Universidade de Aveiro

2015/2016

Departamento de Química

Inês Cláudia Simões Cardoso

Novos sistemas micelares mistos de duas fases aquosas com líquidos iónicos para a extração de ficobiliproteínas de macroalgas

Novel mixed aqueous micellar two-phase systems with ionic liquids for the extraction of phycobiliproteins from macroalgae

Universidade de Aveiro

Departamento de Química

2015/2016

Inês Cláudia Simões Cardoso

Novos sistemas micelares mistos de duas fases aquosas com líquidos iónicos para a extração de ficobiliproteínas de macroalgas

Novel mixed aqueous micellar two-phase systems with ionic liquids for the extraction of phycobiliproteins from macroalgae

Dissertação apresentada à Universidade de Aveiro para cumprimento dos requisitos necessários à obtenção do grau de Mestre em Biotecnologia Industrial e Ambiental, realizada sob a orientação científica do Professor Doutor João Manuel da Costa e Araújo Pereira Coutinho, Professor Catedrático do Departamento de Química da Universidade de Aveiro e co-orientação da Doutora Sónia Patrícia Marques Ventura, Bolseira de Pós-Doutoramento do Departamento de Química da Universidade de Aveiro.

"The important thing in science is not so much to obtain new facts as to discover new ways of thinking about them".

William Bragg

O júri

presidente Professor Doutor João Manuel da Costa e Araújo Pereira Coutinho

Professor Catedrático do Departamento de Química da Universidade de Aveiro.

Doutora Sónia Patrícia Marques Ventura

Bolseira de Pós-Doutoramento do Departamento de Química da Universidade de Aveiro.

Doutor Oscar Rodríguez Figueiras

Investigador, Programa Ramón y Cajal da Universidade de Santiago de Compostela, Espanha.

Agradecimentos

Primeiramente gostaria de agradecer aos meus orientadores, o Prof. Dr. João Coutinho e a Drª. Sónia Ventura pelo excelente acompanhamento e pela disponibilidade que tiveram comigo ao longo deste ano de constantes desafios. Quero agradecer também aos membros do PATh por toda a ajuda e pela receção calorosa, bem como à empresa ALGAplus, parceira neste trabalho. Em especial, quero deixar o meu agradecimento à Filipa, por todo o apoio, amizade, paciência, por todos os ensinamentos e por todo o seu tempo que com certeza farão de mim uma melhor pessoa e que já fizeram de mim uma melhor profissional. Obrigado por acreditares em mim. Agradeço também a todos os amigos que encontrei em Aveiro e que durante estes cinco anos foram a minha segunda família e os responsáveis por me sentir em casa todos os dias. Obrigado em particular à Eduarda, à Patrícia, ao Pedro, à Beatriz, ao David e ao Daniel pelos sorrisos, pela amizade e pelo companheirismo. Sem vocês esta aventura não tinha metade da piada e não deixava metade das saudades. Agradeço sobretudo à Sónia, pela amizade fantástica, pelo apoio incondicional e porque mesmo sem notar tornou os meus dias únicos e especiais, só como uma ‘‘irmã’’ poderia fazer. Por fim, o meu maior agradecimento é dirigido aos meus pais que me apoiaram incondicionalmente durante toda a minha vida e claro, durante esta etapa em particular. Sem vocês nada teria sido possível. Obrigada pelo incentivo, pela paciência, pelos conselhos, pela confiança, pelo carinho e por tudo o que fizeram para que eu nunca me desviasse dos meus sonhos e dos meus objetivos. Fizeram de mim a pessoa que eu sou hoje e como equipa que somos, as minhas vitórias são as vossas vitórias também. Obrigada por nunca me fazerem esquecer que tudo é possível e que a sorte acompanha quem se empenha e trabalha. Quando for grande espero ser tão grande como vocês. Nunca vos poderei agradecer o suficiente.

Palavras-chave Macroalgas, ficobiliproteínas, ficoeritrina, purificação, sistemas micelares de duas fases aquosas, líquidos iónicos, surfactantes, co-surfactantes

Resumo

Atualmente verifica-se um interesse crescente na extração de compostos provenientes de recursos naturais, abundantes e renováveis. As macroalgas constituem uma grande parte da matéria-prima marinha barata e renovável, apresentando na sua constituição diversos compostos bioativos de elevado valor acrescentado, nomeadamente as ficobiliproteínas. Este tipo de investigação é crucial para países como Portugal que, dada a sua vasta área costeira, têm a vantagem de possuir uma extensa oferta de biomassa marinha, permitindo a sua valorização. Contudo, os métodos de extração e purificação convencionais têm de ser melhorados e otimizados, visto serem estas as etapas responsáveis pelos maiores custos associados à comercialização destes compostos. Assim, este trabalho objetiva o desenvolvimento de sistemas micelares de duas fases aquosas usando líquidos iónicos como co-surfatantes para a avaliação do seu potencial como método de purificação de ficobiliproteínas. Numa primeira fase, este trabalho pretende alargar o conjunto de sistemas micelares de duas fases aquosas, dado o número muito reduzido destes sistemas em literatura. Desta forma, diversos copolímeros serão usados como surfatantes não-iónicos em combinação com duas famílias distintas de líquidos iónicos (imidazólios e fosfónios), atuando estes como co-surfatantes, na criação de sistemas micelares de duas fases aquosas convencionais e mistos para posteriormente serem aplicados na extração de ficobiliproteínas, em particular da R-ficoeritrina, a partir de algas vermelhas da espécie *Gracilaria sp.* Os principais resultados obtidos neste trabalho apresentam-se como promissores, uma vez que foi possível obter recuperações de R-ficoeritrina de cerca de 80% e seletividades entre 10 e 15, a partir do uso de amostras reais e, portanto mais complexas.

Keywords Macroalgae, phycobiliproteins, phycoerythrin, purification, aqueous micellar two-phase systems, ionic liquids, surfactants, co-surfactants

Abstract Nowadays, the extraction of natural compounds from renewal and abundant resources has raised the industrial interest. In this sense, macroalgae constitute a large part of cheap and renewable marine source of biomass that have shown great potential as a platform for the recovery of several high-added value compounds, namely phycobiliproteins. This type of research is crucial for countries like Portugal, since it is possible to take advantage of their privileged geographical location to access the raw materials (seaweeds) and value them. However, the currently extraction and purification processes need to be improved and optimized since they are primarily responsible for some difficulties in the industrial implementation. Consequently, this work addresses the exploitation of novel aqueous micellar two-phase systems (AMTPS) using ionic liquids (ILs) as co-surfactants for the assessment of their potential in the extraction of phycobiliproteins. Moreover, this work intends to create fundamental knowledge through the design of new AMTPS since currently there is a flagrant lack of systems compared the great amount of surfactants available in the market. Thus, several copolymers were used as nonionic surfactants in combination with two distinct families of ILs, namely the imidazolium and phosphonium, acting as co-surfactants to create conventional and mixed AMTPS for the extraction of phycobiliproteins, in particular R-phycoerythrin, from the red macroalgae *Gracilaria sp*. The initial results are promising since real matrices are used instead of model molecules and it is possible to verify that R-phycoerythrin recoveries are already higher than 80% and selectivities up to 10 and 15.

CONTENTS

LIST OF FIGURES

LIST OF TABLES

LIST OF ABBREVIATIONS

AMTPS: aqueous micellar two-phase systems

ATPS: aqueous two-phase systems

CMC: critical micellar concentration

CPB: hexadecylpyridinium bromide

CPC: hexadecylpyridinium chloride

CTAB: cetyltrimethylammonium bromide

[C_{10}mim]Cl: 1-decyl-3-methylimidazolium chloride

[C_{12}mim]Cl: 1-dodecyl-3-methylimidazolium chloride

[C_{14}mim]Cl: 1-methyl-3-tetradecylimidazolium chloride

[C_{14}Im-C_6-ImC_{14}][Br]$_2$: 3-(1-tetradecyl-3-hexylimidazolium)-1-tetradecylimidazolium dibromide

DTAB: dodecyltrimethylammonium bromide

HLB: hydrophilic/lipophilic balance

IL: ionic liquid

LCST: lower critical solution temperature

[$N_{1,1,14}$-6-$N_{1,1,14}$][Br]$_2$: N-(N-hexyl-N,N-dimethyl-N-tetradecylammonium)-N,N-dimethyl-N-tetradecylammonium dibromide

[$N_{1,1,14,14}$][Br]: N,N-dimethyl-N,N-di(tetradecyl)ammonium bromide

PEO: polyethylene oxide

PPO: polypropylene oxide

[$P_{6,6,6,14}$]Br: trihexyltetradecylphosphonium bromide

[$P_{8,8,8,8}$]Br: tetraoctylphosphonium bromide

[$P_{4,4,4,14}$]Cl: tributyltetradecylphosphonium chloride

[$P_{6,6,6,14}$]Cl: trihexyltetradecylphosphonium chloride

[$P_{6,6,6,14}$][Dec]: trihexyltetradecylphosphonium decanoate

[$P_{6,6,6,14}$][TMPP]: trihexyltetradecylphosphonium bis 2,4,4-(trimethylpentyl)phosphinate

SDS-PAGE: sodium dodecyl sulphate polyacrylamide gel electrophoresis

TTAB: tetradecyltrimethylammonium bromide

UCST: upper critical solution temperature

λ_{max}: maximum absorption wavelength

1. INTRODUCTION

1.1 VALUING MARINE RESOURCES THROUGH BIOTECHNOLOGY

It is well known that oceans occupy approximately 70% of the planet's surface and that they are considered a source of food and energy for humans and their activities [1]. One of the main aims of the Horizon 2020 program is the creation of new technologies and procedures to unlock the seas and oceans' potential value for marine industries, while protecting the environment and preventing the climate change [2].

The majority of the marine biodiversity (about 90%) remains unexplored despite its great potential for applications within the marine biotechnology field (**Figure 1**). These advances allow an expectation of 10% annual growth for this particular sector. The international financial support from Horizon 2020 program and others have been responsible to increase the marine biodiversity and aquatic biomass exploitation in order to offer new sustainable processes, products and services [2]. Additionally, there is also national funding from programs such as CRER 2020, which is a program for the center region of Portugal, in which Aveiro is included [3]. Those programs are crucial to coastal countries, like Portugal, since they can find new ways to benefit from its strategic location and contribute to the region economic growth while the environment is preserved. Moreover, macro and microalgae are very promising examples of aquatic resources that allow a wide range of applications in the blue biotechnology field [4]. Herein, the goal is to use the raw marine bio-resources, extract and/or transform the compounds through biotechnological processes and further apply them in the correspondent sector [4].

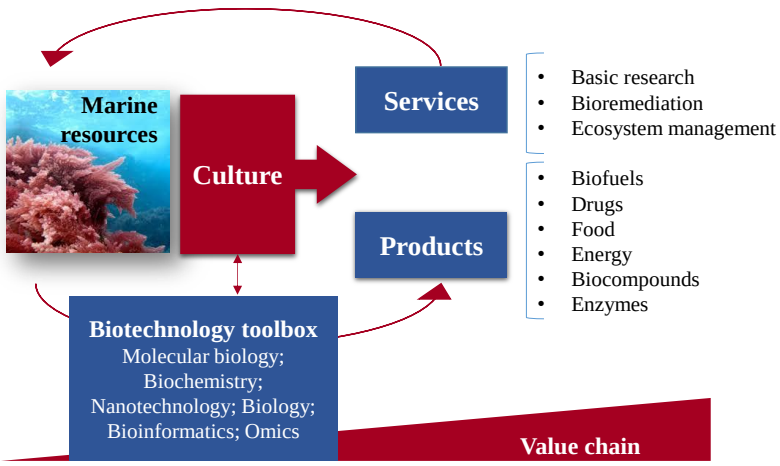


Figure 1 - Marine biotechnology diagram. Adapted from [5].

1.2 - MACROALGAE

Macroalgae, also known as seaweeds, are plant-like organisms frequently found in coastal areas that are usually attached to rocks or other hard substrata. Furthermore, macroalgae are primary producers, *i.e.* they can produce oxygen and organic compounds that serve as the basic trophic level or food for many ecosystem's living beings. Therefore, they have an essential role in the food chain of all aquatic ecosystems [5]. Seaweeds can be naturally found in oceans and seas but they can also be cultivated in a sustainable way, at a large scale in open sea or in aquaculture [6]. They are generally classified as red (Rhodophyta), brown (Phaeophyta) and green/blue algae (Clorophyta) [1,7]. Additionally, macroalgae are divided into distinct groups accordingly to their properties like pigmentation, nature of photosynthetic storage products and other morphological features [8]. Some of them can extend several miles into the sea as far as the sunlight is available, being the red macroalgae the deepest living macroalgae [6].

Rhodophyta is composed of around 8000 different species [7], being considered as an important source of many biologically active metabolites, with a wide range of biological activities such as antiviral [1,6,7], anti-inflammatory (well-reviewed in [6,7,9]), neurophysiological [6,7], insecticidal [6,7], antimicrobial [1,6,7] and cytotoxic [6,7]. When considering the protein contents of red algae, they vary according to the season, species and field conditions, in which one of the most important factors is the nitrogen input during cultivation. Francavilla and co-workers [10] described that in the case of *Gracilaria gracilis*, the protein content varies between 31% and 45% of dry weight depending on the season, while the red algae *Grateloupia turuturu* display a protein content between 14% and 27.5% of dry weight [11]. In general, red seaweeds display an approximate phycobiliprotein content of about 60% of the soluble proteins of the cell [6,12,13].

1.3 - R-PHYCOERYTHRIN: A PARTICULAR PHYCOBILIPROTEIN

Phycobiliproteins are coloured and fluorescent light harvesting pigments of red algae and cyanobacteria, which can constitute up to 40% of the total cell proteins when these organisms are cultured at low light intensities and in the presence of abundant nutrients [14]. However, statistical analysis showed significant differences in terms of phycobiliprotein concentration accordingly to the seasons [6,15,16,17]. From **Figure 2**, it is possible to conclude that R-phycoerythrin is the most abundant phycobiliprotein with

concentrations ranging between 3.6 and 7.0 mg/g of dry weight [10]. In *Rhodosorus marinus*, which is another red algae specie, the phycoerythrin (further discussed) content was found to be 8% of the algae dry weight [12].

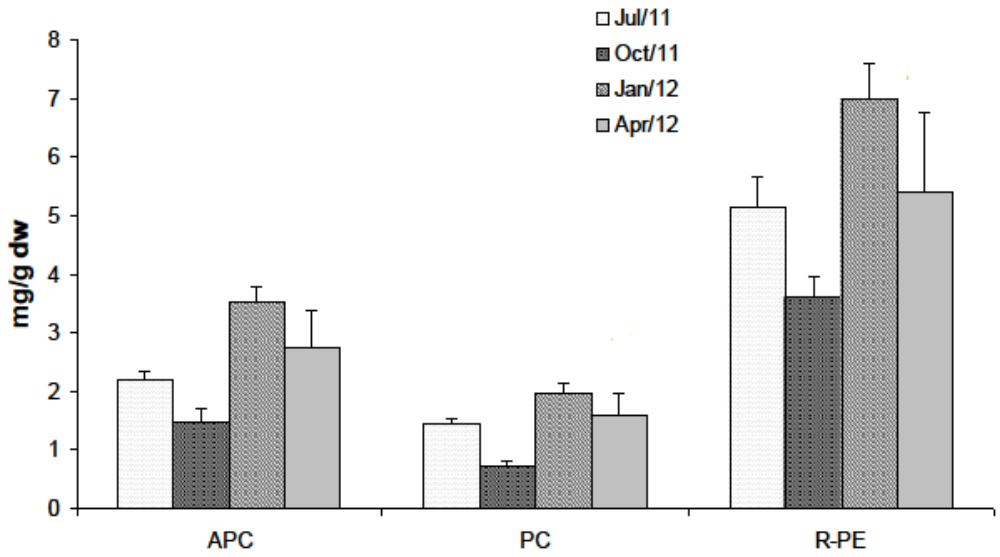


Figure 2 - Concentration of phycobiliproteins (mg/g dry weight): APC, allophycocyanin; PC, phycocyanin and R-PE, R-phycoerythrin in *Gracilaria gracilis* [10].

Stepping back to the phycobiliproteins, which are reasonably stable proteins composed of an open chain of tetrapyrrole prosthetic groups covalently linked to the apoprotein that form supramolecular structures called phycobilisomes. These structures are located on the external structure of the thylakoid membranes, in the stroma [18] (**Figure S.I. 1 - A** in Supporting Information). These unique structures display two substructures – the core and the rods, which are composed of allophycocyanins and hexamers disks of different phycobiliproteins, respectively [19]. From the three monomers (αβ) association results the building block of these structures – the trimer $(\alpha\beta)_3$ – these trimers by their turn, assemble into hexamers with linkage polypeptides (**Figure S.I. 1** in Supporting Information).

The presence of covalently attached prosthetic groups, known as chromophores (via thioether bonds to cysteine residues), is responsible for the phycobiliproteins colours. For phycobiliproteins in particular, the chromophores are called phycobilins and thus, the visible absorption spectra of individual phycobiliproteins are on account of the specific bilins attached to the protein since they confer the spectral properties of phycobiliproteins [14,19]. The four types of chromophores found in red algae, are classified as phycoerythrobilin (λ_{max} = 560 nm), phycocyanobilin (λ_{max} = 620-650 nm), phycobiliviolin

(λ_{max} =575 nm) and phycourobilin (λ_{max} = 450nm) [20,21]. Moreover, factors such as conformation, environment and interchromophore interactions of the native protein play an essential role in the differentiation of the spectra [14,22]. In this sense, phycoerythrins are easily differentiated since their light absorption properties are different among them. In other words, spectral differences between phycoerythrins are due to the presence of different types of bilin prosthetic groups [23]. Thus, R-phycoerythrin has a special absorption spectrum (**Figure 3 – A** and **B**) in its native state with a three-peak absorption maxima at 499, 545, 565 nm [24], which allows the concentration and purity determination using spectroscopic methods. In fact, chromophores allow the efficient transmission of light energy in phycobilisomes, with an efficiency close to 90% by absorbing light in spectral zones where chlorophyll *a* cannot, towards the reaction centres. The light energy transmition occurs according to the following pathway: phycoerythrin/ phycoerythrocyanin, phycocyanin, allophycocyanin and chlorophyll *a* [25]. Therefore, this mechanism allows the survival of living organisms at low light intensity regions, since they act as an efficient photon collector.

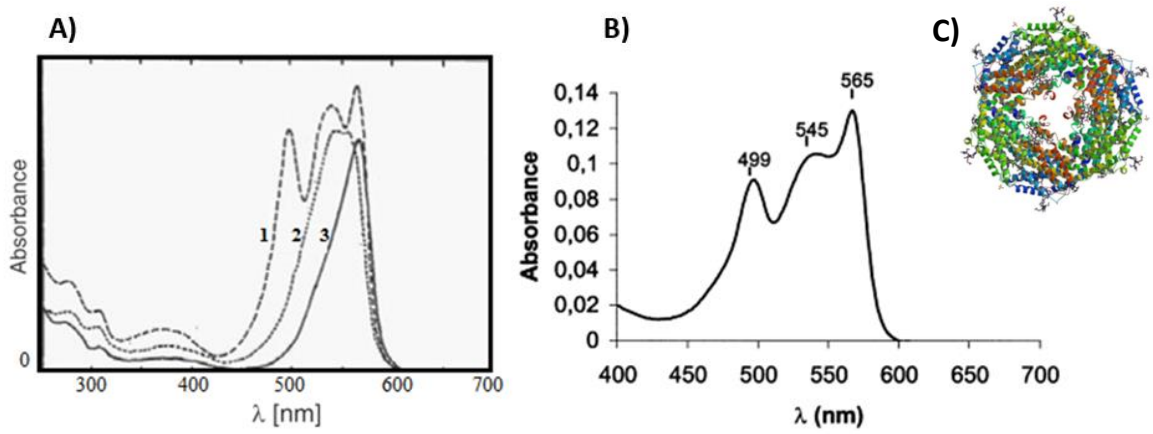


Figure 3 - A) Absorption spectra of different types of phycoerythrin: 1, R-phycoerythrin; 2, B-phycoerythrin; 3, C-phycoerythrin [26]. **B)** Absorption spectra of R-phycoerythrin from *Palmaria palmata* [24]. **C)** Crystal structure of the biological assembly of R-phycoerythrin at 2.2 Angstroms from *Gracilaria chilensis*. Adapted from [27].

Phycoerythrin, which is the most significant pigment found in red macroalgae, has a PDB ID code of 1EYX and usually displays a molecular weight between 240 and 260 kDa [28] (**Figure 3 – C**). Additionally, its subunits (α, β, γ) have also different molecular weights: α = 18 -20 kDa, β = 19-21 kDa and γ = 30 kDa [24]. Depending on the species, different forms of phycoerythrin can occur: R-phycoerythrin, B-phycoerythrin or C-

phycoerythrin. Its stability depends on the location of the γ subunit, which is placed in the center of the molecule and is linked to the trimers $(\alpha\beta)_3$ [29]. The stability of phycoerythrin changes according to some parameters such as pH, light exposure time and temperature (**Table S.I. 1** in the Supporting Information). Thereby, modifications in the solution acidity can lead to disturbance of electrostatic forces and hydrogen bonds involved in the protein proper folding. During the pH induced denaturation of R-phycoerythrin, conformational modifications of the chromophore–protein complexes allow a stronger exposure to solvent and thus, changes in the distance between chromophores that consequently leads to the protein denaturation [30]. Munier and co-workers [31] also showed that, in a pH range between 4 and 10, no colour change was detected in R-phycoerythrin. However, in extremely acidic buffer solutions, the absorption spectra of R-phycoerythrin (**Table S.I. 1 – Figure A** in the Supporting Information) was severely modified. On the other hand, at alkaline pH, the R-phycoerythrin concentration was stable between pH 7 and 10. As far as fluorescence stability is concerned, it is maintained between the pH 3 and 10, and under extreme conditions, the fluorescence was greatly modified (**Table S.I. 1 – Figure D** in Supporting Information) [30,31]. Additionally, many other studies have shown similar results for R-phycoerythrin pH stability in other red algae species [24,30,32–34]. Besides the pH, the light exposure time has also great effect on the R-phycoerythrin stability, which can be confirmed by observing the absorption and fluorescence spectra of R-phycoerythrin presented in **Figure B** and **E** (**Table S.I. 1)** in Supporting Information. Regarding the fluorescence data (**Figure E** (**Table S.I. 1)** in Supporting Information), a significant intensity reduction was detected with the increase of the light exposure time. Moreover, the absorption and fluorescence spectra depicted in **Figures C** and **F** from **Table S.I. 1** (Supporting Information), showed that no significant variation in absorbance or fluorescence was observed up to 40ºC.

1.3.1– R-phycoerythrin applications

The global market of pure phycobiliproteins is estimated in US$50 million with yearly growths of around 10%. Moreover, highly pure R-phycoerythrin represent a very profitable and wide market [4,17] especially when applied in pharmaceuticals, cosmetics, food products, biomedical research and clinical diagnostics [35,36]. This protein is normally expensive and even more when conjugated with antibodies. In **Table 1** are listed some red seaweed product's costs in order to stress the importance of this protein.

Furthermore, the prices change with companies, quantity supplied, purity, protein stability and source [37].

