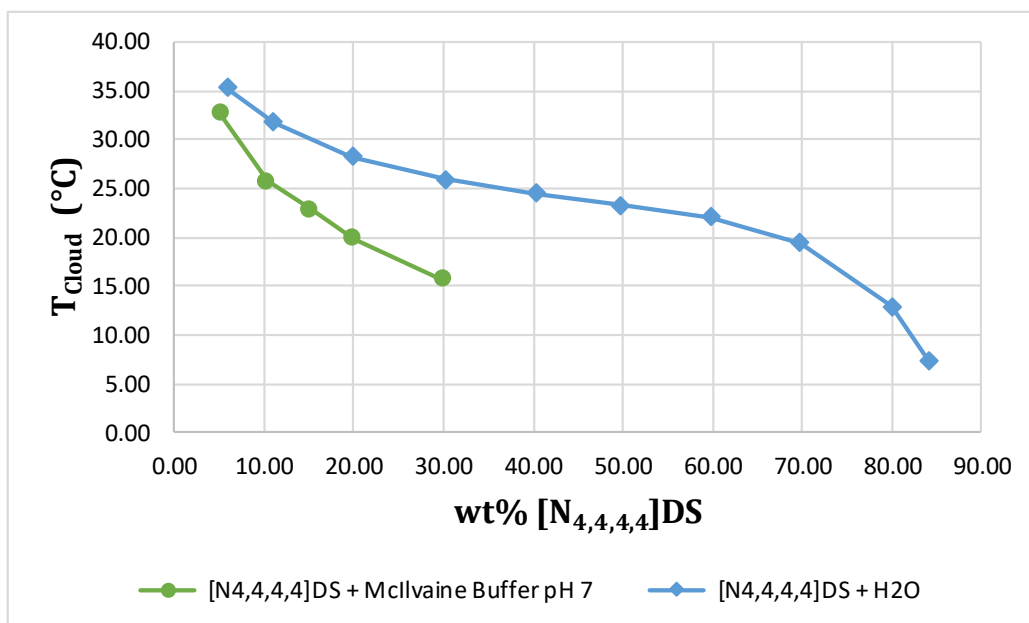
This higher less rigid structure, free-volume and polarizability allows the compound to rearrange its bulk structure to accommodate more molecules of solute and thus, present a wider miscibility region. Moreover, the ammonium structure is more hydrophilic nature, which may also contribute for the results of T_{cloud}.[62] It should be noted that the justification discussed is only an assumption of what may be happening to justify this phenomenon, since no quantum calculations were made to support this. For systems with $[N_{4,4,4,4}]$DBS it was also not possible to determine the cloud points, since they seem to occur at very low temperatures.

3.2. Purification of R-PE using AMTPS in batch regime

After the determination of the cloud point curves of the proposed systems, the second stage of this work was their application as the biphasic system to use to develop the

purification of R-PE in discontinuous regime. For this study, systems with lower cloud points were taken into account, since the protein of interest is thermolabile and thus, sensitive to the temperature conditions required promoting the AMTPS preparation. In this sense, the AMTPS based in Tergitol 15-S-7 and $[N_{4,4,4,4}]DS$ with the McIlvaine buffer solution of pH≈7 were used.

Cardoso and her co-workers[19] evaluated the conventional system with different concentrations of surfactant concluding that a surfactant concentration of 10 wt% allows to reach higher R-PE purification and higher selectivity. Thus, the feed stream mixing point for the AMTPS was defined with a surfactant concentration of 10 wt%. This concentration allows to obtain a high recovery of R-PE in the phase of interest, that is, in the micelle-poor phase, while minimizing contamination by the remaining proteins and other phycobiliproteins.[19] The authors[19] further evaluated the algae extract concentration stating that a concentration of 10 wt% allowed to obtain a greater selectivity and a reduction in the extent of the precipitation at the interface. Therefore, phycobiliproteins were more isolated in the micelle-poor phase, and the contaminating proteins migrated to the micelle-rich phase, as intended.[19] For the mixed AMTPS, a system composed of IL acting as co-surfactant, a concentration of 0.3 wt% was defined based on the same study.[19]

The extraction time was also defined based on Cardoso *et al.* study[19], which was set for 4 hours for both the conventional and mixed AMTPS. However, for the AMTPS in which the SAILs, as the main surfactant, synthesized were used, a longer extraction time was required, this being approximately of 12 hours. However, for this same system, it was not possible to obtain a "conventional" extraction since the $[N_{4,4,4,4}]DS$ system do not separate into two phases, as can be seen in **Figure 7 – (A)**. The system composed of $[N_{4,4,4,4}]DS$ formed 4 phases, which generated more complexity in the batch extraction of the R-PE. However, as depicted in **Figure 7 – (A),** if during the extraction time the system had been left in an inverted flask, it would have been easier to extract the bottom phase. Also, by noticing the color of the bottom phase which was supposed to correspond to the micelle-poor phase, the characteristic pink is noted to be dimmed, which indicates that the concentration of R-phycoerythrin occurred. Thus, quantifying this same phase, and from a mass balance it would be