Table 1 - Red seaweed products cost [38].

Red seaweed bioproduct	Cost
Fresh raw material (Nori, for example)	1 200€ / wet tone
Dried raw material (Nori, for example)	16 000€ / dry tone
Food grade R-phycoerythrin extract	176€ / L
Pure R-phycoerythrin	126€ / mg
R-phycoerythrin antibodies	1 300€ / mg

As previously mentioned, R-phycoerythrin shows thermal stability up to 40ºC and it is also stable in a pH range between 4 and 10 [24,31]. These characteristics are favourable to use phycoerythrin as a colorant in pharmaceutical and food industries. Therefore, there are many patents regarding the use of this pigment as red/pink food colorants (well-reviewed in [39]), namely in jellified desserts and dairy products. Moreover, the unique spectral properties and fluorescence emission make this pigment suitable for a large number of biotechnological applications [17], such as molecular probes (as fluorescin), since phycoerythrin has at least 20 times more fluorescence yield than other pigments usually used in molecular probes. Phycoerythrin also displays a very high molar absorption coefficient (about 2.4×10^6 $M^{-1}cm^{-1}$ for a 240 kDa protein), which justifies its high sensitivity [40]. In addition, this pigment emits in the red-orange spectral zone, where the background noise is lower than for other spectrum areas [36]. Thus, the combination of the mentioned properties makes phycoerythrin suitable for several techniques such as flow cytometry, immunophenotyping and microscopy [39,41].

R-phycoerythrin seems not only to be a good probe in fluorescence resonance energy transfer for the proximity/interaction evaluation between two molecules [24], but also a decent internal marker in electrophoretic techniques, like sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) and non-denaturant electrophoresis, since R-phycoerythrin subunits (with low molecular weight) carry chromophoric groups characterized by a particular deep rose colour [42]. Furthermore, there are already a lot of patents (well-reviewed in [39]) regarding the use of phycobiliproteins as fluorescence labels, markers and tags, energy transfer agents, signal generators, image contrast agents,

diagnostic tools, bioluminescent agents, kits and other general health applications. Moreover, R-phycoerythrin has also some interesting biological activities, such as the anticancer[39], antioxidant [43,44], antidiabetic [44], immunosuppressive and antihypertensive properties [45].

1.3.2– Phycobiliproteins extraction and purification procedures

Phycobiliproteins (R-phycoerythrin in particular) have a variety of applications and offer interesting industrial perspectives. Due to the difficulties in their purification, these pigments are rather expensive and obtaining them as pure compounds is a potentially attractive challenge [38,46]. In this sense, there is a growing interest in creating extraction and purification methodologies that are not only more efficient, but also less expensive and, as much as possible, environmentally friendly [46–49]. Thus, several studies have shown that phycobiliproteins (and consequently, phycoerythrin) can be extracted by soaking seaweeds in water, causing an osmotic shock in the algal cells. However, this type of extraction brings some disadvantages, such as the partial degradation of phycobiliproteins by proteases and the high economic impact of the procedure, mainly due to the time required by the procedure [36]. Another strategy tested was the extraction of phycobiliproteins in diluted sodium phosphate buffer at a pH range between 6.8 and 7.4 from fresh, frozen or dried algae [50].

The majority of the extraction methods are based on cell wall breakage and thus, the pigments are more exposed to the solvent's presence and action [51], which can also be helped by the grind of the macroalgae biomass in liquid nitrogen, since this process will facilitate the cells destruction. Despite the mentioned advantages, the high cost of these procedures is still an obstacle for large scale (industrial) implementation. Therefore, alternative extraction methods are required in order to reduce as much as possible the costs of the procedures, while maintaining or even increasing their efficiency and yields of extraction. In this sense, some investigation has been performed [37,51–53] considering the application of enzymes to promote the enzymatic hydrolysis of the cell walls, however, it is well known that enzymes represent a considerable cost in the enzymatic processes. Nevertheless, the benefit occurred by their use is also important since the enzymes can be chosen according to the nature of the algal cell wall to be used as cell disrupters [50,53,54]. Some authors have also reported the optimization of enzymatic hydrolysis conditions, such

as pH [55] , temperature [50,55], enzyme concentrations [53], for protein or peptide recovery [50,53,54]. As such, enzymatic tissue disruption may be an efficient alternative for releasing many classes of compounds usually confined and inaccessible due to the high polysaccharide content (alginates, carrageenan, agar and xylans), limiting the access of the solvent [56]. Another factor that strongly affects the protein bioavailability is the covalent bond between xylan and the glycoprotein complexes [57]. To overcome these problems, new techniques have been applied in the field, namely the enzymolysis and microwave-assisted extractions [17]. The first one improves the catalytic efficiency, increases the specificity and preserves the original state of active compounds, whereas the second one requires less solvent and increases the extraction rate. However, the high cost of enzymes for industrial applications is still an obstacle for the implementation of this technique as a pre-treatment step of the algae extract [37]. Nonetheless, several studies have been made in order to evaluate the benefits of using enzymes to recover bioproducts from algae extracts [37] and R-phycoerythrin in particular [17,51]. Dumay and co-workers [50] recently found out that R-phycoerythrin extraction using enzymes (xylanases) from *Palmaria palmata* was 62 times greater than without enzyme treatment and the protein purity index was 16 times superior [50].

Regarding the economic and industrial interest of phycobiliproteins, new methodologies are required to replace those recently described using osmotic shock or enzymatic disruptions. Thus, several studies have shown that phycobiliproteins (and consequently, R-phycoerythrin) can be extracted by soaking seaweeds in water, causing an osmotic shock in the algal cells. However, this type of extraction brings some disadvantages, such as the partial degradation of phycobiliproteins by proteases and the high economic impact of the procedure, mainly due to the time required by the procedure [36]. Another strategy tested was the extraction of phycobiliproteins in diluted sodium phosphate buffer at a pH range between 6.8 and 7.4 from fresh, frozen or dried algae [50].

Some authors have already proposed to isolate phycobiliproteins and purify R-phycoerythrin in a cheaper, more efficient and safer way. Usually, these purification procedures are a combination of some techniques to reach higher purification ratios. In one of the typical examples, phycobiliproteins (particularly phycoerythrin) are purified by ammonium sulphate precipitation [58–63]. Herein, high charges of the salt are introduced, normally between 25 and 85% (**Table S.I. 2** from Supporting Information), varying with

the algae species and the protein solubility [61,62], causing the precipitation of certain proteins [61]. It should also be stressed that this *salting-out* phenomenon lacks the ability to precisely isolate (by precipitation) a specific protein, so another method is required to end the purification [63]. Meanwhile, more sophisticated methodologies are being investigated, such as ion-exchange chromatography [65,68,70–72,75], which retains molecules on the column according to specific ionic interactions; gel filtration [34], wherein the separation is carried out based on the size of the molecules in solution [67] and affinity chromatography, which takes advantage of the high affinity of some proteins for their specific surface chemical groups [68]. Another purification method example is the expanded-bed chromatography, wherein the adsorbents with a high-density matrix are used to form a stable expansion at high feed flow rates [69,70]. Lastly, it is also possible to perform a hydroxyapatite chromatography, which is known as a 'pseudo-affinity' chromatography [69].

In most cases, the combination of these techniques seem to be promising to increase the purity index of the target protein [17]. **Table S.I. 2** from Supporting Information shows a compilation of R-phycoerythrin extraction and purification procedures already established and presented in literature, as well as their respective extraction yields, purity index and extraction conditions. Briefly from this table, it is possible to notice that nearly all the purification stages include a chromatographic procedure, a high resolution process, thus conferring high purity to the target molecule. However, it is also an expensive method that displays low productivity, as well as the need for more than one procedure, which is normally associated with higher losses of the target protein [46,71]. These facts contribute greatly to the high costs of the purification processes. In this sense, liquid-liquid extraction appears as an attractive alternative procedure since several steps can be combined into a single operation and thus, conferring lower costs [72]. In general, liquid-liquid extraction is the transfer of certain compounds from one phase to another when two immiscible liquids are brought into contact with each other [80,84]. However, it is carried out using environmental hazardous organic solvents and, therefore, is generally not suited for biomolecules extraction due to some problems associated with the phenomena of denaturation and/or chemical degradation of the compound [48,72,74]. To overcome these drawbacks, aqueous two-phase systems (ATPS) [46] have been emphatically applied over the last decades. ATPS display two aqueous distinct phases conventionally constituted by

either two water soluble polymers [76–78], a polymer and an inorganic salt [78–80], a two-salts combination [78,81], carbohydrates [82], amino-acids [83] and surfactants [84,85]. These systems are promising methodologies since they are mainly composed of water. Thereby, they are considered as more biocompatible as they provide mild extraction conditions [48]. In this sense, they have been applied in the extraction, separation, concentration and purification of several biological compounds, such as proteins [54,83,89-92], antibodies [71,88,89], nucleic acids [71,90], antibiotics [91–93], colorants [94] and alkaloids [95]. However, polymer-based ATPS possess high viscosities and limitations related to the high cost of some polymers that are usually applied, namely dextran. Moreover, when corrosive inorganic salts are employed, there are limitations regarding the equipment maintenance and the wastewater treatment [96]. Later on, and in order to minimize environmental impacts while improving extraction efficiencies, ionic liquids (ILs) were incorporated into the ATPS [78].

Generally, ILs are molten salts composed of a large organic cation and an organic or inorganic anion, conferring them an asymmetric structure. Therefore, most of them display negligible vapour pressure and low flammability which makes their application advantageous giving rise to their "*greener solvent*" character, when compared with the organic solvents [87, 106]. They present a high ability to solvate a huge variety of solutes, high thermal stability, lower toxicity (compared to the organic solvents and also depending on the cation chosen) and high ionic conductivity [97]. They are claimed as ''*designer solvent"*, since ILs can be synthesized accordingly to its final application [98]. Thereby, it is also possible to adjust parameters like the hydrophobicity nature, biodegradation rate [98] and water miscibility in particular [99–101]. Additionally, and despite the fact that they promote safer and more biocompatible environments for the extraction and purification processes [102], their use brings some disadvantages, namely the high cost of these solvents. Thus, they should be applied in aqueous solutions at low concentrations in order to control both the extraction costs and the lower toxicity character of the systems. In addition to all the above unique properties of the ILs and even though imidazolium-based long alkyl side chain ILs have been used for decades, only a few years back they started to be recognized as tensioactive (and amphiphilic) compounds (**Figure 4**). Moreover, some studies showed that ILs are able to form micelles in aqueous solutions [103–107], since the IL anion or cation central part are the charged hydrophilic head group whereas the cation

alkyl side chain is the hydrophobic ‘tail’ domain [108]. Micellization in solutions where ILs act as surface active agents has been quite studied [108–113]. Bowers and co-workers [114] investigated the possibility of ILs to self-aggregate when they have longer alkyl chains with at least eight carbons, $[C_8mim]^+$. Furthermore, aqueous solutions of alkylimidazolium-based ILs, $[C_nmim]$-X, where n = 8–14 and X = Br, Cl, BF_4, or PF_6 are the most studied [115]. ILs are thus being considered as potential ‘‘*biocompatible surface active agents*’’ to replace the conventional cationic and anionic surfactants commonly used. Although, it can be considered that very little is known about the surface active and micellization properties of these special ILs, it is always an interesting subject for investigation in the interface sciences area [106,108,116].

Despite this, the ILs ability to affect the surfactant micellization characteristics has already been studied for IL surfactant combinations, including mixtures of several ILs with ionic and nonionic surfactants [117–119]. The addition of ILs to a surfactant aqueous solution can promote the increase [120–122] or decrease [123–125] of the critical micellar concentration (CMC). Moreover, the IL addition also affects the aggregation number according to the IL’s alkyl side chain, cation and anion [114]. In this sense, mixed micelles composed of surfactants and ILs were studied and considered to be more versatile than those from one single surfactant [134,135]. Considering all the advantages of the use of mixed micelles, the aqueous micellar two-phase systems (AMTPS) with ILs acting as co-surfactants emerged as a promising alternative extraction methodology [126].

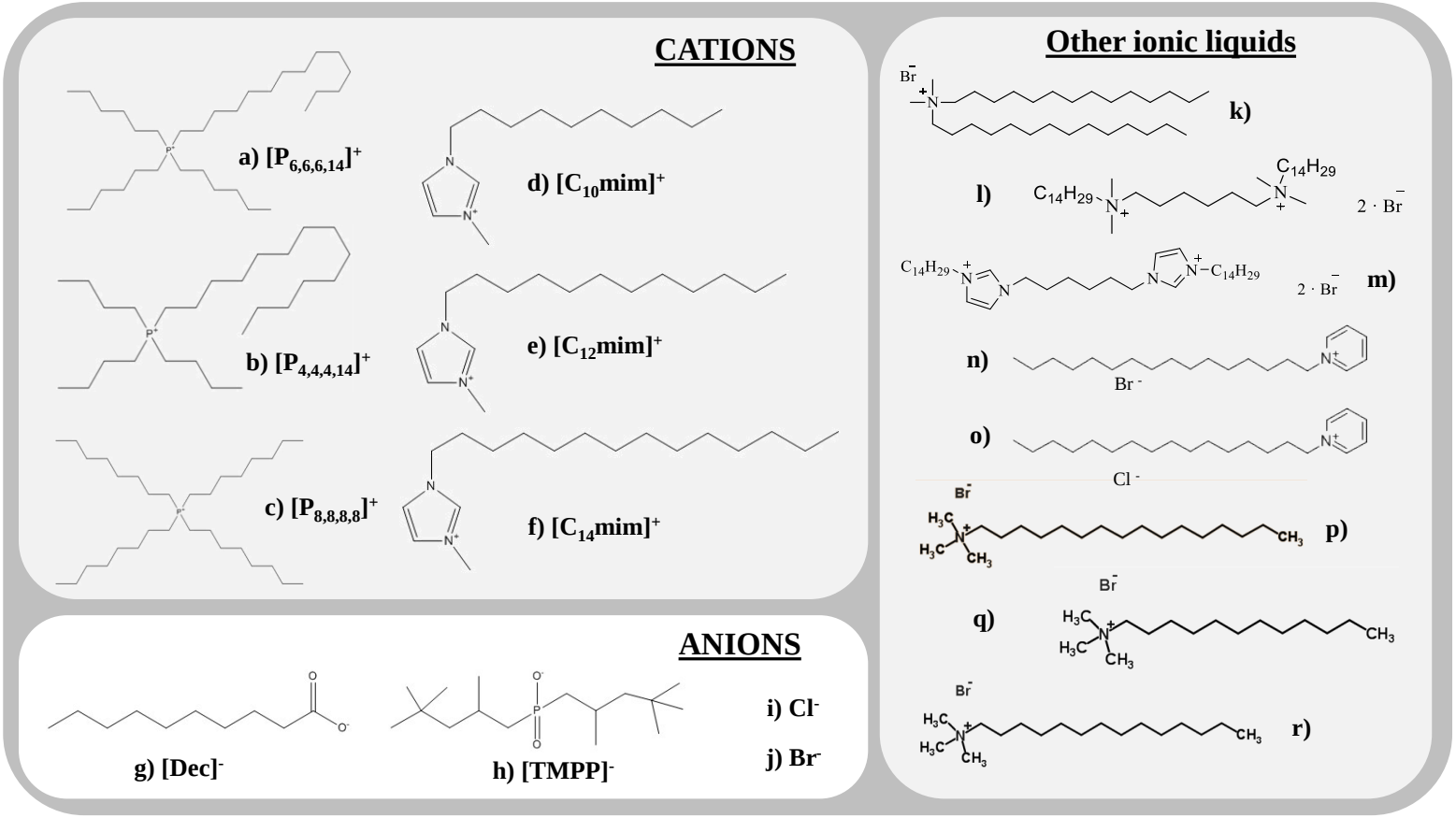


Figure 4 - Tensioactive ILs belonging to the phosphonium family, namely, **a)** and **j)** trihexyltetradecylphosphonium bromide; **a)** and **i)** trihexyltetradecylphosphonium chloride; **a)** and **h)** trihexyltetradecylphosphonium bis 2,4,4-(trimethylpentyl)phosphinate; **a)** and **g)** trihexyltetradecylphosphonium decanoate; **b)** and **i)** tributyltetradecylphosphonium chloride; **c)** and **j)** tetraoctylphosphonium bromide. ILs belonging to the imidazolium family, namely, **d)** and **i)** 1-decyl-3-methylimidazolium chloride; **e)** and **i)** 1-dodecyl-3-methylimidazolium chloride; **f)** and **i)** 1-methyl-3-tetradecylimidazolium chloride. There are other ILs, namely **k)** *N*,*N*-dimethyl-*N*,*N*-di(tetradecyl)ammonium bromide $[N_{1,1,14,14}]Br$, **l)** *N*-(*N*-hexyl-*N*,*N*-dimethyl-*N*-tetradecylammonium)-*N*,*N*-dimethyl-*N*- tetradecylammonium dibromide $[N_{1,1,14}\text{-}6\text{-}N_{1,1,14}]Br_2$, **m)** 3-(1-tetradecyl-3-hexylimidazolium)-1-tetradecylimidazolium dibromide $[C_{14}Im\text{-}6\text{-}C_{14}Im]Br_2$, **n)** hexadecylpyridinium bromide (CPB), **o)** hexadecylpyridinium chloride (CPC), **p)** cetyltrimethylammonium bromide (CTAB), **q)** dodecyltrimethylammonium bromide (DTAB) and **r)** tetradecyltrimethylammonium bromide (TTAB).

1.4- 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 owing to their ability of keeping the native conformation and activity of a wide range of biomolecules [127], including DNA [128], antibiotics [103] and viruses [126]. This is mainly due to the fact that these systems do not interact very much with the molecules, which is clearly an advantage of these systems when compared with the conventional ATPS. The first use of AMTPS as separation systems was reported by Watanabe and Tanaka [129] for the concentration of zinc ions. Afterwards, Bordier and collaborators [130] demonstrated the efficiency of Triton X-114-based AMTPS to separate proteins based on their hydrophobicity, which was recently verified by Vicente and co-workers [127]. Currently, this surfactant is the most widely used in the formation of AMTPS due to its low cloud point, low commercially cost and negligible toxicity [117,141–143]. In this sense, the separation of proteins and other compounds of high-added value using AMTPS have been gaining relevance and interest from the scientific community [89,92,94–96].

Experimentally speaking, AMTPS are composed of surfactants aqueous solutions that at certain critical concentrations and temperatures, known as the cloud point or T_{cloud} [47,49,136], becomes turbid and then separates spontaneously into two macroscopic phases [137]. From the macroscopic phase separation (that is above the surfactant CMC and cloud point) results a micelle-rich and a micelle-poor phase, which is illustrated in **Figure 5 – A**). The micelle-rich phase can occupy the top phase or the bottom phase according to the surfactant [47,49] and usually hydrophobic compounds can be extracted into the micelle-rich phase, while hydrophilic ones are retained in the aqueous phase (micelle-poor phase). When hydrophobic compounds are bigger than the micelle size, then they are recovered in the micelle-poor phase owing to the exclusion-volume effect of the micelles [136,138,146]. Moreover, the phase separation behaviour of surfactants can be described by the graphical representation known as the binodal curve, which is obtained by plotting the different cloud points *versus* the respective surfactant concentrations (**Figure 5 - B**).

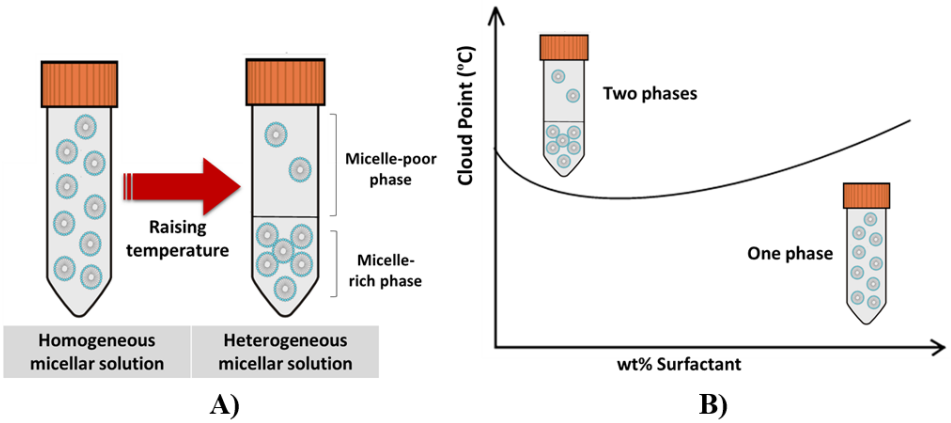


Figure 5 - Illustration of a typical AMTPS two-phase separation: **A**) is the representation of the spontaneous separation of phases by raising the temperature of the system while **B**) represents the phase diagram with the respective binodal curve.

This curve represents the boundary between the conditions at which the system presents a single phase (below/outside the curve) or two macroscopic phases (above/inside the curve) [137]. The separation of phases occur either through cooling or upon heating the system and is characterized by an upper critical solution temperature (UCST) or a lower critical solution temperature (LCST), respectively, or a combination of both (**Figure 6**) [138].

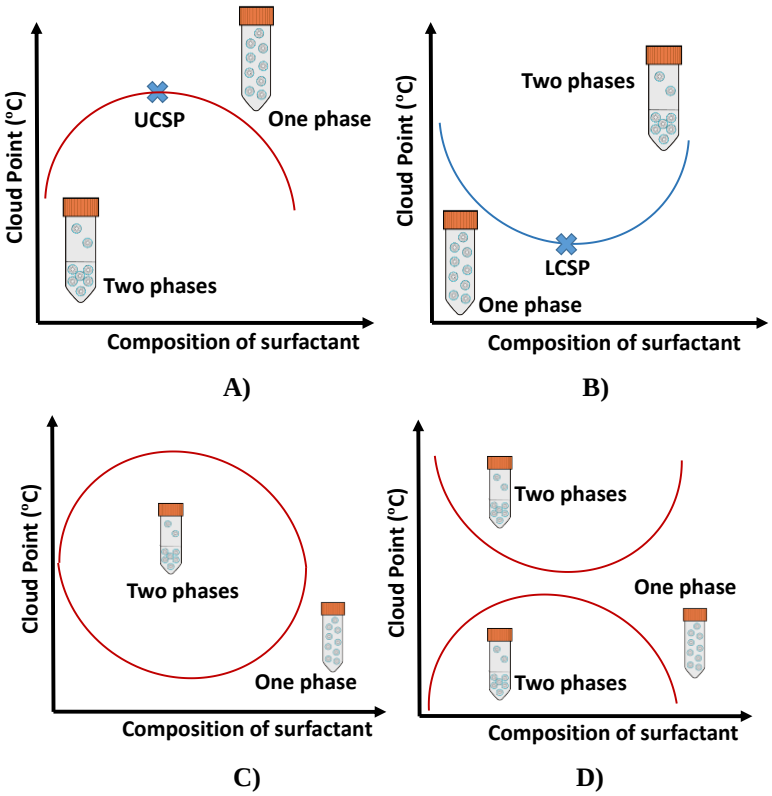


Figure 6 - Possible phase diagrams through LCSP and UCSP representation: **A**) UCSP; **B**) LCSP; **C**) Phase separation region (island) and **D**) Miscibility gap. Adapted from [138].

The formation of these AMTPS is a reversible process by reducing the micelle's solubility, which can be achieved by decreasing the temperature and below the cloud point. Hydrogen bonding between the solvent and the oxygen atoms in the polyoxyethylene chain is responsible for keeping the micelles solubilized in the aqueous solution. It is also fundamental to add that, this occurs in the most common systems, *i.e*. the ones that exhibit a LCST. As the temperature increases, the hydrogen bonding decreases and as a result, there is the formation of intermicellar aggregation [138–140]. With the entropy increase, the micelles fuse together, becoming bigger and migrate to form the top or the bottom phase (depending of the surfactant) as the concentrated micellar phase. The clouding phenomena can be explained by a conformational change in the surfactant polyoxyethylene chain, which normally implies the decrease in the polarity of ethylene oxide unit and thus, the reduction of its hydrophobicity [47].

1.4.1– Surfactants and Copolymer surfactants

Surfactants are used on a daily basis both in academia and industries since these compounds lower the interfacial tension between two liquids or between a liquid and a solid (well-reviewed in [141]). Surfactants are amphiphilic molecules, and as such, they present a hydrophilic region ('head'), which can be ionic or nonionic, and a hydrophobic region ('tail'), which means that they are soluble in both water and organic solvents [141]. When surfactant molecules are dissolved in water at concentrations above the CMC, they are able to self-assemble and form micelles. At this point, the hydrophobic 'tails' are situated in the interior of the aggregate in order to minimize their contact with water while the 'heads' remain on the surface in order to maximize the contact with water [49,142].

The micellization phenomenon occurs when the intermolecular forces, namely hydrophobic, steric, electrostatic, hydrogen bonding and van der Walls interactions, reach their balance [142]. The main attractive forces results from the hydrophobic effect conferred by the hydrophobic tails while the main opposing force are due to the steric interactions between hydrated nonionic heads and electrostatic interactions between charged heads with similar charge [142]. As a result of the different surfactants and the distinct balance between the intermolecular forces, micelles can display several forms, such as spherical, cylindrical or planar [49,141–143]. It is assumed that all types of surfactants exhibit the ability to form two distinct phases when induced by temperature [141] and depending on specific structural features they can be classified as anionic,

cationic, nonionic and amphoteric/zwitterionic surfactants. Anionic surfactants display a negatively charged group like sulphonate, sulphate or carboxylate. This kind of compounds are sensitive to water hardness, *i.e.* the calcium and magnesium concentration on the water, and the most common applications are for cleaning purposes [144]. On the other hand, cationic surfactants possess a hydrophilic part that is positively charged (e.g. with a quaternary ammonium ion). Usually, they are used for softeners but they can also be applied as disinfectants, due to their bactericidal properties [141]. Amphoteric/zwitterionic surfactants display a pH dependent charge of the hydrophilic region. In other words, they can act as an anionic surfactant in an alkali solution or as cationic surfactant in an acidic solution [144]. Lastly, there are nonionic surfactants with a non-charged hydrophilic region (e.g. copolymers) [144]. Nonionic copolymers, also known as block copolymers, are represented by the combination of two different and quite common families of polymers, namely the poly(ethylene oxide) (PEO) and the poly(propylene oxide) (PPO). The presence of both hydrophilic PEO and hydrophobic PPO blocks in the same monomer of surfactant results in a self-arrangement of PEO/PPO copolymers in order to minimize the contact of PPO with water [96,113]. The PEO block dissolves well in aqueous media since it is mostly hydrophilic, while the PPO block does not dissolve well due to its hydrophobic character. Nevertheless, it is this difference in polarities that allows them to self-assemble into micelles within an aqueous solution and depending on their hydrophilic-lipophilic balance (HLB) – **Figure 7**, conferred by the PEO and PPO polymers, respectively, they can display different cloud points.

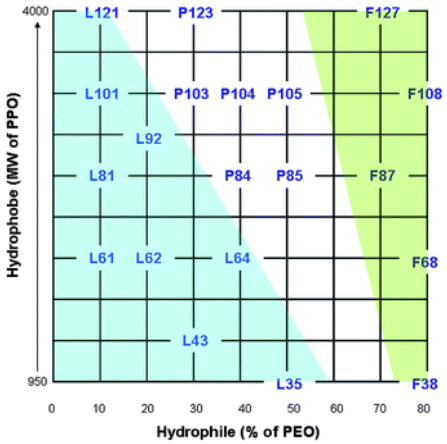


Figure 7 - HLB grid for the Pluronic series. The blue area corresponds to a surfactant in a liquid form, the green area corresponds to a solid form surfactant and the white area corresponds to a surfactant in an intermediate form [116].

This is an important parameter to be taken into account while selecting a surfactant for a purification process [139,145]. These particular nonionic surfactants display a huge advantage over the conventional surfactants, since amphiphilic characteristics can be modified by molecular architecture according to the final application. In other words, the HLB, the total molecular weight and the block sequence can be modified in order to obtain different behaviours in solution [138–140]. However, there is an upper limit on the PPO block size so that the solubility in water can be ensured. Both PEO and PPO homopolymers present a LCST in water [138], *i.e.*, water becomes a worst solvent while the temperature increases. This fact explains a heat-induced formation of aggregates and the consequent phase separation. In this sense, the phase separation of PEO/PPO copolymers in aqueous solution occur at even higher temperatures [139]. These promising block copolymer surfactants are available as the Pluronic family [146]. Herein, it should be stress that the PEO corresponds to the polyethylene glycol (PEG) monomer whereas PPO is the monomer of polypropylene glycol (PPG). Homopolymers, such as PEG and PPG, PEO/PPO copolymers, secondary alcohol ethoxylates, nonylphenol ethoxylates (usually known by their commercial name – Tergitol 15-S-series and Tergitol NP-series, respectively) and some others are also inserted in the nonionic category of surfactants. Then, it is crucial to emphasize their ability to promote the phase separation, also induced by temperature [144].

As far as phase formation is concerned, it is important to stress that the cloud point of a given nonionic surfactant can be modified by the addition of some additives or by increasing the system pressure. The presence of shorter saturated hydrocarbons and neutral electrolytes (such as chlorides and carbonates), in the aqueous micellar solution decreases the cloud point of the system due to their *salting-out* effect. By contrast, *salting-in* electrolytes, such as nitrates and iodides usually increase the cloud point [104]. These results imply that the addition of buffered solutions also play a major role in the system, since these control the conformational structure and chemical speciation of the molecules being purified, while maintain the pH controlled.

According to what is reported in the literature and taking into account the wide variety of surfactants commercially available [146,147], the lack of information about AMTPS is very clear. In this context, this work aims at the creation of fundamental knowledge and databases in the design and comprehension of other AMTPS, an area that is

practically unexplored (for more details see **Table S.I. 3** from Supporting Information). This considers not only the conventional AMTPS formed by several common surfactants and copolymers, but also the novel AMTPS that use tensioactive ILs as co-surfactants, a very recent studied class of micellar systems [127]. In this research, it was possible to verify that the ILs display an important effect on the cloud point, *i.e.* the cloud point can be significantly reduced by the ILs that possess a more hydrophobic character (these and other advantages may be consulted in the SWOT analysis present in **Table 2**).

Table 2 - SWOT analysis regarding new AMTPS under this work and intended for R-phycoerythrin from red macroalgae.

Strengths	**Weaknesses**
Usually are less viscous systems; More versatile and efficient systems, since it is possible to develop systems to favour the enrichment of a specific compound or a class of compounds; Use more biocompatible solvents, which brings lower toxicity; AMTPS have the ability to keep the native conformation and activities of biomolecules, particularly proteins, since they do not interact with the biomolecules; Reduces substantially the number of purification steps.	Temperature depending systems; Cost is directly associated with yield; Cost of the solvents involved; Limited access to more sensitive techniques, which enable the deeper understanding of the AMTPS underlying interactions; Flagrant lack of binodal curves with the immense list of surfactants available in the market.
Opportunities	**Threats**
The addition of certain additives (such as ILs) can improve the AMTPS's performance, namely selectivity and modify the cloud points; Possibility to recycle solvents.	Easily reproducible systems.

1.5- SCOPE AND OBJECTIVES

Phycobiliproteins and in particular, R-phycoerythrin are the principal pigments found in red macroalgae, one of the cheaper and added-value biomass present in Portugal. However, the currently extraction and purification protocols need to be improved and optimized since they are quite expensive and time consuming. In this sense, This work aims at the development of a more efficient, fast, selective and sustainable purification technology to recover and purify phycobiliproteins from the red macroalga *Gracilaria sp*. In order to accomplish this objective, several AMTPS using ILs as co-surfactants will be investigated. In this new proposed class of AMTPS, it is expected the crucial role of the ILs incorporation not only in the phase separation, but also regarding the improvement of the operational conditions and the general performance of the extraction process [47]. Therefore, different AMTPS will be designed, composed of several copolymers, as nonionic surfactants, in combination with two distinct families of ILs (imidazolium and phosphonium) acting as co-surfactants. These will be posteriorly applied on the purification/fractionation of phycobiliproteins, in particular R-phycoerythrin, from *Gracilaria sp*. This work plan is developed in close collaboration with ALGAplus, a company located in Ílhavo (Aveiro) dedicated to the production of seaweed and seaweed-based products in an integrated multi-trophic aquaculture.

2. EXPERIMENTAL SECTION

In this section, methodologies are described regarding the design of several binodal curves belonging to distinct surfactants in presence and absence of ILs as co-surfactants, and the development of an alternative and more efficient methodology for the extraction of phycobiliproteins, namely R-phycoerythrin from red macroalgae *Gracilaria sp.*

2.1– MATERIALS AND METHODS

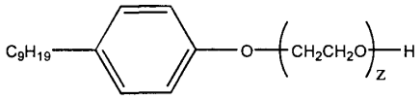
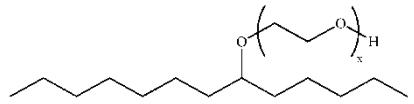
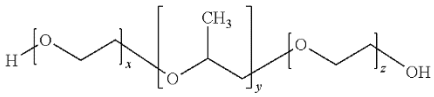
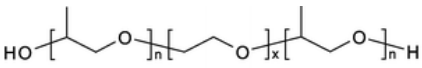
2.1.1 – Materials

The phosphonium-based ILs (**Figure 4**), namely trihexyltetradecylphosphonium chloride [$P_{6,6,6,14}$]Cl (purity = 99.0 wt%), trihexyltetradecylphosphonium bromide [$P_{6,6,6,14}$]Br (purity = 99.0 wt%), trihexyltetradecylphosphonium decanoate [$P_{6,6,6,14}$][Dec] (purity = 99 wt%), trihexyltetradecylphosphonium bis (2,4,4-trimethylpentyl)phosphinate [$P_{6,6,6,14}$][TMPP] (purity = 93.0 wt%), tetraoctylphosphonium bromide [$P_{8,8,8,8}$]Br (purity = 95.0 wt%) and tributyltetradecylphosphonium chloride [$P_{4,4,4,14}$]Cl were kindly offered by Cytec. The imidazolium-based ILs (**Figure 4**) 1-decyl-3-methylimidazolium chloride [C_{10}mim]Cl (purity > 98 wt%), 1-dodecyl-3-methylimidazolium chloride [C_{12}mim]Cl (purity > 98 wt%) and 1-methyl-3-tetradecylimidazolium chloride [C_{14}mim]Cl (purity > 98 wt%) were acquired from Iolitec (Ionic Liquid Technologies, Heilbronn, Germany). The other ILs tested, namely hexadecylpyridinium bromide (CPB) (purity = 97.0 wt%), hexadecylpyridinium chloride monohydrate (CPC) (purity = 99.0 wt%) were supplied from Sigma-Aldrich and *N*-(*N*-hexyl-*N*,*N*-dimethyl-*N*-tetradecylammonium)-*N*,*N*-dimethyl-*N*-tetradecylammonium dibromide [$N_{1,1,14}$-6-$N_{1,1,14}$][Br]$_2$, 3-(1-tetradecyl-3-hexylimidazolium)-1-tetradecylimidazolium dibromide [C_{14}Im-6-ImC_{14}][Br] and *N*,*N*-dimethyl-*N*,*N*-di(tetradecyl)ammonium bromide [$N_{1,1,14,14}$][Br] were synthesized in-house (University of Aveiro) and the structures were confirmed by nuclear magnetic resonance (NMR).

The nonionic surfactants (**Table 3**): Pluronic L-31 (M_n ~ 1,100; PEG, 10 wt%), Pluronic L-35 (M_n ~ 1,900; PEG, 50 wt%), Pluronic L-61 (M_n ~ 2,000; PEG, 10 wt%), Pluronic L-81 (M_n ~ 2,800; PEG, 10 wt%), Pluronic 17R4 (M_n ~ 2,700; PEG, 40 wt%), Pluronic 31R1 (M_n ~ 3,300; PEG, 10 wt%), Pluronic 10R5 (M_n ~ 2,000; PEG, 50 wt%), Tergitol NP-7, Tergitol NP-10, Tergitol 15-S-7, Tergitol 15-S-9, Tween 20, Tween 80, Brij C10, Brij L4, Brij 93, Brij 98 were purchased from Sigma-Aldrich and Triton X-114 was

supplied from Acros Organics (all present a purity ≥ 99%). The McIlvaine buffer components, namely sodium phosphate dibasic heptahydrate $Na_2HPO_4 \bullet 7H_2O$ (purity ≥ 99%), citric acid monohydrate $C_6H_8O_7 \bullet H_2O$ (purity ≥ 99%), and ammonium sulphate (purity ≥ 99%) were acquired at Panreac AppliChem.

Table 3 - Examples of Tergitol and Pluronic surfactant families commercially available and their characteristics [148].

Surfactant	Structure	HLB	CMC (ppm at 25°C)	Average MW	% PEG
Tergitol NP-7	 Tergitol NP-7: z =7 / Tergitol NP-10: z =10	12.0	39	528	-
Tergitol NP-10		13.2	55	642	-
Tergitol 15-S-7	 Tergitol 15-S-7: x=7 / Tergitol 15-S-9: x=9	12.1	38	515	-
Tergitol 15-S-9		13.3	52	607	-
Normal Pluronic					
Pluronic L-31	 PEG - PPG - PEG	1-7	n.d.	1100	10
Pluronic L-61		1-7	n.d.	2000	10
Pluronic L-81		1-7	n.d.	2800	10
Pluronic L-35		18-23	n.d.	1900	50
Reverse Pluronic					
Pluronic 31R1	 PPG – PEG - PPG	1-7	n.d.	3300	10
Pluronic 17R4		7-12	n.d.	2700	40
Pluronic 10R5		12-18	n.a.	2000	50

n.d. – no data available

2.1.2 – Experimental methodology

2.1.2.1 - Design of the binodal curves

The binodal curves of the AMTPS composed of nonionic surfactants and McIlvaine buffer (**Table 4**), in absence and presence of ILs as co-surfactants were set using the cloud point method [149]. The AMTPS cloud point with a known composition were determined

by visually identifying the turbidity of the systems, which occurs during their heating. At this temperature, the systems pass from a clear appearance to a cloudy one. Firstly, a cloud point screening of different nonionic surfactants was carried out using 1 wt% of surfactant and 99 wt% of McIlvaine buffer at pH 7.0, or distilled water, in order select the best systems to be further studied, and their respective binodal curves designed. In this context, each AMTPS display specific amounts of each component: 0.5 to 17.5 wt% of surfactant, 0 or 0.3 wt% of each IL tested, being the system completed with the McIlvaine buffer (pH 7.0) up to a final volume of 10 mL. The systems were heated between 0ºC and 100ºC in a temperature controlled water bath with a precision of ± 0.01ºC (ME-18V Visco-Thermostat, Julabo). For each system, replicas were taken into account and the respective standard deviations determined. Then, the experimental binodal curves were obtained by plotting the cloud point *versus* the surfactant concentration (mass units). This procedure was repeated to study the pH effect upon the binodal curves using the conventional Tergitol 15-S-7 AMTPS, in which the pH ranged between 3.0 and 8.0 (**Table 4**). The knowledge acquired from them allows the selection of strategic mixture points (in the biphasic region) to be further applied in the phycobiliproteins extraction and purification.

Table 4 - McIlvaine buffer composition to a final volume of 1L [150].

pH required	Volume (mL) of Na_2HPO_4 solution (0.2M)	Volume of citric (mL) acid solution (0.1M)
3.0	205.50	794.50
4.0	385.50	614.50
5.0	515.00	485.00
6.0	631.50	368.50
7.0	823.50	176.50
8.0	972.50	27.50

2.1.2.2 – Phycobiliproteins extraction and purification

Fresh *Gracilaria sp.* (**Figure 8**) was kindly provided by ALGAplus Ltda, a company specialized in the production of marine macroalgae, located in Aveiro, Portugal. The macroalgae were collected from an aquaculture environment in different months of the year, namely February, March, April and May (2016). After collecting the macroalgae, the samples were cleaned and washed with fresh and distilled water, at least 3 times each. Algae were weighted and then stored in a freezer at -20ºC until utilization.

Figure 8 - Fresh *Gracilaria sp.* collected in the ALGAplus tanks.

2.1.2.3 - Solid-liquid and liquid-liquid extractions of phycobiliproteins

The algal samples, were previously grounded while frozen with liquid nitrogen, homogenized in distilled water (with a solid/liquid ratio of 0.7) at room temperature and placed in an incubator shaker (IKA KS 4000 ic control) for 20 minutes, at 250 rpm and room temperature, while being protected from light exposure to allow the solid-liquid extraction. Then, the solution was filtered and, subsequently, the filtrate originated was centrifuged in a Thermo Scientific Heraeus Megafuge 16 R Centrifuge at 3500 rpm for 30 minutes, at 4ºC. The resultant pellet was discarded while the phycobiliproteins-rich supernatant was collected and the proteins precipitated with ammonium sulphate at 30 wt % for 4 hours at 4ºC followed by 1 hour centrifuging also at 4ºC.

For the phycobiliproteins extraction using AMTPS, falcon tubes were weighed with specific amounts of each component: 10 wt% of phycobiliproteins crude extract, 10 wt% of surfactant and 0.3 wt% of IL in the case of mixed AMTPS, being the system completed with McIlvaine buffer up to a final volume of 10 mL. The systems were homogenized for at least 2 hours using a tube rotator apparatus model 270 from Fanem®, at 36 rpm. Then, they were left in a temperature above the cloud point (40ºC) for 12 hours in the initial optimization study and then for 4 hours for the remaining studies, allowing the thermodynamic equilibrium to be reached, and thus completing the separation of the phases as well as the migration of the proteins. At the conditions adopted in this work, the systems resulted in a micelle-rich (bottom) and a micelle-poor (top) phases. Both phases were carefully separated and collected for the measurement of volume, weight composition, quantification of both the phycobiliproteins and total proteins. The UV spectroscopy was elected to quantify each molecule at 565 nm and 280 nm, respectively, using a Molecular Devices Spectramax 384 Plus | UV-Vis Microplate Reader. The

analytical quantifications were performed in triplicate. 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, **Figures S.I. 2** and **S.I. 3** in Supporting Information. The integrated extraction methodology adopted to test the purification of phycobiliproteins is summarized in **Figure 9**.

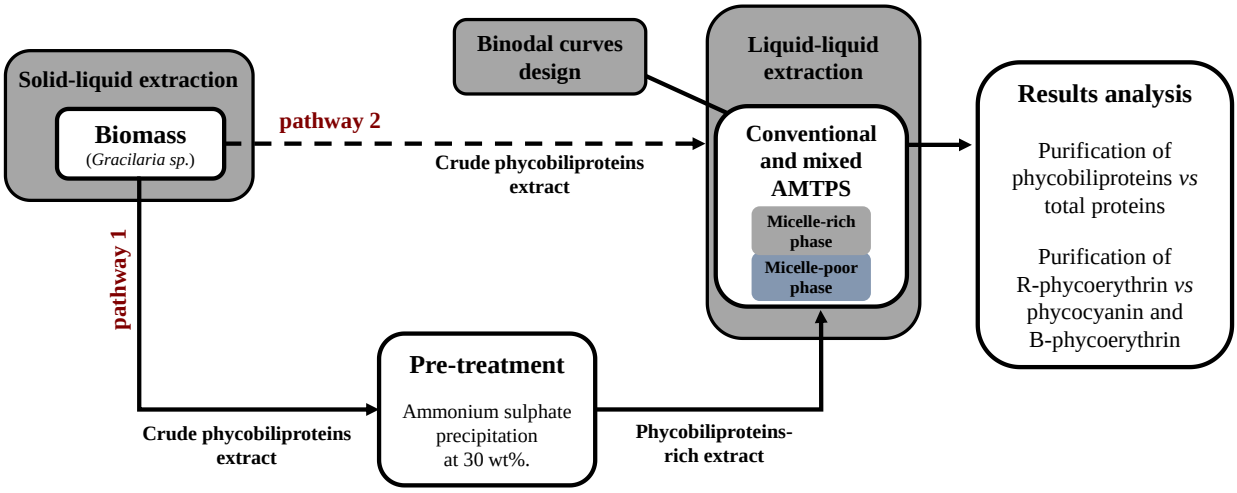


Figure 9 - Schematic representation of the experimental methodology adopted to test the phycobiliproteins extraction and purification.

The partition coefficients for R-phycoerythrin were calculated as the ratio between the amount of R-phycoerythrin present in the micelle-poor (bottom) and the micelle-rich (top) phases, as described in **Eq. 1**.

$$K_{R-phycoerythrin} = \frac{[R-phycoerythrin]_{bot}}{[R-phycoerythrin]_{top}} \quad \textbf{(Eq.1)}$$

where $[R\text{-phycoerythrin}]_{bot}$ and $[R\text{-phycoerythrin}]_{top}$ are, respectively, the concentration of R-phycoerythrin (in g/L) in the bottom and top phases. It should be stressed that the concentration of R-phycoerythrin in each phase was determined based on a calibration curve previously established in Supporting Information (**Figure S.I. 2**). The recovery (R) parameters of R-phycoerythrin and the total protein content towards the bottom (R_{bot}) and the top (R_{top}) phases were determined following **Eqs. 2** and **3**:

$$R_{bot} = \frac{100}{1+\left(\frac{1}{R_v \times K}\right)} \quad \textbf{(Eq. 2)}$$

$$R_{top} = \frac{100}{1 + R_v \times K} \quad \textbf{(Eq. 3)}$$

where R_v stands for the ratio between the volumes of the bottom and top phases. Finally, the selectivity ($S_{R\text{-phycoerythrin/total proteins}}$) of the AMTPS herein developed was described as the ratio between the K values found for R-phycoerythrin and the total proteins, as indicated by **Eq. 4**:

$$S_{R-\text{phycoerythrin}/\text{ total proteins}} = \frac{K_{R-\text{phycoerythrin}}}{K_{\text{total proteins}}} \quad \textbf{(Eq. 4)}$$

In addition, it was determined the phycocyanin contamination index (**Eq. 5**) through the ratio between the absorbance at 620nm and 565nm, which belongs to the phycocyanin and R-phycoerythrin wavelengths, respectively. If this ratio is lower than 0.05, it can be considered that there is no significant contamination by phycocyanin [31]. In an analogous manner, the B-phycoerythrin contamination index (**Eq. 6**) was obtained by the ratio between the absorbance at 565nm and 495nm, which corresponds to the R-phycoerythrin and B-phycoerythrin wavelengths, respectively. In this case, if the obtained value is lower than 1.5, it can be stated that there is no significant contamination with B-phycoerythrin [31].

$$\text{Phycocyanin contamination index} = \frac{Abs_{620}}{Abs_{565}} \quad \textbf{(Eq. 5)}$$

$$\text{B} - \text{phycoerythrin contamination index} = \frac{Abs_{565}}{Abs_{495}} \quad \textbf{(Eq. 6)}$$

3. RESULTS DISCUSSION

3.1 – DESIGN OF THE BINODAL CURVES OF CONVENTIONAL AND MIXED AMTPS

The first step of this work consisted on the cloud point determination of several nonionic surfactants at 1 wt%, either in distilled water or in the McIlvaine buffer pH 7.0, in order to evaluate the effect of the buffer in the system's cloud points. Therefore, different families of nonionic surfactants were tested, namely Brij, Tween, Pluronic and Tergitol. In the particular case of the Brij family, it was found that these surfactants present some solubilisation issues at this concentration, and in this sense, this family of surfactants will not be applied in further studies. Additionally, the Tween family was also excluded from further studies due to its high cloud points (> 75ºC), which incapacitates its use for the biomolecules extraction and purification. Meanwhile, from **Figure 10**, it can be concluded that the use of the McIlvaine buffer (pH 7.0) in the AMTPS formation induces a significant reduction in the systems cloud points since the addition of salts results in the *salting-out* phenomenon [151]. In other words, upon the addition of a salt, the water molecules will preferably interact with these ions and therefore, which will destroy the hydrated structure of the surfactant , consequently promoting the formation of micelles [151]. As such, it is here stated that the use of this buffer would be beneficial in the formation of AMTPS owing not only to its ability to lower the cloud point temperatures, but also to create a more suitable environment for the phycobiliproteins purification.