possible to evaluate if it would really be a more selective system. As a matter of experience, the same system with $[N_{4,4,4,4}]DBS$ (**Figure 7 – (B)**) was also tested, although it was not possible to measure the cloud point. For this system it was also necessary a time of 12 hours of extraction, having subsequently formed 3 phases. Though, since these systems were more complex, they were not taken into account for the development of the continuous separation unit.
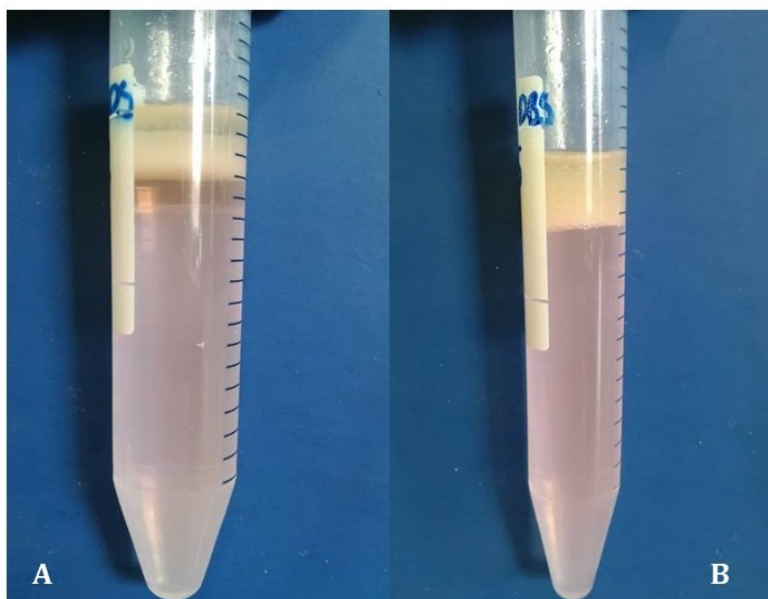
Figure 7. Picture of AMTPS composed of $[N_{4,4,4,4}]DS$ (A) and $[N_{4,4,4,4}]DBS$ (B) forming more than two phases.

Thus, it is already concluded that the system using $[N_{4,4,4,4}]DS$ as SAIL would be possible to be used, but would not be industrially feasible, since it requires a much longer extraction time compared to the conventional system (with Tergitol 15-S- 7) and the mixed system. However, for the conventional AMTPS *versus* mixed AMTPS, the purification of R-PE was experimentally investigated, and the concentration of R-PE and total proteins determined for both phases (**Table 4**). From these results, it was possible to calculate and posteriorly analyze several parameters (**Table 5**).

Table 4. Concentrations (mg/mL) obtained after purification of R-PE by applying the conventional and mixed AMTPS.

		Conventional AMTPS			Mixed AMTPS		
[R-PE]$_{extract}$ (mg/mL)		0.031					
[Total proteins]$_{extract}$ ± 0.02 (mg/mL)		2.52					
		1	2	3	1	2	3
[R-PE] (mg/mL)	Bot Phase	0.024	0.026	0.027	0.029	0.031	0.032
	Top Phase	0.003	0.003	0.002	0.003	0.003	0.003
[Total proteins] ± 0.02 (mg/mL)	Bot Phase	3.23	3.20	3.34	3.77	3.60	3.47
	Top Phase	5.84	6.17	1.67	4.84	6.23	2.41

Table 5. Parameters of selectivity, recovery and purity obtained for conventional and mixed AMTPS, with the respective standard deviation.

System	Conventional AMTPS	Mixed AMTPS
	Average	Average
Selectivity	15.95 ± 0.11	17 ± 2
Mass balance R-PE (%)	47.61 ± 1.96	50 ± 4
Rec (R-PE) bot (%)	90.17 ± 1.52	91.48 ± 1.17
Rec (R-PE) top (%)	9.83 ± 1.52	8.52 ± 1.17
Rec (total proteins) bot (%)	35 ± 2	40.21 ± 1.20
Rec (total proteins) top (%)	65 ± 2	59.79 ± 1.20
Purity Factor	0.26 ± 0.01	0.29 ± 0.02

From the data reported in **Table 5**, it can be seen that there are no big discrepancies between the conventional and mixed AMTPS, although in the mixed AMTPS, the values of selectivity and purity are slightly higher.

3.3. Development of the Separation Unit for Continuous Purification

Initially, the setup was imagined as being composed by two jacketed columns, one operating as a mixer and the other as a decanter. The mixing unit was projected to operate at room temperature (303.15 K) in order to promote the feed of the unit with

one stream composed by the crude extract rich in the phycobiliproteins and a second stream composed by the surfactant solution (Tergitol 15-S-7 + buffer), in specific conditions generating a monophasic stream in which the contaminant proteins would diffuse (mass transfer) to the Tergitol 15-S-7 rich phase thus allowing the R-PE purification. This unit would allow to achieve equilibrium within the column specifications by manipulating the residence time and operating temperature. After achieving the equilibrium at the column exit, the stream would be fed to a separation unit, operating at 313.15 K to promote a phase change transition and thus allowing the separation of the target protein from the contaminant proteins present inside the Tergitol 15-S-7 micelles on the surfactant rich phase. However, to evaluate if one could achieve equilibrium within the column specifications a kinetic study was performed prior to operating the separation in a continuous mode. A kinetic study where the concentration of the total proteins and the R-PE concentration in the protein-rich phase as function of time and at 313.15 K was evaluated using a UV-1800 | SHIMADZU UV spectrophotometer. Starting from the concentration of this protein-rich phase the absorbance was measured at intervals of 25 seconds once a second top phase, composed of water and Tergitol 15-S-7, was added to the system. The compositions of both phases were selected carefully in order to assure that water mass transfer between phases was mitigated and only the diffusion of the proteins was occurring. Thus, from the mass transfer phenomenon, it was expected that a decrease in the absorbances would be observed as function of residence time until they reach a constant value, as depicted in **Figure 8**.
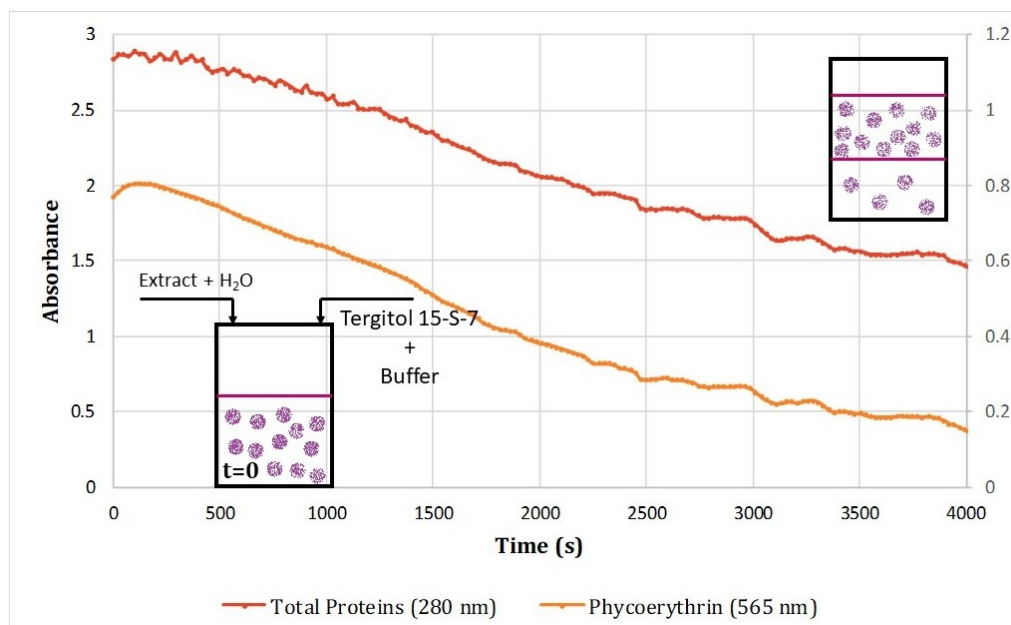
Figure 8. Representative scheme of the mass transfer of the system, with the respective graphic illustration of the absorbance as function of residence time.

As depicted in **Figure 8**, one could estimate that 4 hours are necessary to reach equilibrium. Since operating our mixing and separation unit to achieve such long residence time would lead to very low and unfeasible flow rates the idea of operating with a mixing unit was discarded and adopted a process composed only by a separation unit.

With this new setup in mind and once the optimum operational conditions were established, the continuous purification process was evaluated. As described in the experimental description the separation units were operated at 313.15 K. The temperature of this bath was defined taking into account the cloud point associated with the defined mixing point (10 wt% surfactant - Tergitol 15-S-07, 10 wt% extract and 80 wt% McIlvaine buffer pH 7) (**Figure 9**), *i.e.,* for the 10 wt% point of Tergitol 15-S-7, the cloud point is 308.78 K, so at a temperature of 313.15 K, it is guaranteed that the solution is separated into two phases, being possible to promote the R-PE purification.
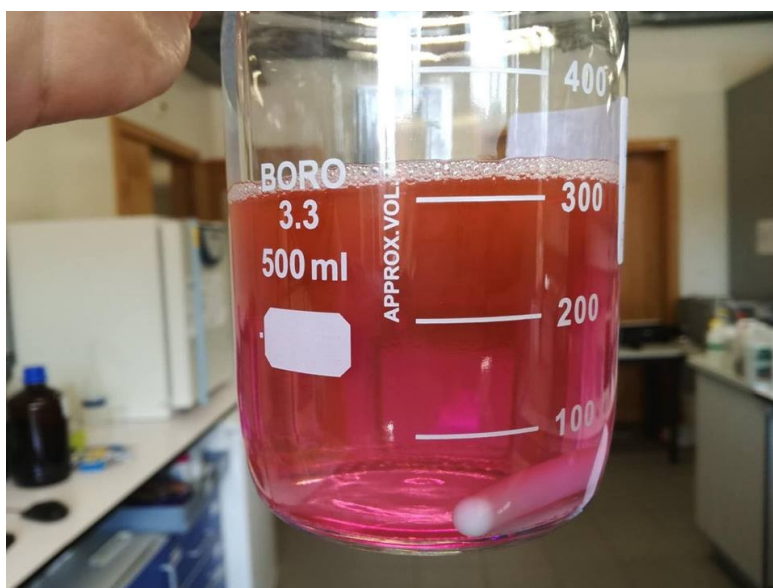
Moreover, a tracer study was carried out in order to analyze the flow along the
columns. The use of the tracer will allow to measure a given perturbation in the
system, such perturbation that was done in a single impulse, that is, with the system
with distilled water, a certain amount of tracer inert, in this case KOH (0.1M), was
introduced in a single impulse and it was measured the conductivity over time
(**Figure 10**), and subsequently, the conductivity values were recorded at 1 second
intervals at the exit of the reactor, with a SevenExcellence | Multiparameter | Mettler
Toledo conductivity meter.
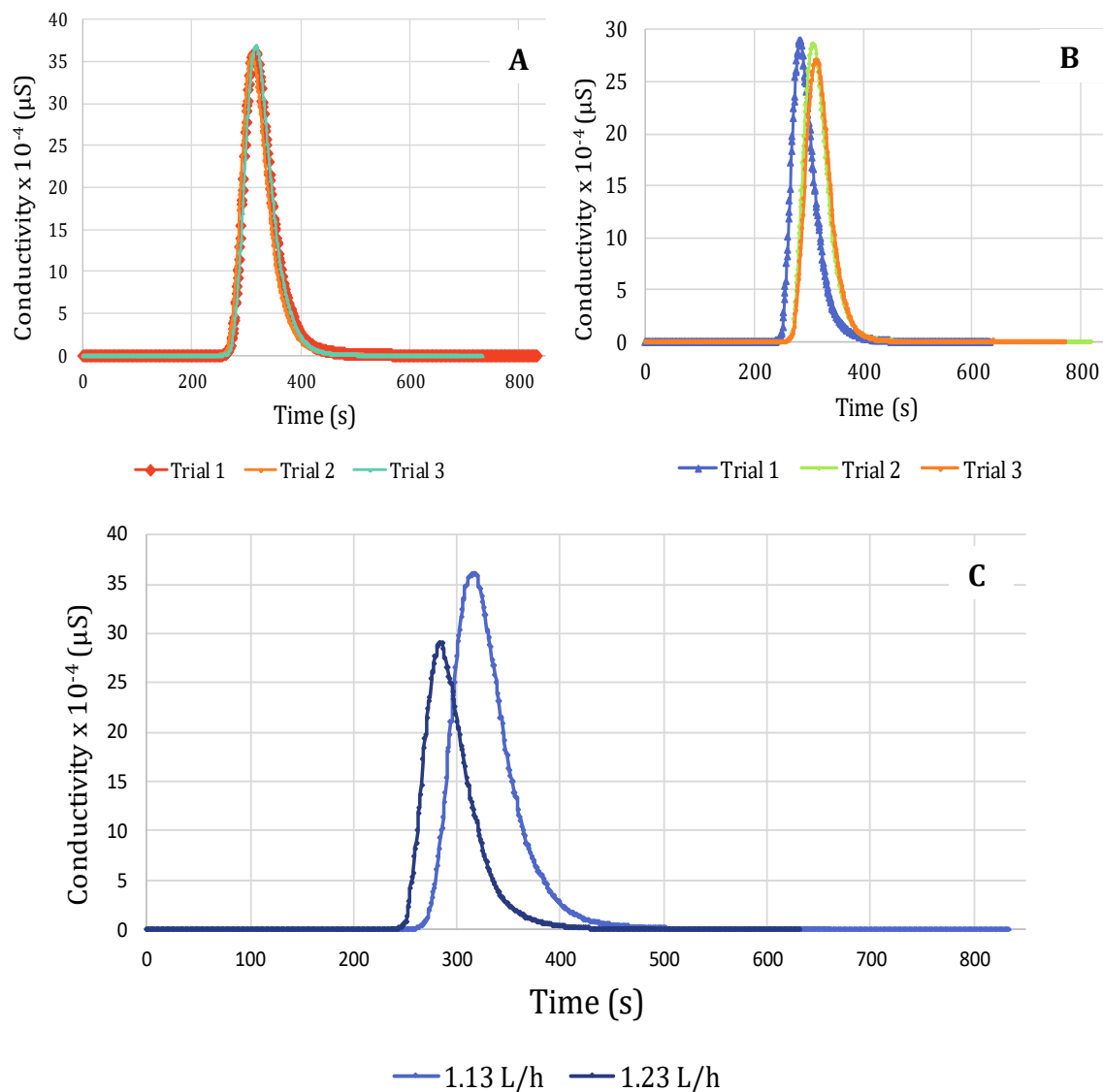
Figure 10. Illustrative figure representing the tracer study performed, where **A** represents the
3 trials for a flow rate of 1.13 L/h; **B** represents the three tests for a flow rate of 1.23 L/h; and
C represents a comparison between the first two tests of each flow, 1.13 L/h and 1.23 L/h.