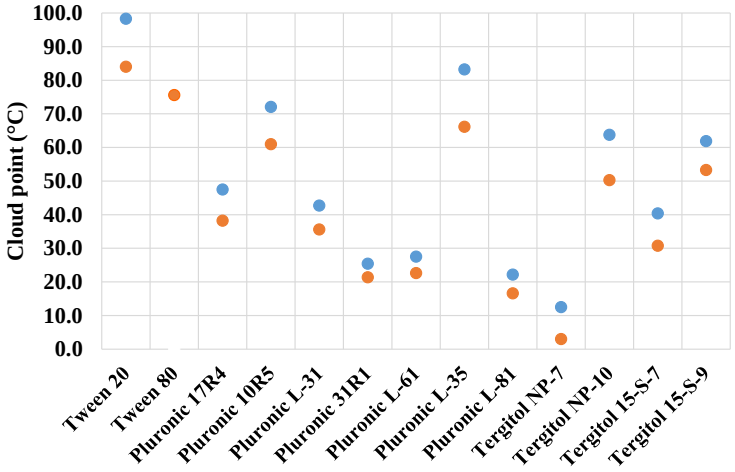


Figure 10 - Nonionic surfactant screening at 1 wt% of surfactant: ● systems in distilled water; ● systems in McIlvaine buffer pH 7.0.

Once the screening was complete, the determination of the binodal curves of distinct AMTPS in absence and presence of several ILs as co-surfactants was carried out. This part of the work allows the creation of a more complete database regarding the binodal curves for nonionic surfactants but it is also relevant to identify and select the more suitable systems to be applied in the phycobiliproteins extraction. In this sense, two distinct families of ILs were strategically chosen, namely the imidazolium ($[C_{10}mim]Cl$, $[C_{12}mim]Cl$ and $[C_{14}mim]Cl$) and phosphonium ($[P_{6,6,6,14}]X$, X = Cl, Br, trihexyltetradecylphosphonium bis (2,4,4-trimethylpentyl)phosphinate and Decanoate; $[P_{4,4,4,14}]Cl$ and $[P_{8,8,8,8}]Br$), as presented in **Figure 4** (chemical structures available). These ILs possess distinct chemical features so different effects can be evaluated: namely the influence of (i) different cations, (ii) the elongation of the cation's alkyl side chain, (iii) various anions and the (iv) symmetry of the cation. Thus, the binodal curves were determined through the visual identification of the cloud point for all the mixture points with distinct concentrations of a nonionic surfactant, an IL as co-surfactant at a fixed concentration of 0.3 wt% and McIlvaine buffer pH 7.0. Firstly, the binodal curves without any co-surfactant were performed to understand the behaviour of each surfactant and then to compare them with the respective mixed AMTPS. In this context, this study started with the Tergitol family (**Table 3**) through Tergitol NP-10, Tergitol 15-S-7 and 15-S-9 (**Figure 11**). Herein, it can be studied the influence of an aromatic ring in the surfactant structure by comparing the Tergitol NP-10 with the others, besides the effect of the increase in the number of ethoxylate groups in the surfactant alkyl chain by comparing Tergitol 15-S-7 and 15-S-9. Tergitol 15-S-9 displays a higher number of ethoxylate groups in its structure, so it is expected that its cloud points are all higher than those measured in the case of Tergitol 15-S-7, owing to its more hydrophilic character. The experimentally binodal curves (**Figures 11 – II** and **III**) are consistent with this information. Moreover, the binodal curve of the conventional system using Tergitol NP-10 is presented in **Figure 11 – I**) and as can be seen, this surfactant also displays high cloud points due to the high number of ethoxylate groups in its structure. Thereby, in terms of conventional AMTPS, it can be concluded that Tergitol 15-S-7 is the most suitable nonionic surfactant for bioseparation processes. On the other hand, when the mixed AMTPS are taken into account, it can be also concluded by **Figures 11 – II, III and IV** that Tergitol NP-10 is more vulnerable to the presence of ILs in the system then the remaining Tergitol-based surfactants, which

might be explained by the structural differences between Tergitol 15-S-series and Tergitol NP-10.

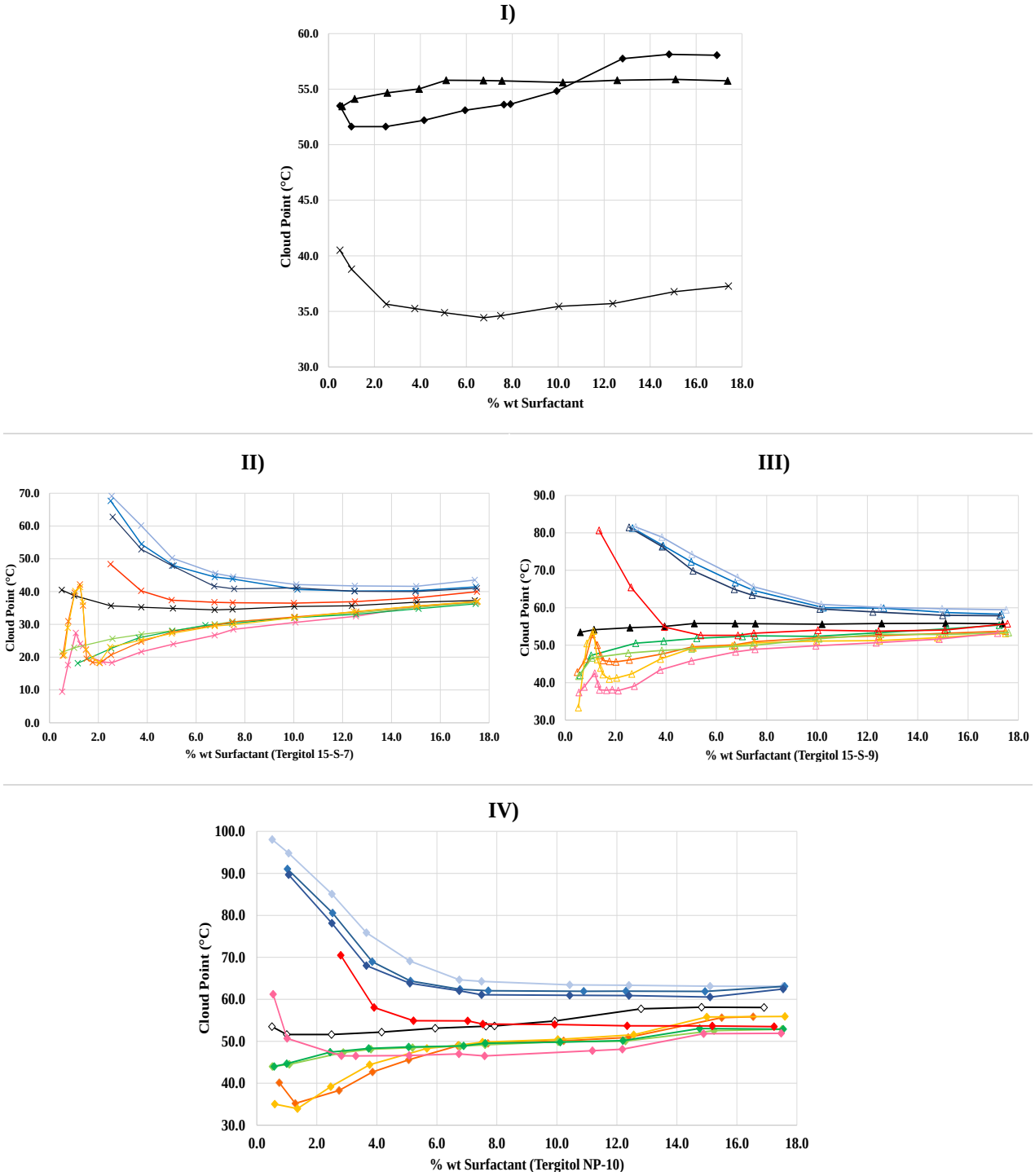


Figure 11 - I) Binodal curves of Tergitol-based AMTPS in absence of ILs, at pH 7.0: X, Tergitol 15-S-7; Δ, Tergitol 15-S-9; ◆, Tergitol NP-10. **II), III), IV)** Binodal curves of Tergitol-based AMTPS with 0.3 wt% of ILs, at pH 7.0: X/▲/♦, $[P_{6,6,6,14}]Cl$; X/▲/♦, $[P_{6,6,6,14}]Br$; X/▲/♦, $[P_{6,6,6,14}][Dec]$; X/▲/♦, $[P_{6,6,6,14}][TMPP]$; X/▲/♦, $[P_{4,4,4,14}]Cl$; X/▲/♦, $[P_{8,8,8,8}]Br$; X/▲/♦, $[C_{10}mim]Cl$; X/▲/♦, $[C_{12}mim]Cl$; and X/▲/♦, $[C_{14}mim]Cl$.

As opposed to Tergitol 15-S-series, Tergitol NP-10 presents an aromatic ring in its structure, which can be mainly responsible for the more pronounced influence of the ILs in the system. A possible explanation for these results lies on the steric hindrance effect caused by the aromatic ring [152–154], since this phenomenon associated with the aromatic rings does not allow the compression of the micellar phase. This way, the ILs have greater ability to interact with the apolar part of the surfactant, consequently displaying a more prominent effect in the cloud points. These results are consistent with those reported by Vicente and co-workers [127], in which it is possible to observe that in the case of nonionic surfactant Triton X-114 that also features an aromatic ring in its structure, the effect of the IL presence is also quite pronounced. It has also been proved that surfactants with ethoxylate groups connected to its aromatic ring lead to steric hindrance effects [155] owing to the flexibility of the ethoxylate groups. In fact, the micellization process is driven by a balance involving electrostatic interactions related to the charged head groups and the hydration level of the surfactant chain [151]. In this sense, the cloud point increase with the ILs addition that possess a more hydrophilic nature since there is a greater hydration shell around the micelles and therefore, a greater amount of energy is required to promote the two-phase separation [156]. On the other hand, ILs with a stronger hydrophobic nature lead to smaller micellar hydration shells and thus, enhancing the ability to promote phase separation at lower temperatures [151].

As previously mentioned, the IL was kept at a fixed concentration of 0.3 wt%, varying only the surfactant concentration from 0.5 to 17.5 wt%. By comparing **Figures 11 – II, III** and **IV)**, it is possible to notice that the presence of different ILs affects the phase separation either by decreasing or increasing the cloud point, as expected according to what was recently reported [127]. In a general way, the binodal curves (**Figures 11 and 16**) show that the IL effect is less pronounced at higher concentrations of surfactant since at higher (surfactant concentrations above 6 wt%) the effect of the surfactant is increasingly dominant. Nonetheless and as aforementioned, the imidazolium-based ILs ([C_{10}mim]Cl, [C_{12}mim]Cl and [C_{14}mim]Cl) and the particular case of [$P_{4,4,4,14}$]Cl, which is the most hydrophilic phosphonium-based IL, induce an increase in the cloud point. On the other hand, the remaining and most hydrophobic phosphonium-based ILs ([$P_{6,6,6,14}$]X, X= Cl, Br, trihexyltetradecylphosphonium bis (2,4,4-trimethylpentyl)phosphinate and Decanoate, [$P_{4,4,4,14}$]Cl and [$P_{8,8,8,8}$]Br), usually provide significant reductions in the cloud

point. In summary, it can be stated that the geometry of the cation, the anion and the length of the alkyl chain seem to present a significant effect on the cloud points at lower surfactant concentrations, however, the hydrophobicity or hydrophilicity of the system is the most dominant effect. For this reason, these effects were summarized in **Table 5,** based on the analysis of the system at a surfactant concentration of 1 wt%.

Regarding the anion effect, it can be seen that $[Dec]^-$ (decanoate) and $[TMPP]^-$ (bis (2,4,4-trimethylpentyl)phosphinate) are anions that provide a stronger effect compared with the anions Cl^- and Br^-. On the other hand, it is possible to verify that the imidazolium cation cause the greatest impact on the systems' cloud points (higher cloud points) compared to the phosphonium cation since this cation led to a bigger difference in the cloud point when compared with the conventional system. In particular, the effects are more significant by reducing the size of the alkyl side chain of the imidazolium-based IL. Moreover, the cation $[P_{4,4,4,14}]^+$ reduces more the temperature than $[P_{6,6,6,14}]^+$. Concerning the cation geometry, it can be stated that the symmetry favors higher variations in the systems' cloud points compared to the conventional AMTPS. Additionally, and in order to evaluate the pH effect on the binodal curves, the conventional Tergitol 15-S-7 AMTPS was used and its formation tested at distinct pH, namely from 3.0 to 8.0. This was only possible through the use of the McIlvaine buffer, since this buffer display a wide range of pH. The results from **Figure 12**, suggest that the increase in the pH leads to a decrease in the system cloud points as a result of the increase in Na_2HPO_4 concentration in the system (**Table 4**), since this salt with a relatively high *salting-out* ability [157]. All these evidences demonstrate the versatility of these systems due to the cloud point manipulation according to the intended purpose with the proper choice of the surfactant, the additives, the buffer solution and consequently the pH of the system. Moreover, it should be pointed out that these changes in the cloud points are relevant from both operational and economic points of view. In fact, very low amounts of ILs can be used to significantly modify the cloud point of the AMTPS.

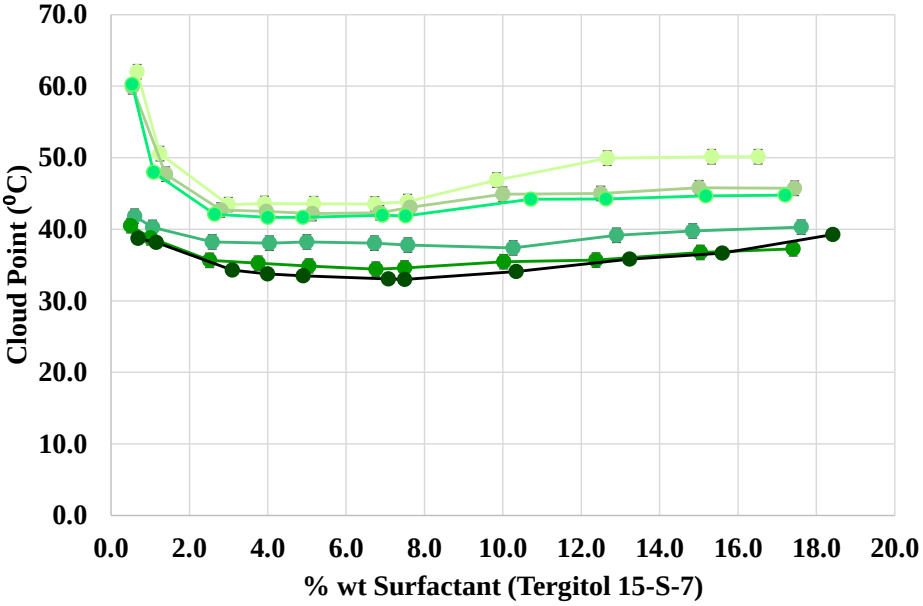


Figure 12 - Binodal curve for the studied pH values for the conventional AMTPS using Tergitol 15-S-7: ●, pH 3.0; ●, pH 4.0; ●, pH 5.0; ●, pH 6.0; ●, pH 7.0; ●, pH 8.0.

The use of Pluronic surfactants under the same conditions applied for the Tergitol family was carried aiming at the formation of AMTPS. Furthermore, the Pluronic family can be divided in into two major groups, the normal (L) and the reverse (R) ones, owing to the position of the PEG and PPG. Therefore, normal Pluronic (L-31, L-35, L-61 and L-81) are constituted by PEG-PPG-PEG, while reverse Pluronic (17R4, 31R1 and 10R5) are constituted by PPG-PEG-PPG [139]. The characteristics of each Pluronic is presented in **Table 3** of Materials and Methods. In this work, it was found that the reverse Pluronics with a more hydrophobic nature (90 and 60 wt% of PPG, respectively 17R4 and 31R1) present nonstandard micellization at higher concentrations (above 15 wt%). This behaviour is reversible with the temperature increase and, only at higher temperatures it is possible to occur a normal phase separation. Moreover, it is also noteworthy to mention that these systems display a temporary turbidity that do not lead to phase separation so this temperature was not considered as the real cloud point, instead the cloud points registered in the binodal curves are the ones that led indeed to the phase separation. Nonetheless, these atypical results could be explained by the heterogeneity in these copolymer's composition [158]. In contrast, Pluronic 10R5 presents a higher PEG content (50 wt%) and shows a normal behaviour, since it is a more hydrophilic copolymer. By observing **Figure 13 – I**, it is possible to conclude that reverse copolymers (Pluronic 31R1, 17R4 and 10R5),

with increasing PEG content in the copolymer, also increase the hydrophilicity of the system. Consequently, the cloud points are higher, which is consistent with what would be expected [139], and follow the increasingly cloud point trend: Pluronic 31R1 < Pluronic 17R4 < Pluronic 10R5 (**Figure 13 – V, VI, VII**). Meanwhile, Pluronics with higher PEG content, such as Pluronic L-35, exhibit higher cloud points (**Figure 13 – IV**), following the tendency: Pluronic L-61 < Pluronic L-35, *i.e.* for similar copolymer molecular weight, the cloud points rise by increasing the percentage of PEG in the copolymer constitution.

Besides this effect, the molecular weight of the copolymer also displays a significant impact on the behaviour of these AMTPS. In other words, a fixed content of 10 wt% of PEG (**Figure 13 – II** and **III)** accompanied with an increase in the copolymer molecular weight seems to induce a considerably more hydrophobic system since the cloud points of these systems decrease with the increase of the copolymer molecular weight. This led to the conclusion that the increase of the copolymer molecular weight is probably due to the increase in the PPG molecular weight, which is the only explanation to the hydrophobicity boost. In this sense, the cloud points of these conventional AMTPS follow the trend: Pluronic L-61 < Pluronic L-31. The effect of the ILs addition into the Pluronic family is very similar to what occurs in the Tergitol family, which was previously discussed. However, in the case of Pluronic L-31 and L-61 (**Figure 13 – II** and **III**), the binodal curves of the more hydrophobic ILs were not possible to determine since the systems were already turbid at 0ºC, though without any visible phase separation, independently of the system conditions of time or temperature imposed. Finally, and according to **Figure 13**, it is possible to verify that for all the Pluronics tested, there is a stabilization of the cloud point at higher surfactant concentrations. This phenomenon might be occurring since a structured water-surfactant system appears at high copolymer concentration [159]. This structure breaks with the temperature increase, however some water molecules are still attached to the micellar system. Consequently, it appears a kind of buffer between micelles that hampers the micelles clustering. Therefore, higher temperature is required to remove these “floating” water molecules, which are barriers for the micellar interaction. Eventually, the micellar interaction will reach its limit so that the cloud point remains almost unchanged, even though the surfactant concentration keeps increasing [159]. Moreover, the binodal curves of Pluronic L-81, in absence and presence of ILs, were not possible to determine, since all systems present a ‘‘milky’’ appearance at

all temperatures tested (from 0ºC to 100ºC). This milky aspect has already been mentioned by other authors for this copolymer family [158], which could be attributed to the high content (90%) of PPG, the most hydrophobic part of the copolymer. Once more, the trends associated with the variation of the cation, anion and cations' symmetry in the normal Pluronic family are similar to those observed for the Tergitol family. On the other hand, in the case of reverse Pluronics, it is not possible to establish a trend since each Pluronic behaves differently. However, it can be seen that with the increase of the copolymer PEG content, the impact of the cation's symmetry is more significant (**Table 5**).