This study was done for different flow rates, in order to verify if occurs accumulation along the column, since at very low flow rates greater is the tendency to accumulate. From **Figure 10- C**, that represents the distribution of residence time, it can be seen that, as expected, different flow rates lead to different mean time. The flow rates were determined using a graduate tube with an uncertainty of 0.05 mL in which a specific volume was measured on a specific period of time. Several measurements were made to reduce the associated error, and the mean values were then averaged. Mean residence time is defined as the average time the molecules leaving the system there remain. Thus, for the different flow rates, the mean residence time obtained is present in **Table 6**.

Table 6. Mean residence time experimentally obtained for each flow.

$V \pm 0.05$ (mL)	$t \pm 0.01$ (s)	Q (L/h)	τ (min)	$T_{res.\,exp}$(min)
6.15	18.72			
5.50	15.98			
5.25	17.28	1.13	5.92	8.24
5.90	20.16			
6.30	21.94			
6.20	19.48			
6.80	23.26			
5.60	14.77	1.23	5.46	5.18
5.55	15.75			
6.05	15.05			

Analyzing **Table 6**, it is verified that the higher the flow rate, Q, the lower the residence time. Another parameter to analyze is the occurrence of different physical

phenomena that can occur in the equipment (**Figure 11**). The occurrence of such physical phenomena, such as channeling of the fluid, recycling of the fluid or the creation of stagnant regions in the vessel, induces changes in flow patterns[63]. Thus, these phenomena occurrence indicate that there is a given standard flow that can be analyzed from a study with tracer, in which for each flow pattern a different tracer response occurs.
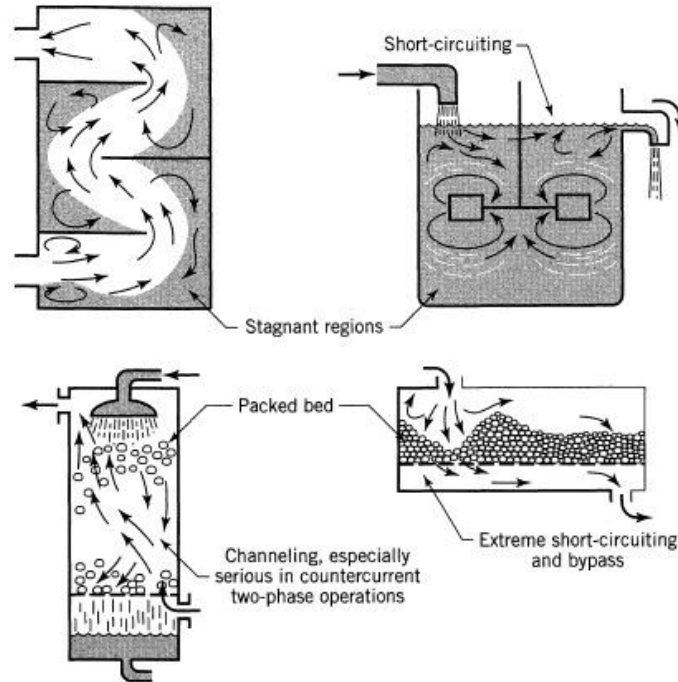
Figure 11. Different flow behaviours that may exist in the process equipment associated with different physical phenomena. *Adapted from* [63].