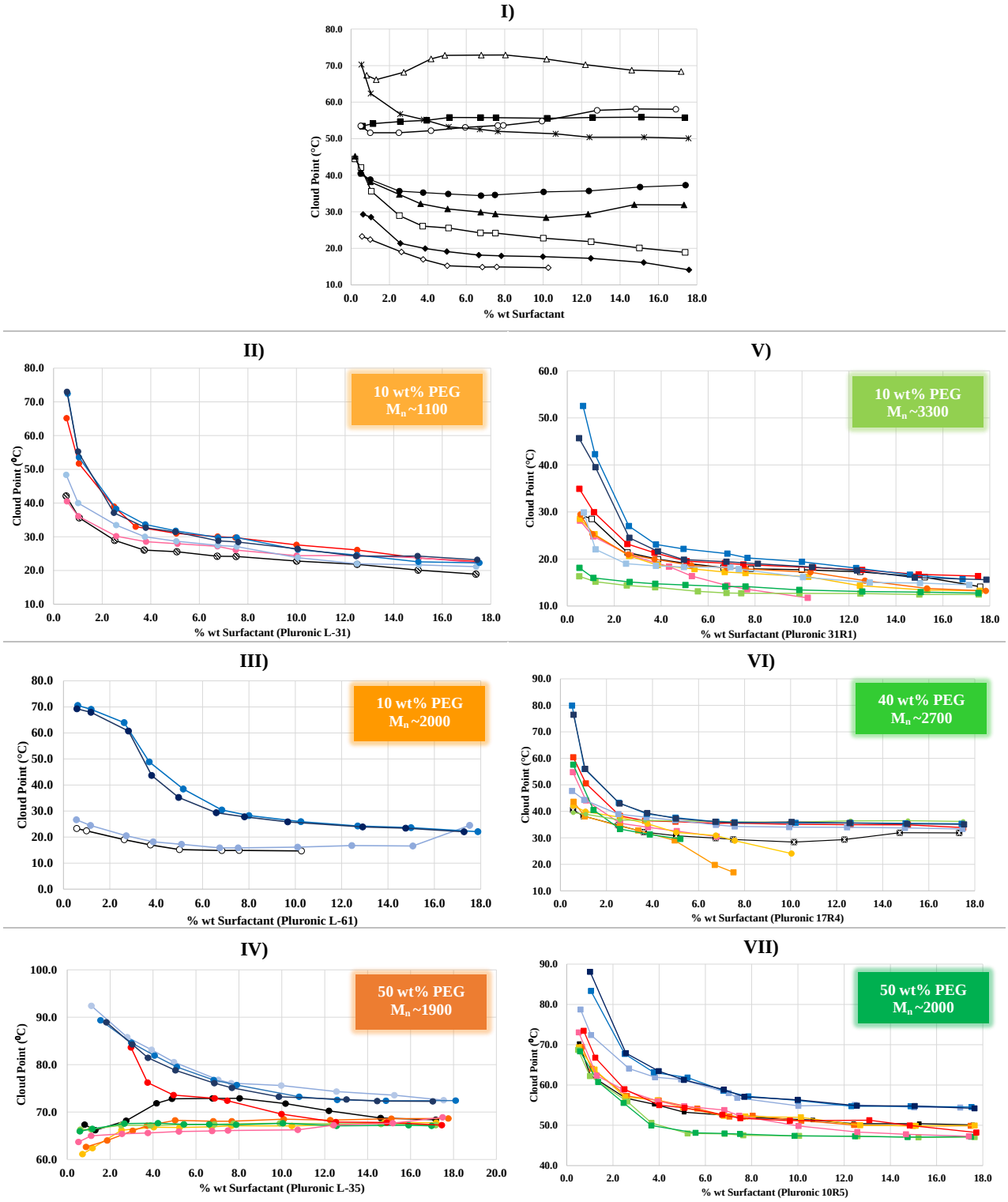


Figure 13 - I) Binodal curve for the studied surfactants in absence of ILs, at pH 7.0: ⊘ Pluronic L-31; o, Pluronic L-61; ●, Pluronic L-35; □, Pluronic 31R1; ⊠, Pluronic 17R4; ■, Pluronic 10R5. **II)**, **III)**, **IV)**, **V)**, **VI)**, **VII)** Binodal curves for the studied ILs at 0.3 wt% with the correspondent surfactant, at pH 7.0: ●/■, $[P_{6,6,6,14}]Cl$; ●/■, $[P_{6,6,6,14}]Br$; ●/■, $[P_{6,6,6,14}][Dec]$; ●/■, $[P_{6,6,6,14}][TMPP]$; ●/■, $[P_{4,4,4,14}]Cl$; ●/■, $[P_{8,8,8,8}]Br$; ●/■, $[C_{10}mim]Cl$; ●/■, $[C_{12}mim]Cl$; ●/■, $[C_{14}mim]Cl$.

Table 5 - Effect of the IL's anion, cation, symmetry and length of alkyl size chain upon the cloud point for mixed AMTPS of Tergitol and Pluronic surfactant families at 1 wt% of surfactant.

Surfactant		PEG (wt%)	Anion effect	Cation effect*	Alkyl side chain length effect*	Cation symmetry effect
Tergitol family	Tergitol 15-S-7	-	$[Dec]^- \approx [TMPP]^- > Br^- > Cl^-$	$[C_{10}mim]^+ > [C_{12}mim]^+ > [C_{14}mim]^+ > [P_{4,4,4,14}]^+ > [P_{6,6,6,14}]^+$	$[C_{10}mim] > [C_{12}mim] > [C_{14}mim]$	$[P_{8,8,8,8}]^+ > [P_{6,6,6,14}]^+$
	Tergitol 15-S-9	-	$[Dec]^- \approx [TMPP]^- > Br^- > Cl^-$	$[C_{10}mim]^+ > [C_{12}mim]^+ > [C_{14}mim]^+ > [P_{4,4,4,14}]^+ > [P_{6,6,6,14}]^+$	$[C_{10}mim] > [C_{12}mim] > [C_{14}mim]$	$[P_{6,6,6,14}]^+ > [P_{8,8,8,8}]^+$
	Tergitol NP-10	-	$[Dec]^- \approx [TMPP]^- > Cl^- \approx Br^-$	$[P_{4,4,4,14}]^+ > [C_{10}mim]^+ > [C_{12}mim]^+ > [C_{14}mim]^+ > [P_{6,6,6,14}]^+$	$[C_{10}mim] > [C_{12}mim] > [C_{14}mim]$	$[P_{8,8,8,8}]^+ > [P_{6,6,6,14}]^+$
Pluronic family	Pluronic L-31	10	Impossible to determine	$[C_{10}mim]^+ \approx [C_{12}mim]^+ \approx [C_{14}mim]^+ \approx [P_{4,4,4,14}]^+$	$[C_{12}mim] \approx [C_{14}mim] > [C_{10}mim]$	Impossible to determine
	Pluronic L-61	10	Impossible to determine	Impossible to determine	$[C_{12}mim] \approx [C_{14}mim] > [C_{10}mim]$	Impossible to determine
	Pluronic L-35	50	$[Dec]^- \approx [TMPP]^- > Cl^- \approx Br^-$	$[P_{4,4,4,14}]^+ > [C_{10}mim]^+ \approx [C_{12}mim]^+ \approx [C_{14}mim]^+ > [P_{6,6,6,14}]^+$	$[C_{14}mim] \approx [C_{12}mim] \approx [C_{10}mim]$	$[P_{8,8,8,8}]^+ \approx [P_{6,6,6,14}]^+$
	Pluronic 31R1	10	$Cl^- \approx Br^- > [Dec]^- \approx [TMPP]^-$	$[C_{12}mim]^+ > [C_{14}mim]^+ \approx [P_{6,6,6,14}]^+ > [C_{10}mim]^+ > [P_{4,4,4,14}]^+$	$[C_{12}mim] \approx [C_{14}mim] > [C_{10}mim]$	$[P_{6,6,6,14}]^+ > [P_{8,8,8,8}]^+$
	Pluronic 17R4	40	$Br^- > Cl^- \approx [Dec]^- \approx Cl^- \approx [TMPP]^-$	$[C_{12}mim]^+ \approx [C_{14}mim]^+ > [P_{4,4,4,14}]^+ > [C_{10}mim]^+ > [P_{6,6,6,14}]^+$	$[C_{12}mim] \approx [C_{14}mim] > [C_{10}mim]$	$[P_{8,8,8,8}]^+ > [P_{6,6,6,14}]^+$
	Pluronic 10R5	50	$[Dec]^- \approx [TMPP]^- > Cl^- \approx Br^-$	$[C_{14}mim]^+ > [C_{12}mim]^+ > [C_{10}mim]^+ > [P_{4,4,4,14}]^+ > [P_{6,6,6,14}]^+$	$[C_{14}mim] > [C_{12}mim] > [C_{10}mim]$	$[P_{8,8,8,8}]^+ \approx [P_{6,6,6,14}]^+$

* For 1 wt% of surfactant, some surfactants show cloud points above 100°C, so the trends were assumed based on the overall binodal curve.

3.2 – EXTRACTION AND PURIFICATION OF PHYCOBILIPROTEINS USING CONVENTIONAL AND MIXED AMTPS

After the determination of the binodal curves, the second part of this work shows the application of the most promising AMTPS in the phycobiliproteins extraction and purification. The systems to test would be those with lower cloud points so that they are able to maintain the proteins' native conformation as well as their stability. In this sense, the nonionic surfactant Tergitol 15-S-7 was selected as a starting point to optimize different extraction conditions, such as the surfactant concentration, extraction time, phycobiliproteins extract concentration, ILs addition and the system pH. All systems are buffered with the McIlvaine buffer pH 7.0 since it not only provides higher extraction efficiencies of phycobiliproteins [160] but it also displays a wide range of pHs. It should also be emphasized that this work is based on the use of the real crude extract rich in phycobiliproteins, and thus a pre-purification step applied to the phycobiliproteins extract was adopted to remove some of the contaminating proteins. Herein, the main goal is to retrieve R-phycoerythrin (protein of interest) in one phase and concentrate the contaminant proteins in the opposite phase. Though, the phycobiliproteins extract also possess two other proteins belonging to the phycobiliproteins family, namely phycocyanin and B-phycoerythrin. In this sense, the priority in this work is to isolate the phycobiliproteins family in one phase, while the non-fluorescent proteins migrate towards the other phase. Simultaneously and if possible, an AMPTS might be able to reduce the R-phycoerythrin contamination with the remaining phycobiliproteins.

The first parameter to be optimized was the surfactant concentration in a conventional AMTPS (**Figure 14**) at pH 7.0. Thus, different concentrations were tested: 4 wt%, 6 wt%, 8 wt% and 10 wt% and it can be concluded that the best surfactant concentration corresponds to 10 wt%. Although the concentration corresponding to 4 wt% of Tergitol 15-S-7 shows a greater recovery of R-phycoerythrin in the micelle-poor phase (bottom phase), this system is also represented by a lower selectivity, since the total contaminant proteins are concentrated in the same phase. Concerning phycobiliproteins, it can easily be seen that the system is not contaminated with B-phycoerythrin (contamination index below 1.50), though all of them are contaminated with phycocyanin

(contamination index higher than 0.05), being the AMTPS composed of 10 wt% of surfactant the best one (**Figure 15**).

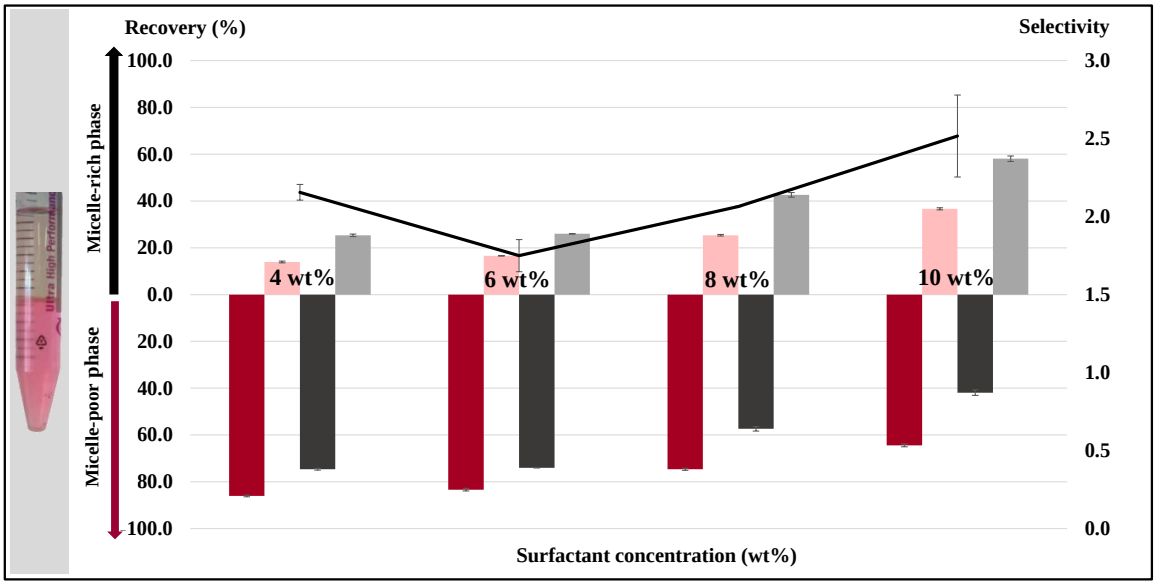


Figure 14 - Study of the surfactant (Tergitol 15-S-7) concentration effect in the phycobiliproteins extraction and purification (20 wt% of extract) using conventional AMTPS at pH 7.0 in an overnight extraction: and , R-phycoerythrin recovery (%) in the micelle-poor micelle-rich phases, respectively; and , total proteins recovery (%) in the micelle-poor and micelle-rich phases, respectively; and the line represents the selectivity.

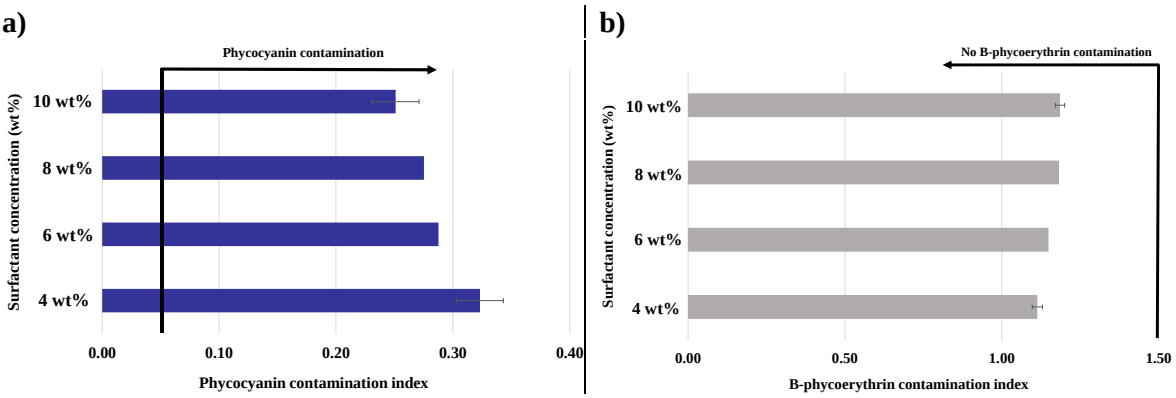


Figure 15 - a) Phycocyanin and **b)** B-phycoerythrin contamination indexes of conventional AMTPS under the following conditions: extraction time: overnight; McIlvaine buffer at pH 7.0 and 20 wt% of extract.

Once stipulated and adopted the best surfactant concentration (10 wt%) at pH 7.0, the purification time was optimized, *i.e.* the time that the system needs to reach the thermodynamic equilibrium and spontaneously separate, while achieving the proteins migration equilibrium. Several extraction and purification times were tested, such as 2, 3, 4 and 5 hours. However, these below 3 hours are not feasible as the thermodynamic equilibrium was not reached, which was easily seen macroscopically (results not shown).

This study is presented in **Figure 16,** where it appears that the best extraction time is 3 hours. From that point, although the R-phycoerythrin recovery in the micelle-poor phase remains constant, the tendency is to gradually loose selectivity. Regarding the presence of phycocyanin (**Figure 17 - a**), the extraction time of 3 hours provides a higher phycocyanin contamination. Though, for further optimizations, it is intended to use systems with higher selectivity. Nevertheless, there is no significant B-phycoerythrin contamination for all the extraction times tested (**Figure 17 - b**).

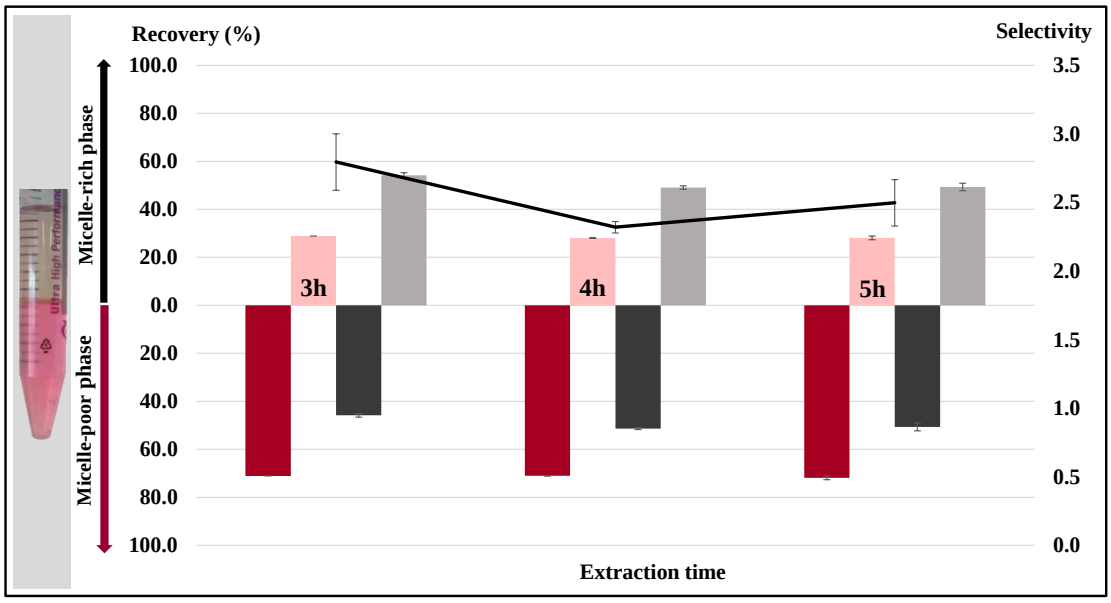


Figure 16 - Study of the extraction and purification time effect in the phycobiliproteins extraction (20 wt% of extract) using conventional AMTPS at pH 7.0: and , R-phycoerythrin recovery (%) in the micelle-poor micelle-rich phases, respectively; and , total proteins recovery (%) in the micelle-poor and micelle-rich phases, respectively; and the line represents the selectivity.

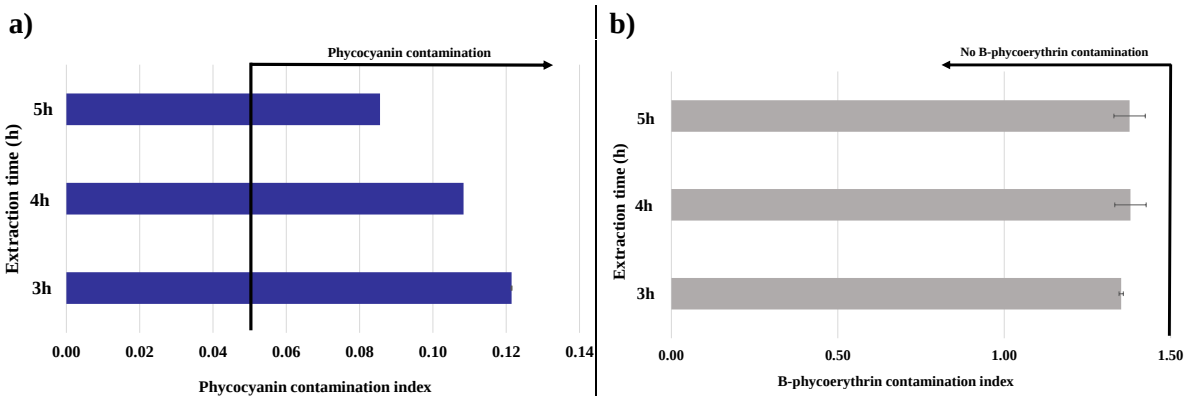


Figure 17 - a) Phycocyanin and **b)** B-phycoerythrin contamination indexes of conventional AMTPS under the following conditions: 10 wt% of Tergitol 15-S-7; McIlvaine buffer at pH 7.0 and 20 wt% of extract.

After the optimization of the surfactant concentration (10 wt%) and time of purification (3 hours), the study of mixed AMTPS was performed, starting with $[P_{6,6,6,14}]Cl$, $[P_{4,4,4,14}]Cl$, $[P_{8,8,8,8}]Br$ and $[C_{14}mim]Cl$, also used to study the phycobiliproteins extract concentration. During the previous optimization studies, 20 wt% of phycobiliproteins extract was used however, it was visible some protein precipitation at the interface. In order to optimize this condition and since this was not overcome with the reduction of the extraction time, the extract concentration was reduced to 10 wt%. In **Figure 18**, it is possible to observe that the reduction in the phycobiliproteins extract concentration in the systems leads to a significant increase in selectivity, while reducing the extent of precipitation. In these conditions, the contaminant proteins start to migrate towards the micelle-rich phase and thus, the phycobiliproteins are more isolated in the micelle-poor phase, with less contaminants. In fact, the reduction of the extract concentration can lead to a considerable selectivity increase from 3 to 10, while using $[P_{8,8,8,8}]Br$ as the co-surfactant. These results are promising since real matrices are used instead of model molecules and it is possible to verify that R-phycoerythrin recoveries are already above 80% and the selectivity is above 6 (**Figure 18**). Thus, further studies using other ILs were carried out with an extract concentration of 10 wt%, which also allowed a significant reduction in protein precipitation at the interface. It should also be highlighted that for the mixed AMTPS, the system requires 4 hours to completely separate instead of the 3 hours used before with the conventional system. Nonetheless, if the standard deviation of selectivity is taken into account, there is no significant difference between 3 and 4 hours for the phase separation, thereby 4 hours can be used without compromise the systems performance. **Figure 19 – a)** shows that the decrease in the extract concentration eliminates the presence of phycocyanin in the R-phycoerythrin-rich phase in the mixed AMTPS with 0.3 wt% of $[P_{4,4,4,14}]Cl$. In addition, there is no significant B-phycoerythrin contamination for all the systems tested (**Figure 19 - b**).

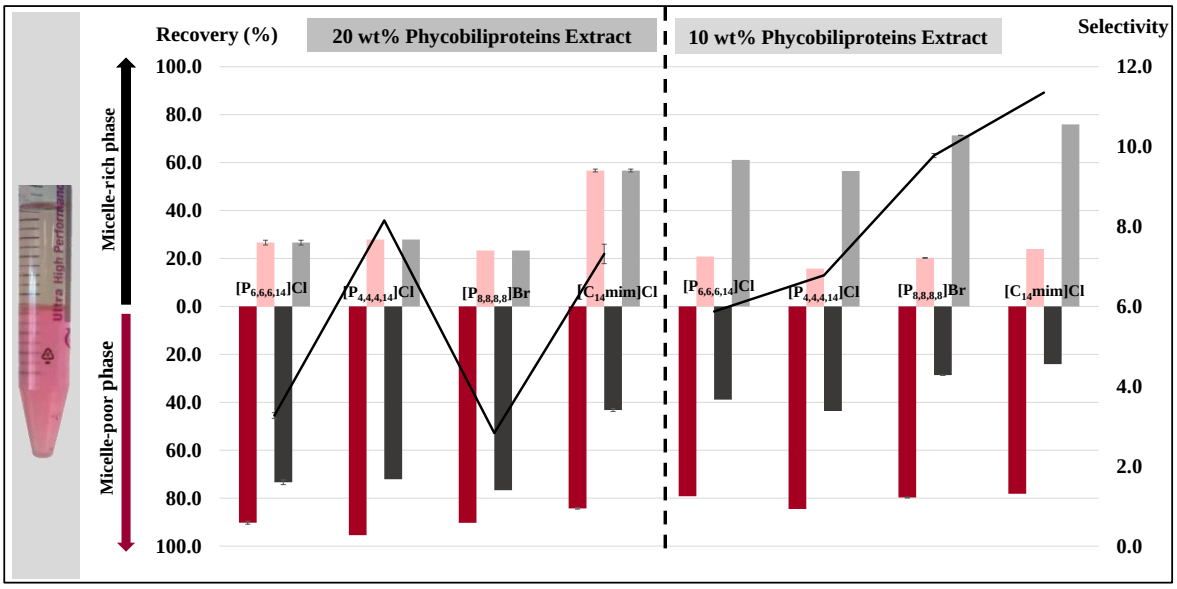


Figure 18 - Study of the extract concentration in the phycobiliproteins extraction and purification using the mixture point composed of 10 wt% Tergitol 15-S-7 + 0.3 wt% of IL and McIlvaine buffer at pH 7.0: ■ and ■, R-phycoerythrin recovery (%) in the micelle-poor micelle-rich phases, respectively; ■ and ■, total proteins recovery (%) in the micelle-poor and micelle-rich phases, respectively; and the line represents the selectivity.