Therefore, it was also possible to verify if there were short-circuit zones, *i.e.*, some of the elements of the fluid pass too fast compared to others, or dead or stagnant zones corresponding to zones, whose elements of the fluid become stagnant. However, in order to evaluate much more concretely if one of these anomalies actually occurred, a comparison of the mean residence time with the time of passage in the system, τ, given by **Eq. 7**, was verified that for the lowest flow occurs dead zones and for the highest occurs short-circuiting, therefore, the flow is not ideal.

$$\tau = \frac{V}{Q_v} \qquad\qquad \textbf{\textit{Eq. (7)}}$$

A good flow characterization in chemical reactors is important when dimensioning these. In theory, tubular reactors are always considered ideal, *i.e.* pug-flow reactor (PFR). However, the study of non-ideal behavior is equally important in order to be aware of reactor operation and scale-up studies. The distribution of residence time (DTR) is one of the techniques used to evaluate the hydrodynamic behavior of a chemical reactor, in which the experimental DTR curves are compared with theoretical flow models. In this work, the obtained DTR experimental curves were compared with 3 models: laminar flow given by **Eq. 8**; "n" CSTR in series from **Eq. 9**; and the asymmetric model that corresponds to a semi-empirical dispersion model given by **Eq.10** and proposed by Pap *et al.*[64].

$$E(t) = \left(\frac{\tau^2}{2t^3}\right) H\left(t - \frac{\tau}{2}\right) \qquad \textbf{Eq. (8)}$$

$$E(t) = \left(\frac{1}{\tau_i}\right)^n \frac{t^{n-1} exp\left(-\frac{t}{\tau_i}\right)}{(n-1)!} \qquad \textbf{Eq. (9)}$$

$$E(t) = h_m \times exp\left\{\left(\frac{4}{a^2} - 1\right)[\ln(1+b) - b]\right\} H\left(t - \left(t_m - \frac{\sigma(4-a^2)}{2a}\right)\right) \qquad \textbf{Eq. (10)}$$

where τ corresponds to the space time; H the unitary step function; τ_i the space time in each tank; n the number of tanks; h_m the peak maximum height; t_m the time at peak maximum; σ the standard deviation; a the asymmetry factor; and b given by the expression $b = \frac{2a(t-t_m)}{\sigma(4-a^2)}$.
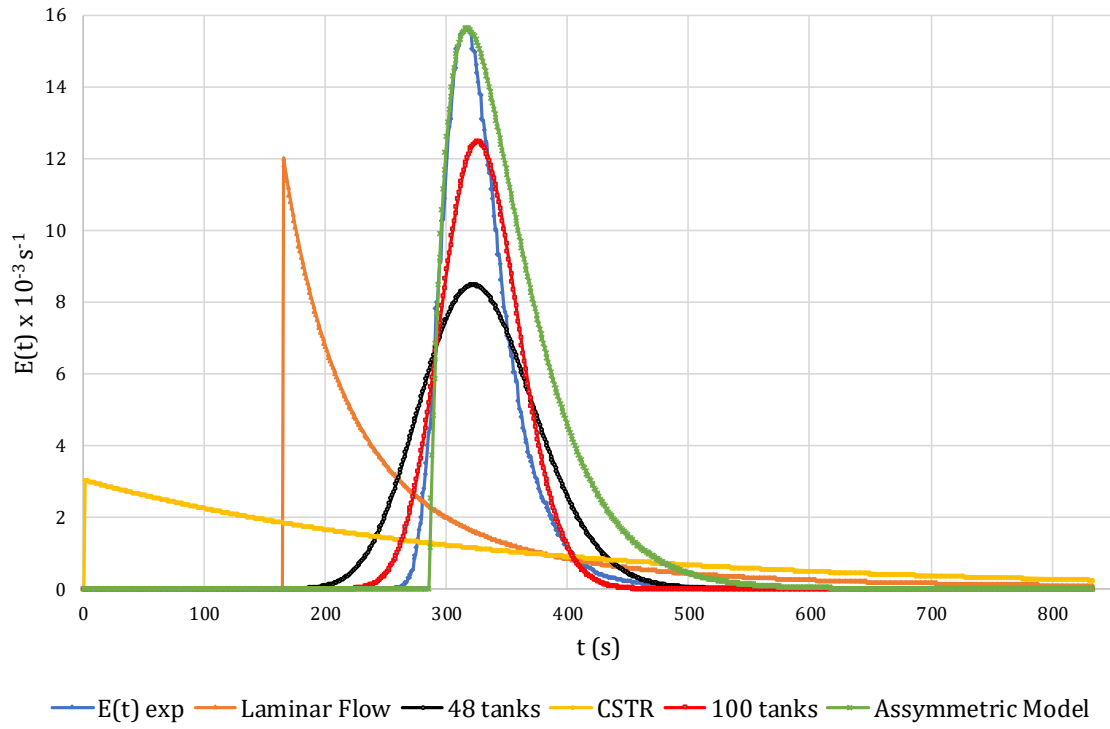
Figure 12. Experimental DTR curves compared to the DTR curves obtained by theoretical models for a flow of 1.13 L/h.
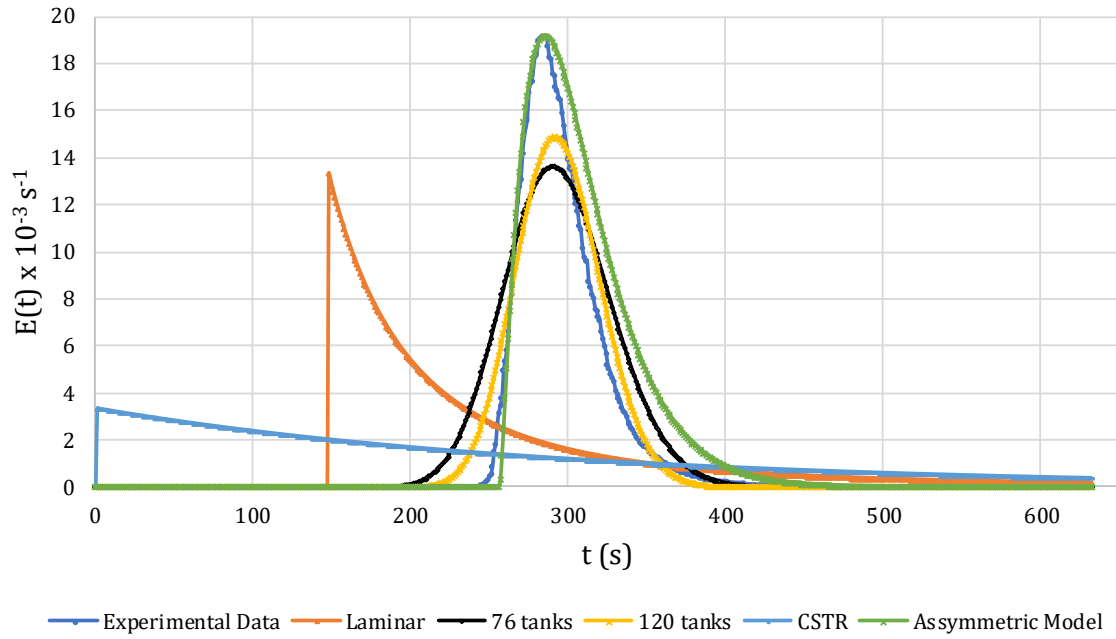
Figure 13. Experimental DTR curves compared to the DTR curves obtained by theoretical models for a flow of 1.23 L/h.