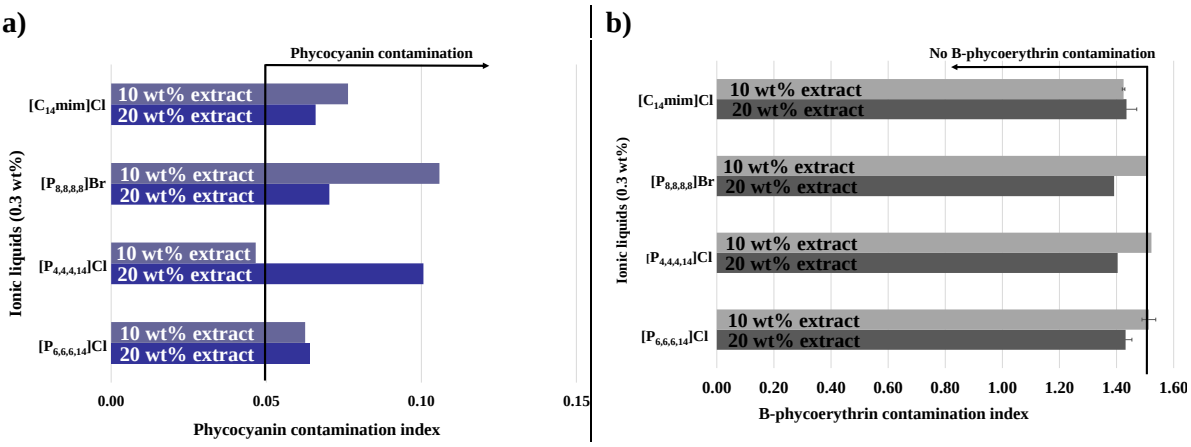


Figure 19 - a) Phycocyanin and **b)** B-phycoerythrin contamination indexes of AMTPS under the following conditions: 10 wt% of Tergitol 15-S-7; McIlvaine buffer at pH 7.0 and 10 wt% or 20 wt% of extract; extraction time of 4 hours.

Under the optimized conditions so far (10 wt % of surfactant, 10 wt % of phycobiliproteins extract and 4 hours of extraction and purification) and by observing **Figure 20**, it can be concluded that the most promising systems for R-phycoerythrin extraction are: $[C_{14}mim]Cl$ > $[P_{6,6,6,14}]$ TMPP > $[P_{6,6,6,14}]Dec$ > $[P_{6,6,6,14}]Br \approx [P_{8,8,8,8}]Br$. Nevertheless, in almost all cases, the addition of a small amount of IL brings enormous advantages compared with the conventional AMTPS. Finally, it appears that the IL's anion

greatly affects the system's behaviour, since there is an increasing in the R-phycoerythrin recovery in the micelle-poor phase according to the following order: Dec < Br ≈ Cl < TMPP. Regarding the cation symmetry there is no significant trend, nonetheless, when the length of the cation's alkyl side chains is increased, both the R-phycoerythrin recovery and selectivity are slightly improved.

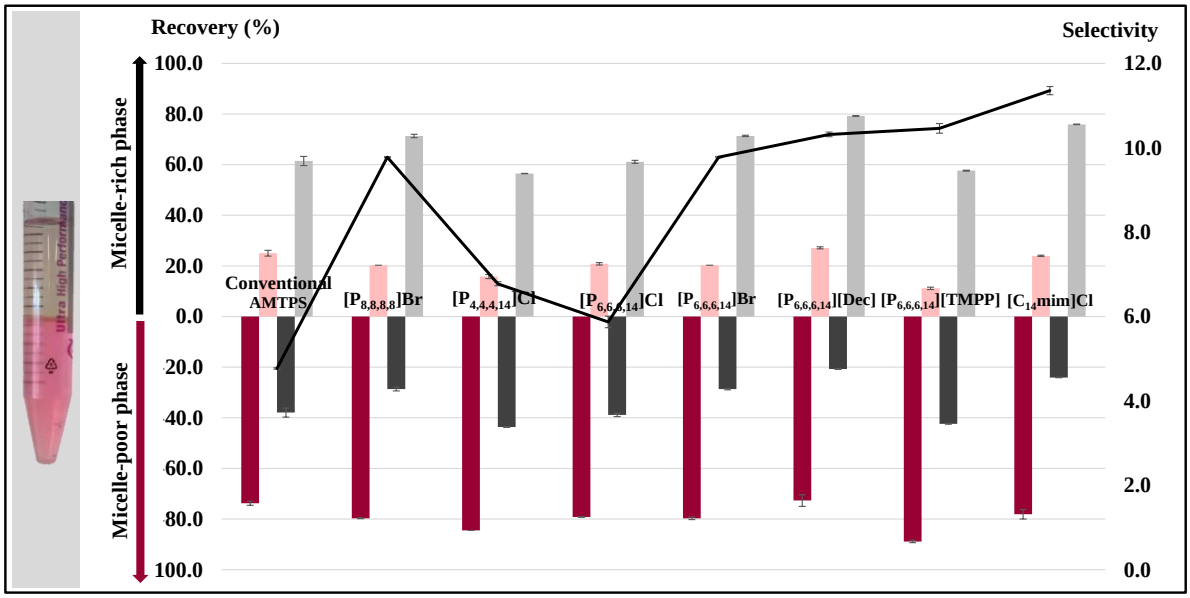


Figure 20 - Study of the IL addition as co-surfactant in the phycobiliproteins extraction and purification using the mixture point composed of 10 wt% Tergitol 15-S-7 + 0.3 wt% of IL and McIlvaine buffer at pH 7.0: and , R-phycoerythrin recovery (%) in the micelle-poor micelle-rich phases, respectively; and , total proteins recovery (%) in the micelle-poor and micelle-rich phases, respectively; and the line represents the selectivity.

Through the observation of **Figure 21**, it can be seen that the only system that is not contaminated at all with phycocyanin in the phycoerythrin-rich phase is the AMTPS with 0.3 wt% of $[P_{4,4,4,14}]Cl$, since its contamination index is below 0.05. Then, the mixed system with $[C_{14}mim]Cl$ presents cumulatively more selective extraction and only possess a small contamination with phycocyanin. On the other hand, the mixed systems with $[P_{6,6,6,14}][TMPP]$ and $[P_{6,6,6,14}][Dec]$ present higher selectivity regarding the elimination of the non-fluorescent proteins from the phycobiliproteins-rich phase, however the R-phycoerythrin in these systems is more contaminated with phycocyanin (contamination index much higher than 0.05). By observing the **Figures 21 - a)** and **b)** it can be stated that there is no significant B-phycoerythrin contamination for all the systems tested if the standard deviation is considered.

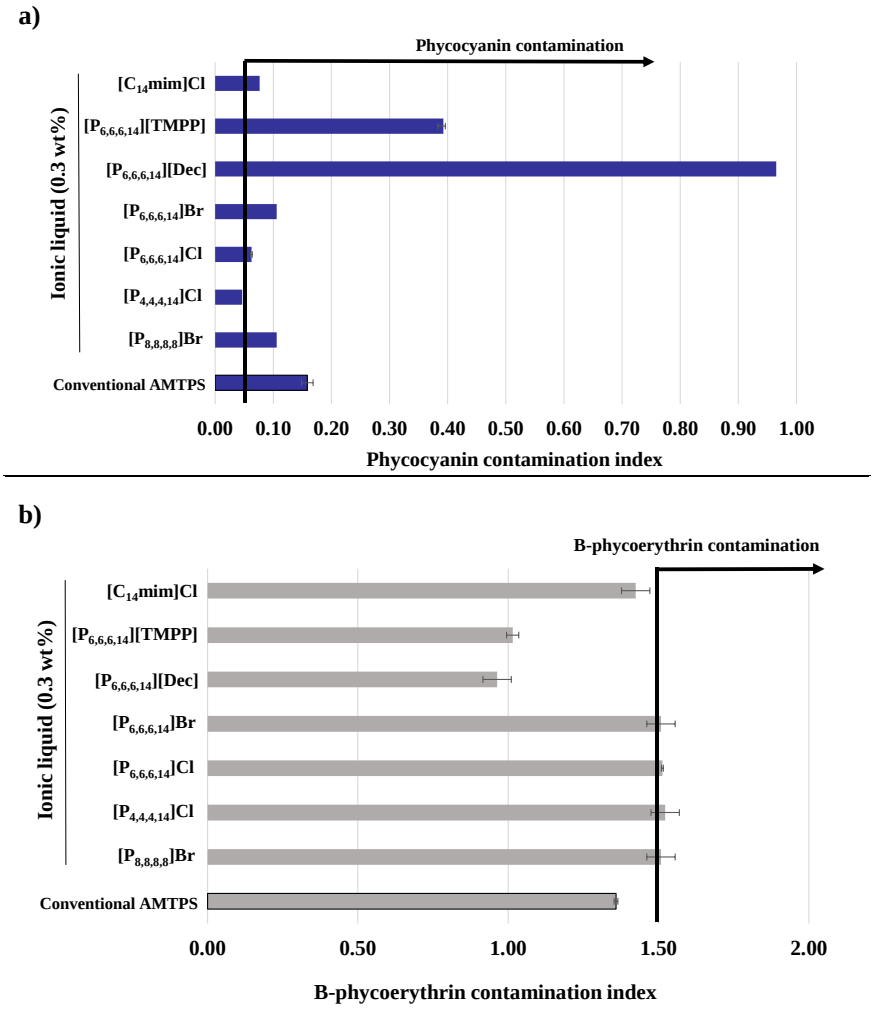


Figure 21 – a) Phycocyanin and **b)** B-phycoerythrin contamination indexes of AMTPS under the following conditions: 10 wt% of Tergitol 15-S-7; McIlvaine buffer at pH 7.0 and 10 wt% of extract.

Then the systems' pH was investigated considering conventional AMTPS at pH 6.0, 7.0 and 8.0, being the main results depicted in **Figure 22**. From this figure, it is clear that the conventional AMTPS at pH 6 does not bring any extraction benefit, since it is visible that the selectivity is about 7 times smaller compared to that obtained at pH 7.0. Nevertheless, and considering the error associated with the conventional AMTPS at pH 7.0, the system at pH 8.0 could be considered more selective. Meanwhile, and taking into account these results, the same test was carried out with mixed AMTPS (meaning with the ILs incorporation) and the main results show that the use of mixed AMTPS further enhances this trend. Additionally, the conventional AMTPS at pH 8.0 seems to significantly decrease the phycocyanin contamination, while it is not contaminated with B-phycoerythrin. In addition, it is mandatory to highlight that great fluctuations are present in the conventional AMTPS's selectivity data (**Figures 20** and **22**), which could be justified

by the dependence of the systems' performance with the seasonality of the macroalgae. That is, the screening of **Figure 20** was carried with a batch more concentrated in R-phycoerythrin than the screening represented in **Figure 22**.

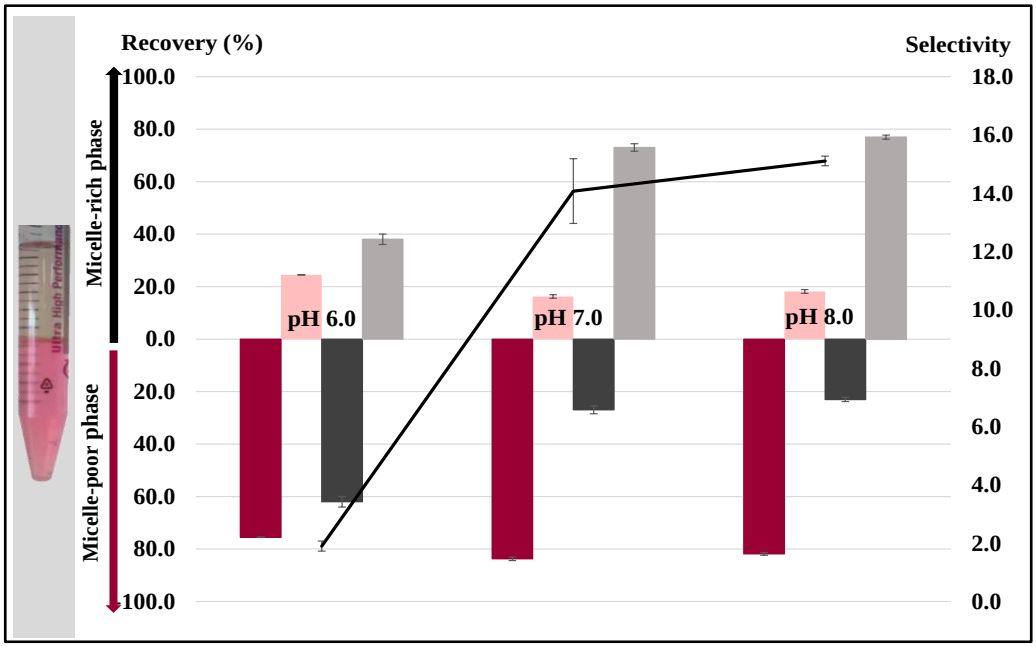


Figure 22 - Study of the system pH in phycobiliproteins extraction and purification using conventional AMTPS composed of 10 wt% Tergitol 15-S-7 and McIlvaine buffer at pH 6.0, 7.0 and 8.0: and , R-phycoerythrin recovery (%) in the micelle-poor micelle-rich phases, respectively; and , total proteins recovery (%) in the micelle-poor and micelle-rich phases, respectively; and the line represents the selectivity.

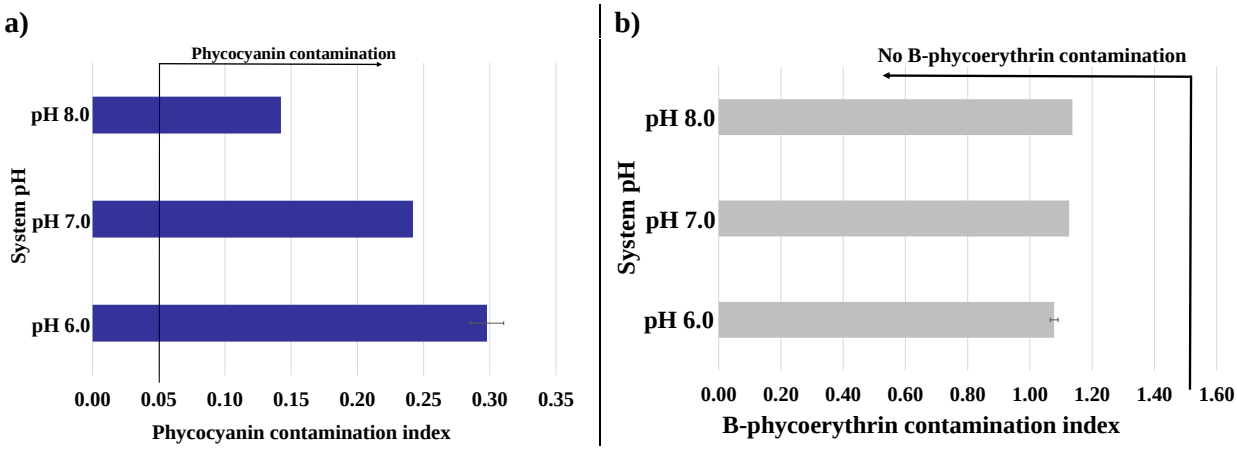


Figure 23 – a) Phycocyanin and **b)** B-phycoerythrin contamination indexes of conventional AMTPS under the following conditions: extraction time of 4 hours; 10 wt% of phycobiliproteins extract and 10 wt% of Tergitol 15-S-7.

Following what was previously mentioned, several extractions were carried out at pH 8.0 with ILs incorporated at a fixed concentration of 0.3 wt%. The cloud points corresponding to each conventional and mixed AMTPS are presented in **Figure S.I. 5** in Supporting Information. According to **Figure 24**, it is possible to conclude that at pH 8.0,

the IL addition to the system did not boost the extraction performances since the conventional AMTPS presents higher recoveries and selectivities. Therefore, purifications using mixed AMTPS and carried out at pH 7.0 are more promising. Regarding to phycocyanin contamination, all the systems display a significant contamination and the mixed AMTPS with $[P_{6,6,6,14}]Cl$ and $[P_{4,4,4,14}]Cl$ are the only ones that present a relevant B-phycoerythrin contamination (**Figure 25**).

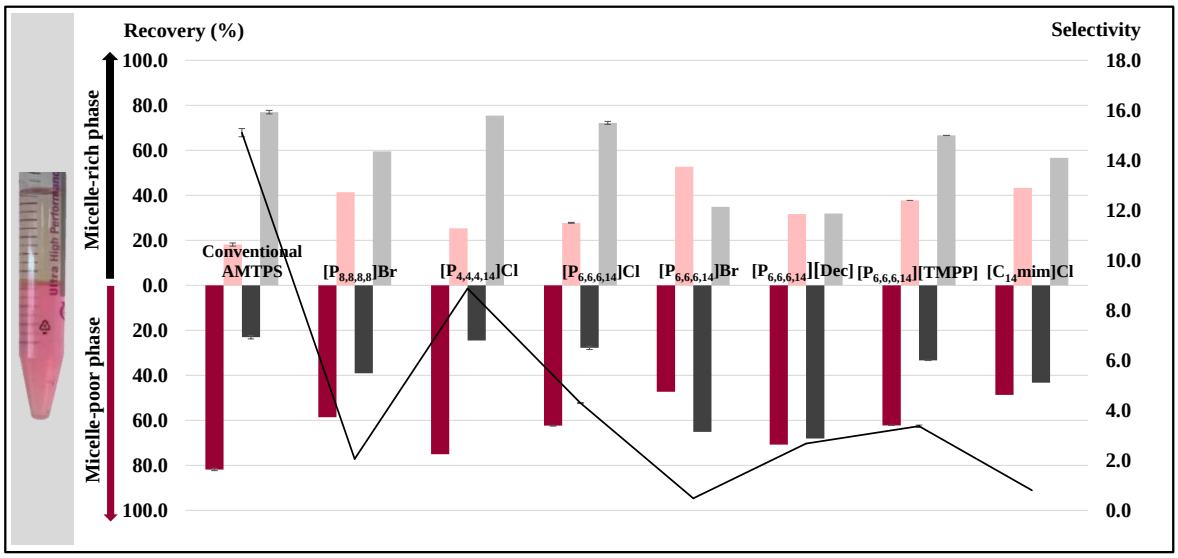


Figure 24 - Study of the IL addition as co-surfactant in the phycobiliproteins extraction and purification using the mixture point composed of 10 wt% Tergitol 15-S-7 + 0.3 wt% of IL and McIlvaine buffer at pH 8.0: and , R-phycoerythrin recovery (%) in the micelle-poor micelle-rich phases, respectively; and , total proteins recovery (%) in the micelle-poor and micelle-rich phases, respectively; and the line represents the selectivity.

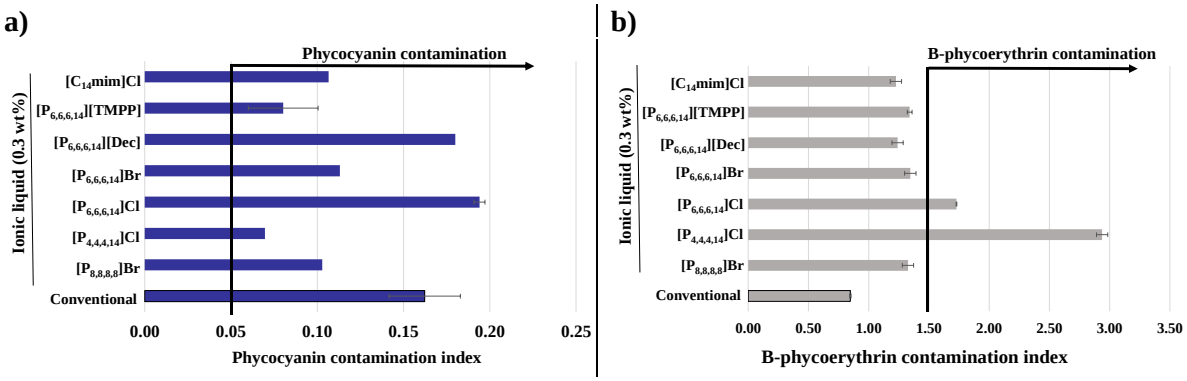


Figure 25 – a) Phycocyanin and **b)** B-phycoerythrin contamination indexes of conventional AMTPS under the following conditions: extraction time of 4 hours; 10 wt% of phycobiliproteins extract and 10 wt% of Tergitol 15-S-7.

Furthermore, and aiming to understand the potential elimination of the pre-purification step using ammonium sulphate (**Figure 9** from Materials and Methods – path 2), while reducing the process' costs, the purification of phycobiliproteins using the

conventional AMTPS at pH 6.0, 7.0 and 8.0 was tested and compared to those obtained until this point. In a first stage, recoveries and selectivities of the conventional AMTPS were compared at pH 6.0, 7.0 and 8.0 (**Figure 26**). However, according to this figure**,** it is possible to conclude that, once again, pH 6.0 does not bring any benefit for the extraction process whereas pH 7.0 and 8.0 showed similar recovery and selectivity results.

Concerning the contaminations (**Figure 27**), the elimination of the extract's pre-purification step greatly reduces the contamination with phycocyanin in the phycoerythrin-rich phase. Though, the absence of this step causes an increase in B-phycoerythrin contamination. Nevertheless, this increase is not sufficient to consider that the micelle-poor phase is contaminated with this protein. As such, the elimination of the pre-purification step using ammonium sulphate does not bring significant differences in terms of the proteins recovery in their respective phases in comparison with recoveries achieved with pre-purified extracts.

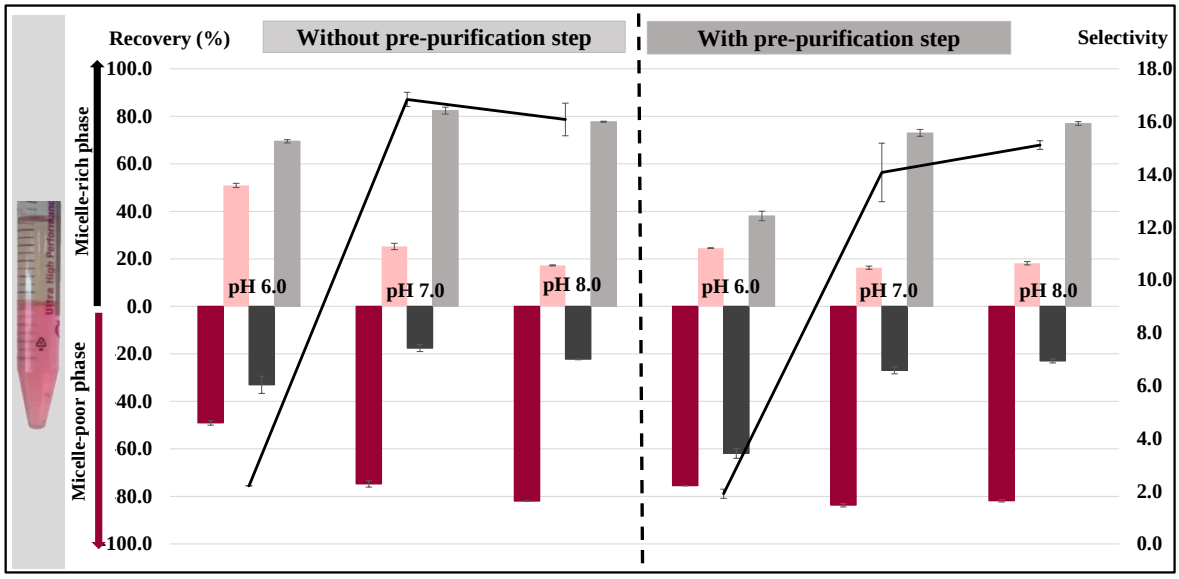


Figure 26 - Study of the system pH in phycobiliproteins extraction and purification using conventional AMTPS composed of 10 wt% Tergitol 15-S-7 and McIlvaine buffer at pH 6.0, 7.0 and 8.0: and , R-phycoerythrin recovery (%) in the micelle-poor and micelle-rich phases, respectively; and , total proteins recovery (%) in the micelle-poor and micelle-rich phases, respectively; and the line represents the selectivity.