After the mixer was discarded, a first trial was performed by testing the system. However, even for the lower flow rate it was found that the system did not achieve separation of the two desired phases. The following conclusion was reached: since the study of the tracer was done, it was assumed that everything would work well with the real mixture, but no, because the study of the tracer was done with water, and therefore a mixture of one single phase. Since the real mixture is a two-phase system, the geometry of the reactor causes mixing zones, on the spyral changing direction, between the two phases. Therefore, to eliminate these mixing points it was necessary to decrease the flow rate; however, changing from the highest (2.55 L/h) to the lowest flow rate (1.13 L/h) this phenomena was not eliminated. Thus, in order to be able to operate at lower flow rates the peristaltic pump was replaced by a syringe pump able to operate at flow rates ranging from 0.1 to 200 mL/h. Already with the necessary changes to the process, a new test was continued, in which it was verified if the unit was able to operate as a separator on the entire range of the syringe pump operating flow rates. Furthermore, it was found that even at the highest flow rate the separation unit allowed to achieve the system equilibrium. Moreover, it was

concluded that operating with a single separation column the intended separation was achieved and thus, the setup was simplified by removing one of the columns. Following the above mentioned procedure, the maximum and highest flow rate possible for the syringe injection pump to operate and assure the separation equilibrium was 200 mL/h; the evaluated flow rate was also the maximum flow rate allowed by the used model.

Finally, in order to characterize the flow along the fluid, a new tracer study was carried out. However, contrary to what was done earlier this was done on the basis of a stepped impulse, having obtained the curve shown below. (**Figure 14**)
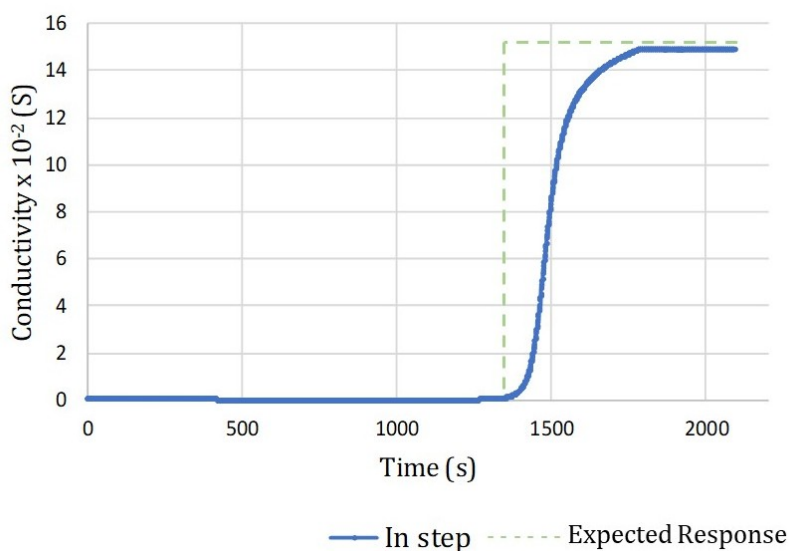
Figure 14. Graphic illustration showing the actual response obtained with the study with the tracer *versus* the response that would be expected to obtain.

Therefore, from the analysis to the curve profile obtained and that expected, **Figure 14**, it is also possible to conclude that dead zones occur along the reactor.

Therefore, aiming at optimizing the new continuous separation process setup, a test was performed at the highest flow rate, 200 mL/h, and at 313.15 K. A monophasic phase containing 10 wt% of McIlvaine buffer, 80 wt% of Tergitol 15-S-7 and 10 wt% crude extract rich in phycobiliproteins, that was allowed to reach equilibrium for 24

hours, were fed to the separation unit and the two phases formed collected separately, and analytically (UV spectroscopy) analysed as function of time in intervals of 5 minutes. The **Table 7** represents a comparison between the R-PE concentrations obtained in batch and discontinuous, being possible to observe that the concentration in the continuous process is bigger than in batch process, which indicates that the use of the continuous process is more advantageous, since it can purify a greater amount of R-phycoerythrin. Lastly, it should also be noted that the studies indicate that it might be possible to continue to increase the flow, as long this does not imply mixing, but rather ensures separation. However, with the set-up studied in this work it would be possible to treat 1 217 kg of fresh seaweed *per* year and to obtain around half a kg of pigment, considering a processing of 24 hours during 7 days a week.

Table 7. Concentrations of R-PE and total proteins obtained for the liquid-liquid extraction using AMTPS for both the continuous and batch regimes.

	Batch Process	Continuous Process
$[\text{R-PE}]_{extract}$ (mg/mL)	0.31 ± 0.01	0.41 ± 0.03
$[\text{Total Proteins}]_{extract}$ (mg/mL)	25.15 ± 0.20	13.11 ± 0.09
$[\text{R-PE}]_{bottom}$ (mg/mL)	0.26 ± 0.01	0.41 ± 0.03
$[\text{Total Proteins}]_{bottom}$ (mg/mL)	6.51 ± 0.20	4.05 ± 0.09
$[\text{R-PE}]_{top}$ (mg/mL)	0.03 ± 0.01	0.0091 ± 0.0003
$[\text{Total Proteins}]_{top}$ (mg/mL)	18.25 ± 0.20	8.89 ± 1.42

4.   CONCLUSIONS

This work aimed the development of a continuous process for the purification of R-PE, using aqueous micellar two-phase systems (AMTPS). This phycobiliprotein has been recognized for its high potential in applications from the pharmaceutical to the energetic sectors, given its characteristic properties, namely fluorescence, light absorption, and biological properties as antioxidant and anticancer. For these same reasons, several companies have shown interest in developing a continuous industrial process that allows the extraction and purification of R-PE with different purity levels, which are dependent on the final application. Thus, in order to achieve this same objective, this work was divided in two main stages: a first that focused on the characterization of the cloud point curves, and a second including the application of the most appropriate AMTPS on the development of a separation unit for the continuous purification of R-PE.

The first stage allowed the identification of the best operating conditions for the development of the separation unit. Thus, several AMTPS were studied by determining their cloud points. In this work, conventional AMTPS with Tergitol 15-S-7 or SAILs as the only surfactant and solvent, and mixed AMTPS with ILs as co-surfactants, were investigated. All systems were measured either with McIlvaine buffer pH≈7 as solvent, rather than with distilled water. Briefly, the cloud point temperatures determined showed that the use of McIlvaine buffer induces lower cloud points compared to systems with distilled water due to the *salting-out* phenomenon promoted by the presence of salts from the buffer as additives. For AMTPS with SAIL, it was only possible to determine cloud points for the $[N_{4,4,4,4}]DS$, since for both $[N_{4,4,4,4}]DBS$ and $P[_{4,4,4,4}]DS$ the cloud points would be very low or high values. As for the $[N_{4,4,4,4}]DBS$ what may justify is the presence of the aromatic ring in the anion, however, as for the $[P_{4,4,4,4}]DS$ what may justify is the central atom.

Given the necessary conditions, the second stage consisted in the development of a purification unit, using the AMTPS, in a continuous regime. In a first step, the process consisted of two columns, a mixer and a decanter, a small decanter and a peristaltic pump, however, from a mass transfer study, it was concluded that it would never be viable to make use of a mixer, since it did not allow the system to reach equilibrium. Then, a study of the flow along the reactor was made, and it was observed that a

much lower flow rate would be required, and therefore it was necessary to replace the peristaltic pump with a syringe pump. After, a test was carried and even with the maximum flow rate of 200 mL/h, the separation of the two phases occurred visually, and it was later analyzed that it was possible to reach the equilibrium. Since even at the maximum flow rate allowed by the pump it was possible to achieve a more efficient purification compared to the batch process. From the results it seems that the limiting step for process optimization became the geometry of the reactor. Thus, from this study it was possible to conclude that it is possible to resort to a continuous process using AMTPS for the purification of R-PE, but again more optimizations are needed.

5. FUTURE WORK

Regarding future work, this thesis open the door for the use of SAILs as the main and only surfactant to promote the AMTPS formation, which should be considered for further studies, not only enlarging the number of SAILs studied but also testing their (AMTPS) application in the purification of phycobiliproteins if the appropriate conditions of separation are assessed. Moreover, regarding to the R-PE purification unit developed in this work (the main focus of this thesis), some challenges appear as future work:

- Evaluate how many separation units would be necessary to obtain a higher degree of purity for R-PE, for the geometry described;
- To determine the maximum flow rate for the geometry described in this process;
- To evaluate for the optimum maximum flow rate, whether the residence time would be the limiting step of the process;
- To test the process for other possible reactor geometries.

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7. Appendix A – Reactions applied on the synthesis of SAILs
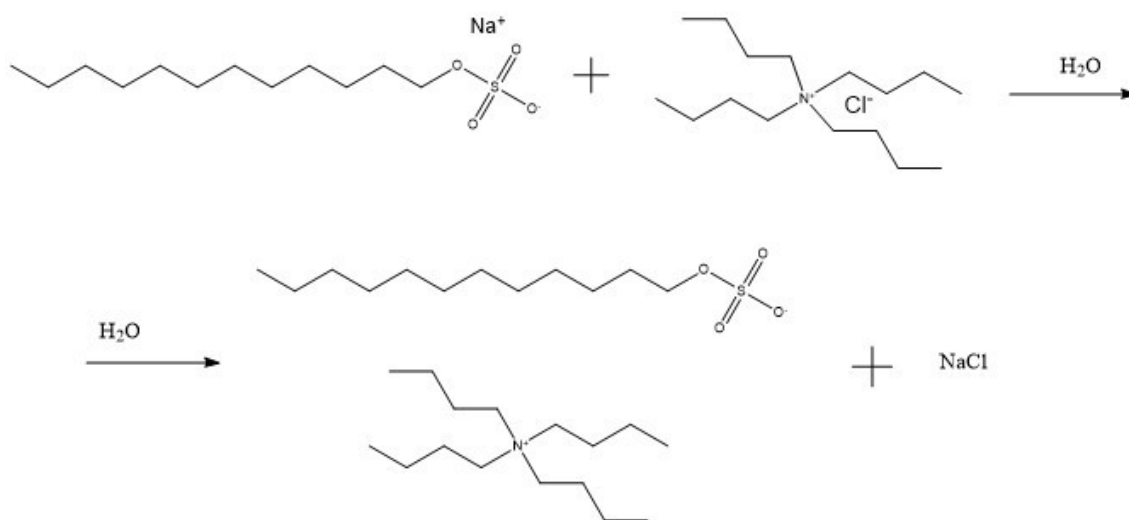
- **[N$_{4,4,4,4}$]DS**