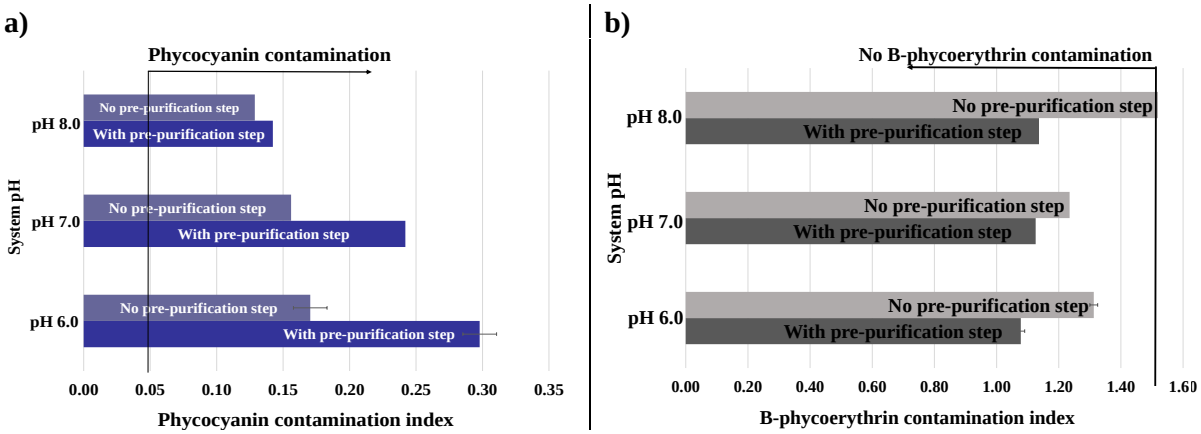


Figure 27 - a) Phycocyanin and **b)** B-phycoerythrin contamination indexes of conventional AMTPS under the following conditions: extraction time of 4 hours; 10 wt% of phycobiliproteins extract and 10 wt% of Tergitol 15-S-7.

So far, all these studies did not led to a complete lack of precipitation at the interphase nor the complete precipitation of only contaminant proteins. Therefore, new ILs were studied aiming at the increase in the selectivity of the purification process. However, it was firstly necessary to know their cloud points. In this sense, there is a screening of all the cloud points for a fixed concentration of 10 wt% of Tergitol 15-S-7 and 0.3 wt% of IL at pH 7.0 in **Figure S.I. 4** from Supporting Information. Herein, pH 7.0 was selected since it provides better recoveries and selectivities without a pre-purification step (reduced costs). Moreover, the ILs here tested were two gemini tensioactive ILs, namely $[N_{1,1,14}\text{-}6\text{-}N_{1,1,14}][Br]_2$ and $[C_{14}Im\text{-}6\text{-}ImC_{14}][Br]_2$, one dialkyl-IL $[N_{1,1,14,14}]Br$ and two pyridinium-based ILs, namely CPC and CPB. The extraction performance of these systems is presented in **Figure 28** through the recovery and selectivity results and it is possible to notice that, once more, none of the ILs tested presented better performances than the conventional AMTPS. Regarding the phycocyanin contamination, only the mixed AMTPS with CPC seems to significantly reduce this contamination. However, none of these mixed AMTPS present a considerable B-phycoerythrin contamination (**Figure 29**).

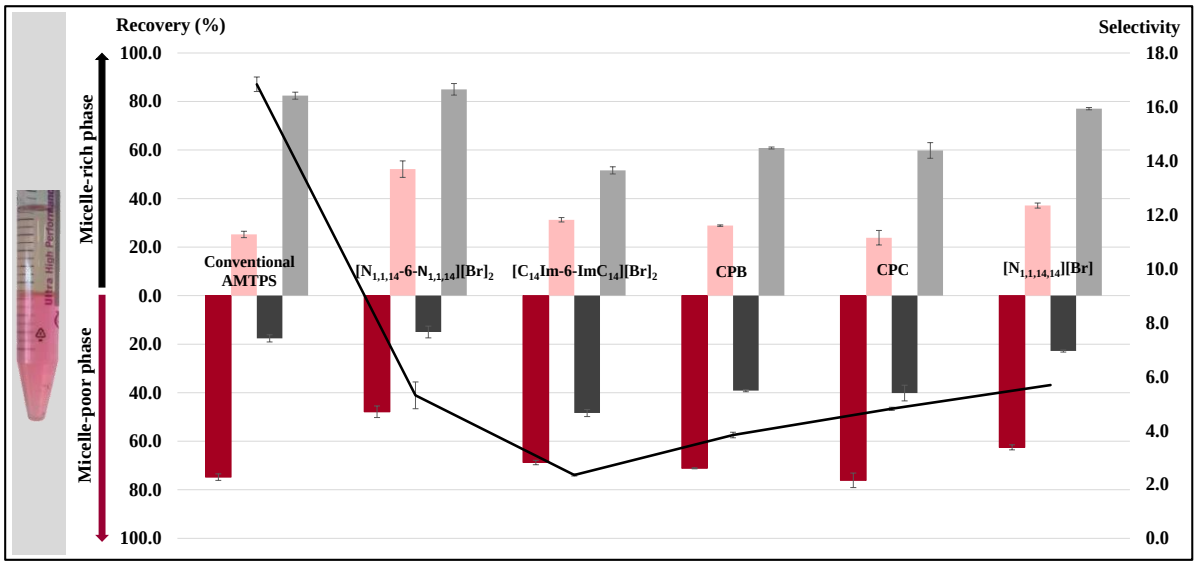


Figure 28 - Study of the IL addition as co-surfactant in the phycobiliproteins extraction and purification using the mixture point composed of 10 wt% Tergitol 15-S-7 + 0.3 wt% of IL and McIlvaine buffer at pH 7.0: and , phycoerythrin recovery (%) in the micelle-poor micelle-rich phases, respectively; and , total proteins recovery (%) in the micelle-poor and micelle-rich phases, respectively; and the line represents the selectivity.

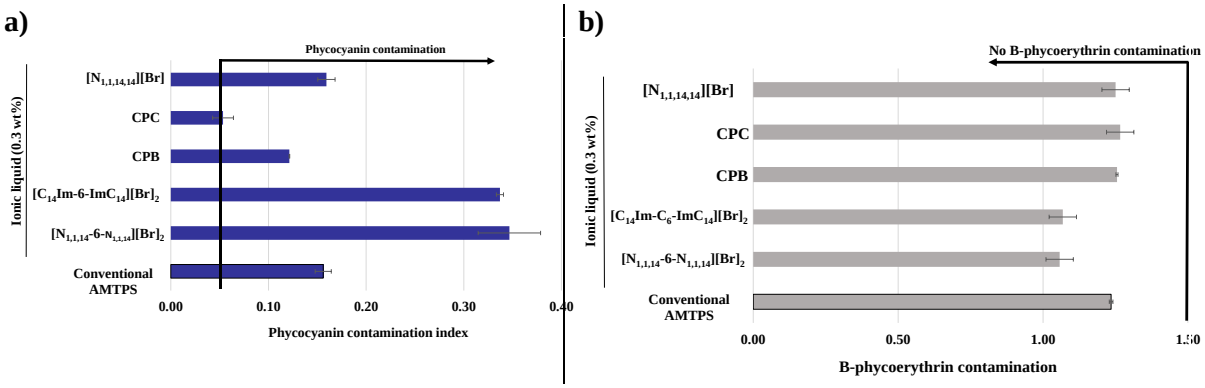


Figure 29 - a) Phycocyanin and **b)** B-phycoerythrin contamination indexes of conventional AMTPS under the following conditions: extraction time of 4 hours; 10 wt% of phycobiliproteins extract and 10 wt% of Tergitol 15-S-7.

4. FINAL REMARKS

This work studies the effect of ILs as co-surfactants on the design of the binodal curves of AMTPS composed of several nonionic surfactants (Tergitol and Pluronic families) and the McIlvaine buffer. One of this work's aims was to increase the database of conventional and mixed AMTPS. Having a larger AMTPS database, it is finally possible to apply these new systems in the extraction and purification of a large number of (bio)molecules adjusting and optimizing the system conditions for each particular case. These results demonstrate that ILs have an important effect on the binodal curves, which is highly dependent on the ILs hydrophobic/hydrophilic nature. In fact, the cloud point can be significantly reduced by selecting ILs possessing a more hydrophobic nature, such as those belonging to the phosphonium family. In a general way, the binodal curves show that the IL effect is more pronounced at lower concentrations of surfactant, since at higher surfactant concentrations (above 6 wt%) the effect of the surfactant is increasingly dominant.

In the particular case of the Tergitol family, as opposed to Tergitol 15-S-series, Tergitol NP-10 presents an aromatic ring in its structure, which can be mainly responsible for the more pronounced influence of the ILs in the system. In relation to the Pluronic family, it is evident that the content of PEG and PPG are crucial for higher hydrophilicity and hydrophobicity of the systems and for the increase and decrease in the cloud points, respectively. Besides this effect, the molecular weight of the copolymer also displays a significant impact on the behaviour of these AMTPS. In other words, a fixed content of PEG accompanied with an increase in the copolymer molecular weight seems to induce a considerably more hydrophobic system since the cloud points of these systems decrease with the increase of the copolymer molecular weight. Moreover, it is also possible to conclude that the increase in the system pH leads to a decrease in their cloud points as a result of the increase in the Na_2HPO_4 concentration of the buffer for the Tergitol 15-S-7-based systems. Regarding the extraction of phycobiliproteins (in particular R-phycoerythrin) and under the optimized conditions so far, it can be concluded that the most promising systems for R-phycoerythrin extraction are: $[C_{14}mim]Cl$, $[P_{6,6,6,14}]$ TMPP, $[P_{6,6,6,14}][Dec]$ and $[P_{8,8,8,8}]Br$, at pH 7.0. These results are promising since real matrices are used instead of model molecules and it is possible to verify that R-phycoerythrin recoveries are already above 80% with selectivity values up to 12. Moreover, in almost all cases, the addition of a small amount of IL brings enormous advantages compared with the

conventional AMTPS since it is possible to increase the systems selectivity from 5 to 12. Finally, it appears that the IL anion, cation symmetry and their alkyl chains size affects the system's behaviour. Moreover, it is possible to conclude that the system pH influences the extraction performance since pH 6.0 does not bring any benefit for the extraction process whereas pH 7.0 and 8.0 showed similar recoveries and selectivities. In addition, the elimination of the pre-purification step using ammonium sulphate does not bring significant differences in terms of the proteins recovery in their respective phases in comparison with recoveries achieved with pre-purified extracts. Nevertheless, the lack of pre-purification step brings some benefits in the elimination of the phycocyanin not to mention a considerable time reduction in the overall process as well as the cost associated.

5. FUTURE WORK

Regarding future work, this thesis presents the opportunity to determine the missing binodal curves for some of the new ILs tested. Additionally, it would also be important to design these same binodal curves at different pHs in order to enlarge these new system's applicability and versatility, as it was here demonstrated that the system pH can be beneficial to improve the extraction performance. Following these studies, it is also relevant the determination of the mixed AMTPS' CMCs and the respective micelles sizes for a better understanding of the interactions of these systems. This way, it is possible to have a more extensive knowledge and complete database since these novel AMTPS can be applied to a plethora of compounds from different types of matrices. They may also overcome the problems displayed by the current technologies for some biomolecules.

Moreover, this thesis showed that, even with the addition of the proper IL to a mixed AMTPS, this single step was still not enough to simultaneously enhance the recovery and purification of the protein of interest in one phase and the non-fluorescent proteins in the opposite phase while boosting the elimination of the remaining phycobiliproteins. In this sense, it would be highly relevant to carry out a purification process with several cycles. That is, it can be employed a mixed AMTPS with $[C_{14}mim]Cl$ at pH 7.0 in one cycle in order to improve the recovery and purification of R-phycoerythrin in the opposite phase of the non-fluorescent proteins. Then, a second cycle using the mixed AMTPS with $[P_{4,4,4,14}]Cl$ also at pH 7.0 should be applied to maximize the elimination of phycocyanin and B-phycoerythrin. This way, the two best systems towards the purification of R-phycoerythrin from the non-fluorescent proteins and from the remaining phycobiliproteins, respectively, are combined together for an improved purification. Finally, once the complete optimization has been concluded, it is mandatory to carry out some protein stability studies through circular dichroism, for example.

6. REFERENCES

[1] S. Chanda, R. Dave, M. Kaneria, K. Nagani, Seaweeds: A novel, untapped source of drugs from sea to combat Infectious diseases, Current Research Technology Education Topics in Applied Microbiology and Microbial Biotechnology, Formatex Research Center, Badajoz, Spain. (2010) 473–480.

[2] Horizon 2020: The EU Framework Programme for Reasearch and Innovation - Aquatic Resources, European Comission. http://ec.europa.eu/programmes/horizon2020/en/area/aquatic-resources (accessed in November 2, 2015).

[3] Centro 2020 (CRER 2020), FEDER FSE. http://www.centro.portugal2020.pt/index.php/programa (accessed November 2, 2015).

[4] Ecorys, Study in support of Impact Assessment work on blue-biotechnology. Revised Final Report, (2014) 214.

[5] L. Pereira, A review of the nutrient composition of selected edible seaweeds, Seaweed: Ecology, Nutrient Composition and Medicinal Uses, 2012.

[6] S.K. Kim, Handbook of Marine Macroalgae: Biotechnology and Applied Phycology, 2011.

[7] A. A. El Gamal, Biological importance of marine algae, Saudi Pharmaceutical Journal (2010) 1–25.

[8] B. Kılınç, S. Cirik, G. Turan, Organic Agriculture Towards Sustainability, InTech. (2014) 735–748.

[9] I. Jaswir, H. Monsur, Anti-inflammatory compounds of macro algae origin: A review, Journal of Medicinal Plants Research 5 (2011) 7146–7154.

[10] M. Francavilla, M. Franchi, M. Monteleone, C. Caroppo, The red seaweed *Gracilaria gracilis* as a multi products source, Marine Drugs 11 (2013) 3754–76.

[11] C. Denis, M. Morançais, M. Li, E. Deniaud, P. Gaudin, G. Wielgosz-Collin, Study of the chemical composition of edible red macroalgae *Grateloupia turuturu* from Brittany (France), Food Chemistry 119 (2010) 913–917.

[12] J.I.S. Khattar, D. Singh, G. Kaur, Algal Biology and Biotechnology, Punjabi University, India, 2009.

[13] W. Jahn, J. Steinbiss, K. Zetsche, Light intensity adaptation of the phycobiliprotein content of the red alga, 161 (1984) 536–539.

[14] A.N. Glazer, Phycobiliproteins - a family of valuable, widely used fluorophores, Journal of Applied Phycology 6 (1994) 105–112.

[15] J. Dumay, M. Morançais, M. Munier, C. Le Guillard, J. Fleurence, Phycoerythrins: Valuable Proteinic Pigments in Red Seaweeds, Elsevier, 2014: pp. 321–343.

[16] J. Fleurence, Seaweed proteins: Biochemical, nutritional aspects and potential uses, Trends in Food Science Technology 10 (1999) 25–28.

[17] A.V. Galland-Irmouli, J. Fleurence, R. Lamghari, M. Luçon, C. Rouxel, O. Barbaroux, Nutritional value of proteins from edible seaweed *Palmaria palmata* (Dulse), Journal of Nutritional Biochemistry 10 (1999) 353–359.

[18] E. Gantt, Structure and function of phycobilisomes: light harvesting pigment complexes in red and blue-green algae, International Review of Cytology 12, 1952.

[19] B. Ke, Photosynthesis Photobiochemistry and Photobiophysics, Media, Springer Science & Business, 2001.

[20] A. N. Glazer, Light harvesting by phycobilisomes, Annual Review of Biophysics and Biophysical Chemistry 14 (1985) 47–77.

[21] A.N. Glazer, Phycobilisome a macromolecular complex optimized for light energy transfer, Biochemical and Biophysical Acta – Review on Bioenergetics. 768 (1984) 29–51.

[22] N. Glazer, C.S. Hixson, Characterization of R-Phycocyanin, Biochemical and Biophysical Acta (BBA) - Reviews on Bioenergetics 2 (1975) 5487–5495.

[23] W.J.S. and K.Z. Jahn, Light intensity adaptation of the phycobiliprotein content of the red alga *Prophyridium*, Planta 161:536-539. 161 (1984) 536–539.

[24] A. V. Galland-Irmouli, L. Pons, M. Luçon, C. Villaume, N.T. Mrabet, J.L. Guéant, One-step purification of R-phycoerythrin from the red macroalga *Palmaria palmata* using preparative polyacrylamide gel electrophoresis, Journal of Chromatography: Biomedical Sciences and Applications 739 (2000) 117–123.

[25] A.N. Glazer, Light guides. Directional energy transfer in a photosynthetic antenna, Journal of Biological Chemistry 264 (1989) 1–4.

[26] S. Roy, C. Llewellyn, E. Egeland, G. Johnsen, Phytoplankton Pigments: Characterization, Chemotaxonomy and Applications in Oceanography, Cambridge University Press, 2011.

[27] C. Contreras-Martel, J. Martinez-Oyanedel, M. Bunster, P. Legrand, C. Piras, X. Vernede, Crystallization and 2.2 Å resolution structure of R-phycoerythrin from *Gracilaria chilensis*: A case of perfect hemihedral twinning, Acta Crystallographica Section D: Biological Crystallography 57 (2001) 52–60.

[28] L. Sun, S. Wang, X. Gong, M. Zhao, X. Fu, L. Wang, Isolation, purification and characteristics of R-phycoerythrin from a marine macroalga *Heterosiphonia japonica*, Protein Expression and Purification 64 (2009) 146–154.

[29] G. Wang, B. Zhou, C. Zeng, Isolation, properties and spatial site analysis of gamma subunits of B-phycoerythrin and R-phycoerythrin, Chinese Academy of Sciences 41 (1998) 9–17.

[30] A. Orta-Ramirez, J.E. Merrill, D.M. Smith, pH Affects the Thermal Inactivation Parameters of R-Phycoerythrin from *Porphyra yezoensis*, Journal of Food Science 65 (2000) 1046–1050.

[31] M. Munier, S. Jubeau, A. Wijaya, M. Morançais, J. Dumay, L. Marchal, Physicochemical factors affecting the stability of two pigments: R-phycoerythrin of *Grateloupia turuturu* and B-phycoerythrin of *Porphyridium cruentum*, Food Chemistry 150 (2014) 400–407.

[32] N.S. Gakkaishi, Effects of pH on the Conformation from *Nori Porphyra sp*, Planta 57 (1991) 899–903.

[33] L. N. Liu, H.-N. Su, S.-G. Yan, S.-M. Shao, B.-B. Xie, X.-L. Chen, Probing the pH sensitivity of R-phycoerythrin: Investigations of active conformational and functional variation, Biochemical and Biophysical Acta – Bioenergetic reviews. 1787 (2009) 939–946.

[34] R. Rossano, N. Ungaro, A. D'Ambrosio, G. Liuzzi, P. Riccio, Extracting and purifying R-phycoerythrin from Mediterranean red algae *Corallina elongata*, Journal of Biotechnology 101 (2003) 289–293.

[35] V. D. Pandey, A. Pandey, V. Sharma, Biotechnological applications of cyanobacterial phycobiliproteins, Applied Biotechnology 2 (2013) 89–97.

[36] J. Fleurence, R-Phycoerythrin from red macroalgae: Strategies for extraction and potential application in Biotechnology, Applied Biotechnology 21 (2003) 63–68.

[37] W. A. J.P. Wijesinghe, Y.-J. Jeon, Enzyme-assistant extraction (EAE) of bioactive components: A useful approach for recovery of industrially important metabolites from seaweeds: A review, Fitoterapia. 83 (2012) 6–12.

[38] J. Dumay, M. Munier, Feasibility Study of the Recovery of R - Phycoerythrin from Red Algae in France, University of Nantes Press (2011).

[39] S. Sekar, M. Chandramohan, Phycobiliproteins as a commodity: trends in applied research, patents and commercialization, Journal of Applied Phycology. 20 (2008) 113–136.

[40] Z.P. Chen, D.L. Kaplan, K. Yang, J. Kumar, K. a Marx, S.K. Tripathy, Two-photon-induced fluorescence from the phycoerythrin protein, Applied Optics 36 (1997) 1655–1659.

[41] D. Isailovic, I. Sultana, G.J. Phillips, E.S. Yeung, Formation of fluorescent proteins by the attachment of phycoerythrobilin to R-phycoerythrin alpha and beta apo-subunits, Analytical Biochemistry 358 (2006) 38–50.

[42] R. Aráoz, M. Lebert, D.P. Häder, Electrophoretic applications of phycobiliproteins, Electrophoresis. 19 (1998) 215–219.

[43] R. Pangestuti, K. Kim, Biological activities and health benefit effects of natural pigments derived from marine algae, Journal of Functional Foods. 3 (2011) 255–266.

[44] C. Fitzgerald, E. Gallagher, D. Tasdemir, M. Hayes, Heart health peptides from macroalgae and their potential use in functional foods, Journal of Agricultural and Food Chemistry 59 (2011) 6829–6836.

[45] R.E. Cian, O. Martínez-Augustin, S.R. Drago, Bioactive properties of peptides obtained by enzymatic hydrolysis from protein byproducts of *Porphyra columbina,* Food Research 49 (2012) 364–372.

[46] S. Raja, V.R. Murty, V. Thivaharan, V. Rajasekar, V. Ramesh, Aqueous Two Phase Systems for the Recovery of Biomolecules – A Review, Science and Technology. 1 (2012) 7–16.

[47] H. Tani, T. Kamidate, H. Watanabe, Aqueous Micellar Two-Phase Systems for Protein Separation, Analytical Sciences 14 (1998) 875–888.

[48] J. A. Asenjo, B. a. Andrews, Aqueous two-phase systems for protein separation: Phase separation and applications, Journal of Chromatography 1238 (2012) 1–10.

[49] C.O. Rangel-Yagui, a. Pessoa-Jr, D. Blankschtein, Two-Phase Aqueous Micellar Systems - an Alternative Method for Protein Purification, Brazilian Journal of Chemical Engineering 21 (2004) 531–544.