Figure 15. Reaction scheme of tetrabutylammonium dodecyl sulphate, [N$_{4,4,4,4}$]DS.
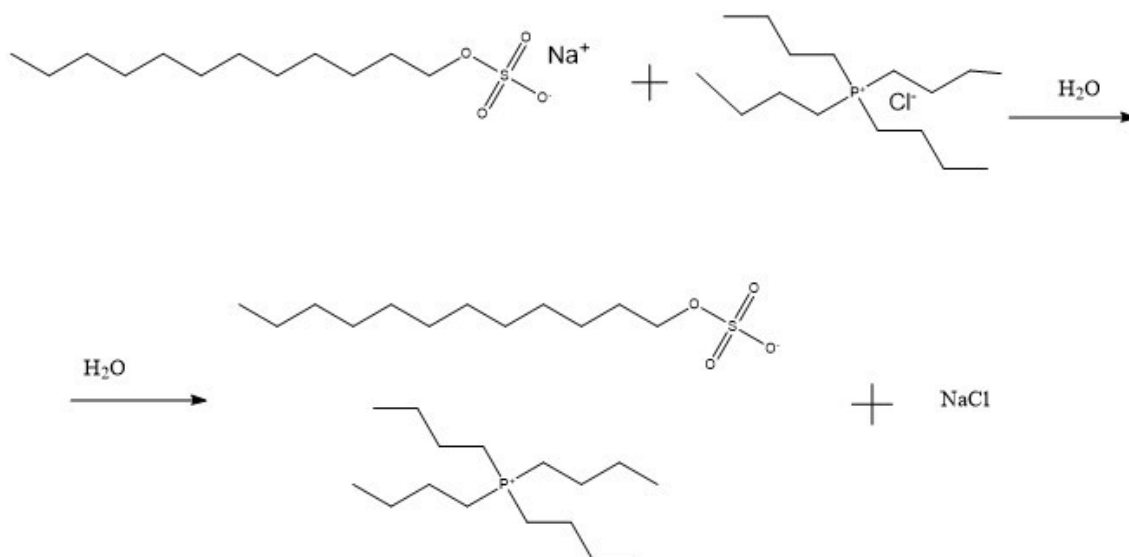
- **[P$_{4,4,4,4}$]DS**

Figure 16. Reaction scheme of tetrabutylphosphonium dodecylsulphate, [P$_{4,4,4,4}$]DS.
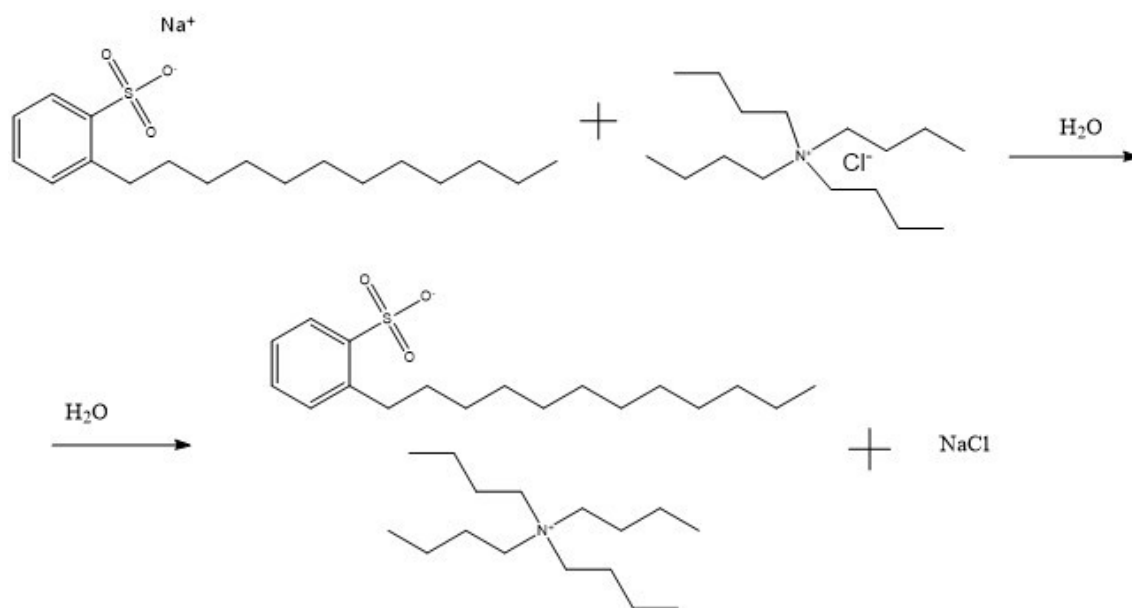
- **[N$_{4,4,4,4}$]DBS**

Figure 17. Reaction scheme of tetrabutylammonium dodecylbenzenesulfonate, [N$_{4,4,4,4}$]DBS.