[50] J. Dumay, N. Clément, M. Morançais, J. Fleurence, Optimization of hydrolysis conditions of *Palmaria palmata* to enhance R-phycoerythrin extraction, Bioresources. Technology 131 (2013) 21–27.

[51] Y. Joubert, J. Fleurence, Simultaneous extraction of proteins and DNA by an enzymatic treatment of the cell wall of *Palmaria palmata* (Rhodophyta), Journal of Applied Phycology 20 (2008) 55–61.

[52] J. Fleurence, E. Antoine, M. Lucon, Method for extracting and improving digestibility of *Palmaria* proteins, Journal of Chemical Engineering 2002.

[53] J. Fleurence, The enzymatic degradation of algal cell walls: A useful approach for improving protein accessibility?, Journal of Applied Phycology 11 (1999) 313–314.

[54] C. Denis, H. Le Jeune, P. Gaudin, J. Fleurence, An evaluation of methods for quantifying the enzymatic degradation of red seaweed *Grateloupia turuturu*, Journal of Applied Phycology 21 (2009) 153–159.

[55] S.U. Kadam, B.K. Tiwari, C.P. O'Donnell, Application of novel extraction technologies for bioactives from marine algae, Journal of Agricultural and Food Chemistry 61 (2013) 4667–75.

[56] Y. Horie, K. Horie, S. K, Physiological differences of soluble and insoluble dietary fibre fractions of brown algae and mushrooms in pepsin activity in vitro and protein digestibility, Journal of Clinical Nutrition (1994).

[57] E. Deniaud, B. Quemener, J. Fleurence, M. Lahaye, Structural studies of the mix-linked beta-(1-->3)/beta-(1-->4)-D-xylans from the cell wall of *Palmaria palmata* (Rhodophyta, International Journal of Biological Macromolecules 33 (2003) 9–18.

[58] N. Senthilkumar, C. Kurinjimalar, R. Thangam, V. Suresh, G. Kavitha, P. Gunasekaran, Further studies and biological activities of macromolecular protein R-Phycoerythrin from *Portieria hornemannii*, International Journal Biological Macromolecules 62 (2013) 107–116.

[59] E. D'Agnolo, R. Rizzo, S. Paoletti, E. Murano, R-Phycoerythrin from the red alga *Gracilaria longa*, Phytochemistry 35 (1994) 693–696.

[60] J.-F. Niu, Z.-F. Chen, G.-C. Wang, B.-C. Zhou, Purification of phycoerythrin from *Porphyra yezoensis Ueda* (Bangiales, Rhodophyta) using expanded bed absorption, Journal of Applied Phycology 22 (2010) 25–31.

[61] J. Niu, M. Xu, G. Wang, K. Zhang, G. Peng, Comprehensive extraction of agar and R-phycoerythrin from *Gracilaria Lemaneiformis* (Bangiales, Rhodophyta), Indian Journal of Marine Sciences 42 (2013) 21–28.

[62] C. Cai, Y. Wang, C. Li, Z. Guo, R. Jia, W. Wu, Purification and photodynamic bioactivity of phycoerythrin and phycocyanin from *Porphyra yezoensis* Ueda, Journal of Marine Sciences 13 (2014) 479–484.

[63] Q. Kaixian, M. Franklin, M. a. Borowitzka, The study for isolation and purification of R-phycoerythrin from a red alga, Applied Biochemistry and Biotechnology. 43 (1993) 133–139.

[64] J.F. Niu, G.-C. Wang, C.-K. Tseng, Method for large-scale isolation and purification of R-phycoerythrin from red alga *Polysiphonia urceolata Grev*, Protein Expression and Purification 49 (2006) 23–31.

[65] G. Wang, Isolation and purification of phycoerythrin from red alga *Gracilaria verrucosa* by expanded-bed-adsorption and ion-exchange chromatography, Journal of Chromatography 56 (2002) 509–513.

[66] A. Jungbauer, R. Hahn, Ion-Exchange Chromatography, 1st ed., Elsevier Inc., 2009.

[67] Harvard, Guide to Gel Filtration or Size Exclusion Chromatography, Harvard Apparatus (2013) 14.

[68] D.S. Hage, Affinity chromatography: a review of clinical applications, Journal of Clinical Chemistry 45 (1999) 593–615.

[69] L.J. Cummings, M. a. Snyder, K. Brisack, Chapter 24- Protein Chromatography on Hydroxyapatite Columns, 463 (2009) 387–404.

[70] P. Gagnon, R. Frost, T. Ogawa, V. Biosystems, B. Laboratories, CHT TM Ceramic Hydroxyapatite — A New Dimension in Chromatography of Biological Molecules, Biorad. (2000) 3–6.

[71] P. Albertsson, Partitioning of Cell Particles and Macromolecules, 2nd edition, Wiley–Interscience, New York, 1996.

[72] A.M.G. Hong Yang, Aqueous Two-Phase Extraction Advances for Bioseparations, Journal of Bioprocessing and Biotechniques 04 (2013) 1–8.

[73] C.I.C. Silvestre, J.L.M. Santos, J.L.F.C. Lima, E. a G. Zagatto, Liquid-liquid extraction in flow analysis: A critical review, Analytical and Chemical Acta. 652 (2009) 54–65.

[74] P.G. Mazzola, A.M. Lopes, F.A. Hasmann, A.F. Jozala, T.C. V Penna, P.O. Magalhaes, Liquid – liquid extraction of biomolecules : an overview and update of the main techniques, Journal of Enzyme and Microbial Technology 157 (2008) 143–157.

[75] F.F. Cantwell, M. Losier, Liquid-liquid extraction, Scince Institute of Brooklyn (2002) 297–340.

[76] A.D. Diamond, J.T. Hsu, Protein Partitioning in PEG / Dextran Aqueous Two-Phase Systems, Bioprocessing Institute of Benthlehem 36 (1990) 1017–1024.

[77] J.M.S. Cabral, Cell partitioning in aqueous two-phase polymer systems. Advanced. Biochemistry Engineer Biotechniques 106 (2007) 151–71.

[78] M.G. Freire, A.F.M. Cláudio, J.M.M. Araújo, J. A P. Coutinho, I.M. Marrucho, J.N. Canongia Lopes, Aqueous biphasic systems: a boost brought about by using ionic liquids, Chemical Society 41 (2012) 4966–95.

[79] X. Zhao, X. Xie, Y. Yan, Liquid–liquid equilibrium of aqueous two-phase systems containing poly(propylene glycol) and salt ($(NH_4)_2SO_4$, $MgSO_4$, KCl, and KAc): experiment and correlation, Thermochimical Acta 516 (2011) 46–51.

[80] C.L.S. Louros, A.F.M. Cláudio, C.M.S.S. Neves, M.G. Freire, I.M. Marrucho, J. Pauly, Extraction of Biomolecules Using Phosphonium-Based Ionic Liquids + K3PO4 Aqueous Biphasic Systems, International Journal of Molecular Sciences 11 (2010) 1777–1791.

[81] N.J. Bridges, K.E. Gutowski, R.D. Rogers, Investigation of aqueous biphasic systems formed from solutions of chaotropic salts with kosmotropic salts (salt-salt ABS), Green Chemistry 9 (2007) 177.

[82] M.G. Freire, C.L.S. Louros, L.P.N. Rebelo, J. A. P. Coutinho, Aqueous biphasic systems composed of a water-stable ionic liquid + carbohydrates and their applications, Green Chemistry 13 (2011) 1536.

[83] M. Domínguez-Pérez, L.I.N. Tomé, M.G. Freire, I.M. Marrucho, O. Cabeza, J. A. P. Coutinho, Extraction of biomolecules using aqueous biphasic systems formed by ionic liquids and aminoacids, Separation and Purification Technologies 72 (2010) 85–91.

[84] A. Gong, X. Zhu, Surfactant/ionic liquid aqueous two-phase system extraction coupled with spectrofluorimetry for the determination of dutasteride in pharmaceutical formulation and biological samples, Fluid Phase Equilibrium 374 (2014) 70–78.

[85] D. Bhatt, K.C. Maheria, J. Parikh, Studies on Surfactant–Ionic Liquid Interaction on Clouding Behaviour and Evaluation of Thermodynamic Parameters, Journal of Surfactants and Detergents 16 (2012) 547–557.

[86] J.C. Merchuk, B. Andrews, J. A. Asenjo, Aqueous two-phase systems for protein separation. Studies on phase inversion, Journal of Chromatography: Biomedical Sciences 711 (1998) 285–293.

[87] F. Hachem, B. a. Andrews, J.A. Asenjo, Hydrophobic partitioning of proteins in aqueous two-phase systems, Enzyme Microbial Technology 19 (1996) 507–517.

[88] P. A. J. Rosa, a. M. Azevedo, I.F. Ferreira, J. de Vries, R. Korporaal, H.J. Verhoef, Affinity partitioning of human antibodies in aqueous two-phase systems, Journal of. Chromatography 1162 (2007) 103–113.

[89] A.M. Azevedo, a. G. Gomes, P. a. J. Rosa, I.F. Ferreira, A.M.M.O. Pisco, M.R. Aires-Barros, Partitioning of human antibodies in polyethylene glycol–sodium citrate aqueous two-phase systems, Separation and Purification Technologies 65 (2009) 14–21.

[90] R. Bott, Fractionation of Nucleic Acids by Dextran - Polyethylene Glycol Two-Phase Systems, Igarss 2014. 280 (2014) 1–5.

[91] A. Soto, A. Arce, M.K. Khoshkbarchi, Partitioning of antibiotics in a two-liquid phase system formed by water and a room temperature ionic liquid, Separation and Purification Technologies 44 (2005) 242–246.

[92] J.F.B. Pereira, F. Vicente, V.C. Santos-Ebinuma, J.M. Araújo, A. Pessoa, M.G. Freire, Extraction of tetracycline from fermentation broth using aqueous two-phase systems composed of polyethylene glycol and cholinium-based salts, Process Biochemistry 48 (2013) 716–722.

[93] Q. Liu, J. Yu, W. Li, X. Hu, H. Xia, H. Liu, Partitioning Behavior of Penicillin G in Aqueous Two Phase System Formed by Ionic Liquids and Phosphate, Separation and Purification Technologies 41 (2006) 2849–2858.

[94] S.P.M. Ventura, V.C. Santos-Ebinuma, J.F.B. Pereira, M.F.S. Teixeira, A. Pessoa, J. A. P. Coutinho, Isolation of natural red colorants from fermented broth using ionic liquid-based aqueous two-phase systems, Journal of Industrial of Microbial Biotechnology 40 (2013) 507–516.

[95] M.G. Freire, C.M.S.S. Neves, I.M. Marrucho, J.N. Canongia Lopes, L.P.N. Rebelo, J. A. P. Coutinho, High-performance extraction of alkaloids using aqueous two-phase systems with ionic liquids, Green Chemistry 12 (2010) 1715.

[96] Y. Liu, Y.L. Yu, M.Z. Chen, X. Xiao, Advances in Aqueous Two-Phase Systems and Applications in Protein Separation and Purification, Journal of Chemical Engineering Technology (2011).

[97] R. Feng, D. Zhao, Y. Guo, Revisiting Characteristics of Ionic Liquids: A Review for Further Application Development, Journal of Environmental Protection 01 (2010) 95–104.

[98] H. Passos, M.G. Freire, J. A. P. Coutinho, Ionic liquid solutions as extractive solvents for value-added compounds from biomass, Green Chemistry 16 (2014) 4786–4815.

[99] M.G. Freire, P.J. Carvalho, R.L. Gardas, I.M. Marrucho, J.A. P. Coutinho, Mutual Solubilities of Water and the [$C_n$mim][Tf_2N] Hydrophobic Ionic Liquids, Society. (2008) 1604–1610.

[100] J.G. Huddleston, A.E. Visser, W.M. Reichert, H.D. Willauer, G. a. Broker, R.D. Rogers, Characterization and comparison of hydrophilic and hydrophobic room temperature ionic liquids incorporating the imidazolium cation, Green Chemistry 3 (2001) 156–164.

[101] H. Zhao, S. Xia, P. Ma, Use of ionic liquids as “green” solvents for extractions, Journal of Chemical Technology Biotechnology 80 (2005) 1089–1096.

[102] M. Martins, Phycobiliproteins extraction from the red macroalga *Gracilaria vermiculophylla*, University of Aveiro, 2015.

[103] V.C. Santos, F.A. Hasmann, A. Converti, A. Pessoa, Liquid–liquid extraction by mixed micellar systems: A new approach for clavulanic acid recovery from fermented broth, Biochemical Engineering Journal 56 (2011) 75–83.

[104] T. Saitoh, H. Tani, T. Kamidate, H. Watanabe, Phase separation in aqueous micellar solutions of nonionic surfactants for protein separation, Trends in Analytical Chemistry 14 (1995) 213–217.

[105] A. Sadaghiania, Clouding of a nonionic surfactant: The effect of added surfactants on the cloud point, Journal of Colloid Interface Sciences. 144 (1991) 191–200.

[106] D. Shaw, T. Corocoran, Surface Activity and Micelle Formation, Green Chemistry (2002).

[107] P. Taylor, W.L. Hinze, E. Pramauro, A Critical Review of Surfactant-Mediated Phase Separations (Cloud-Point Extractions): Theory and Applications, A Critical Review of Surfactant-Mediated Phase Separations, Journal of Analytical Chemistry (1993) 37–41.

[108] J. Łuczak, J. Hupka, J. Thöming, C. Jungnickel, Self-organization of imidazolium ionic liquids in aqueous solution, Journal of Colloids and Surfaces 329 (2008) 125–133.

[109] G. Fei, L. Jie, Z. Liqiang, Y. Li, L. Zhen, L. Ganzuo, Aqueous Solution Measured By Isothermal Titration Microcalorimetry, Journal of Analytical Chemistry (2011) 1–13.

[110] M. Joskowska, J. Hupka, Imidazolium Ionic Liquids in Mineral Processing, Energy. 42 (2008) 223–236.

[111] C. Jungnickel, J. Łuczak, J. Ranke, J.F. Fernández, A. Müller, J. Thöming, Micelle formation of imidazolium ionic liquids in aqueous solution, Journal of Colloids and Surfaces 316 (2008) 278–284.

[112] Z. Miskolczy, K. Sebök-Nagy, L. Biczók, S. Göktürk, Aggregation and micelle formation of ionic liquids in aqueous solution, Chemical Physical Letture 400 (2004) 296–300.

[113] N. A. Smirnova, E. A. Safonova, Ionic Liquids as Surfactants, Russ, Journal of Physical Chemistry A 84 (2010) 1695–1704.

[114] J. Bowers, C.P. Butts, P.J. Martin, M.C. Vergara-Gutierrez, R.K. Heenan, Aggregation behavior of aqueous solutions of ionic liquids, Langmuir 20 (2004) 2191–2198.

[115] N. A. Smirnova, E. A. Safonova, Micellization in solutions of ionic liquids, Colloid Journal 74 (2012) 254–265.

[116] N. V. Sastry, N.M. Vaghela, V.K. Aswal, Effect of alkyl chain length and head group on surface active and aggregation behavior of ionic liquids in water, Fluid Phase Equilibrium 327 (2012) 22–29.

[117] M.S. Álvarez, M. Rivas, F.J. Deive, M. a. Sanromán, a. Rodríguez, Ionic liquids and non-ionic surfactants: a new marriage for aqueous segregation, RSC Advances 4 (2014) 32698.

[118] F. Comelles, I. Ribosa, J.J. Gonzalez, M. Garcia, Interaction of Nonionic Surfactants and Hydrophilic Ionic Liquids in Aqueous Solutions: Can Short Ionic Liquids Be More than a Solvent? Nonionic Surfactant / Hydrophilic Imidazolium IL interaction interaction, Journal of Colloids and Surfaces (2012) 6–14.

[119] M.U. Araos, G.G. Warr, Structure of nonionic surfactant micelles in the ionic liquid ethylammonium nitrate, Langmuir 24 (2008) 9354–9360.

[120] L.G. Chen, H. Bermudez, Charge screening between anionic and cationic surfactants in ionic liquids, Langmuir. 29 (2013) 2805–2808.

[121] F. Comelles, I. Ribosa, J.J. Gonzalez, M. Garcia, Interaction of Nonionic Surfactants and Hydrophilic Ionic Liquids in Aqueous Solutions: Can Short Ionic Liquids Be More than a Solvent?, Langmuir (2012) 6–14.

[122] R. Pramanik, S. Sarkar, C. Ghatak, V.G. Rao, S. Mandal, N. Sarkar, Effects of 1-butyl-3-methyl imidazolium tetrafluoroborate ionic liquid on Triton X-100 aqueous micelles: Solvent and rotational relaxation studies, Journal of Physical Chemistry B 115 (2011) 6957–6963.

[123] M. Blesic, M.H. Marques, N. V. Plechkova, K.R. Seddon, L.P.N. Rebelo, A. Lopes, Self-aggregation of ionic liquids: micelle formation in aqueous solution, Green Chemistry 9 (2007) 481.

[124] K. Behera, S. Pandey, Interaction between ionic liquid and zwitterionic surfactant: A comparative study of two ionic liquids with different anions, Journal of Colloid Interface Sciences 331 (2009) 196–205.

[125] N. A. Smirnova, A. A. Vanin, E. A. Safonova, I.B. Pukinsky, Y. a. Anufrikov, A.L. Makarov, Self-assembly in aqueous solutions of imidazolium ionic liquids and their mixtures with an anionic surfactant, Journal of Colloid Interface Sciences 336 (2009) 793–802.

[126] C. Liu, D.T. Kamei, J. a King, D.I. Wang, D. Blankschtein, Separation of proteins and viruses using two-phase aqueous micellar systems, Journal of Chromatography B Biomedical Sciences 711 (1998) 127–138.

[127] F. A. Vicente, L.P. Malpiedi, F. A. e Silva, A. Pessoa, J. A. P. Coutinho, S.P.M. Ventura, Design of novel aqueous micellar two-phase systems using ionic liquids as co-surfactants for the selective extraction of (bio)molecules, Separation and Purification Technology 135 (2014) 259–267.

[128] F. Mashayekhi, A.S. Meyer, S. A. Shiigi, V. Nguyen, D.T. Kamei, Concentration of mammalian genomic DNA using two-phase aqueous micellar systems, Biotechnological Bioengennering 102 (2009) 1613–1623.

[129] H. Watanabe, H. Tanaka, A non-ionic surfactant as a new solvent for liquid-liquid extraction of zinc (II) with 1-(2-pyridylazo)-2-naphthol, Talanta 25 (1978) 585–589.

[130] C. Bordier, Phase separation of integral membrane proteins in Triton X-114 solution, Journal of Biological Chemistry 256 (1981) 1604–1607.

[131] A. A. Gouda, A.S. Amin, Cloud-point extraction, preconcentration and spectrophotometric determination of trace quantities of copper in food, water and biological samples, Spectrochemistry Acta - Molecular Spectroscopy 120 (2014) 88–96.

[132] T. Gu, P. A. Galera-Gómez, Clouding of Triton X-114: The effect of added electrolytes on the cloud point of Triton X-114 in the presence of ionic surfactants, Journal of Colloids Surfaces 104 (1995) 307–312.

[133] M.S. Álvarez, F. Moscoso, A. Rodríguez, M. a. Sanromán, F.J. Deive, Triton X surfactants to form aqueous biphasic systems: Experiment and correlation, Journal of Chemical Thermodynamics 54 (2012) 385–392.

[134] D.T. Kamei, J. a. King, D.I.C. Wang, D. Blankschtein, Separating lysozyme from bacteriophage P22 in two-phase aqueous micellar systems, Biotechnological Bioengeneering 80 (2002) 233–236.

[135] P.M. Duque Jaramillo, H. a. Rocha Gomes, F.G. De Siqueira, M. Homem-De-Mello, E.X.F. Filho, P.O. Magalhães, Liquid-liquid extraction of pectinase produced by *Aspergillus oryzae* using aqueous two-phase micellar system, Separation and Purification Technology 120 (2013) 452–457.

[136] C. Liu, Y.J. Nikas, D. Blankschtein, Novel Bioseparations Using Two-Phase Aqueous Micellar Systems, Separation and Purification Technology 52 (1996) 185–192.

[137] C.L. Liu, Y.J. Nikas, D. Blankschtein, Novel bioseparations using two-phase aqueous micellar systems, Biotechnological Bioengeneering 52 (1996) 185–92.

[138] Center of National Research, USA, Phase transitions in polymeric and micellar systems (2013) 1689–1699.

[139] P. Alexandridis, Poly(ethylene oxide)/poly(propylene oxide) block copolymer surfactants, Journal of Colloid Interface Sciences 2 (1997) 478–489.

[140] A. Huff, K. Patton, H. Odhner, D.T. Jacobs, B.C. Clover, S.C. Greer, Micellization and phase separation for triblock copolymer 17R4 in H_2O and in D_2O, Langmuir. 27 (2011) 1707–12.

[141] M. Mishra, P. Muthuprasanna, K.S. Prabha, P.S. Rani, I. a S. Babu, I.S. Chandiran, Basics and potential applications of surfactants - A review, International Journal of Pharmaceutical Research 1 (2009) 1354–1365.

[142] Charles Tanford, Hydrophobic Effect - Formation of Micelles and Biological Membranes, 2nd Edition, Wiley, 1980.

[143] R. Nagarajan, Association of nonionic polymers with micelles, bilayers, and microemulsions, Journal of Chemical Physics 90 (1989) 1980.

[144] Jean-Louis Salager, Surfactants: Types and Uses, in: Laboratory of formulation, interfaces, rheology and processes, 1999: p. 49.

[145] J.Y.T. Chong, X. Mulet, L.J. Waddington, B.J. Boyd, C.J. Drummond, Steric stabilisation of self-assembled cubic lyotropic liquid crystalline nanoparticles: high throughput evaluation of triblock polyethylene oxide-polypropylene oxide-polyethylene oxide copolymers, Soft Matter 7 (2011) 4768.

[146] Sigma-Aldrich, Sigma-Aldrich Surfactants, (2015). https://www.sigmaaldrich.com/materials-science/material-science-products.html?TablePage=9541703 (accessed October 1, 2015).

[147] Dow, Dow - Surfactants, (2015). http://www.dow.com/surfactants/products/ (accessed October 1, 2015).

[148] Sigma, Sigma Aldrich Company (2015). www.sigmaaldrich.com (accessed November 15, 2015).

[149] T. Inoue, H. Ohmura, D. Murata, Cloud point temperature of polyoxyethylene-type nonionic surfactants and their mixtures, Journal of Colloid Interface Sciences 258 (2003) 374–382.

[150] Common Buffers and Stock Solutions. Current Protocols in Food Analytical Chemistry, John Wiley and Sons, 2001.

[151] V.C. Santos-Ebinuma, A.M. Lopes, A. Converti, A. Pessoa, C.D.O. Rangel-Yagui, Behavior of Triton X-114 cloud point in the presence of inorganic electrolytes, Fluid Phase Equilibrium 360 (2013) 435–438.

[152] A. Denicourt-Nowicki, B. Léger, A. Roucoux, N -Donor ligands based on bipyridine and ionic liquids : an efficient partnership to stabilize rhodium colloids.

Focus on oxygen-containing compounds hydrogenation, Physical Chemistry (2011) 13510–13517.

[153] R. Hayes, G.G. Warr, R. Atkin, At the interface: solvation and designing ionic liquids, Physical Chemistry (2010).

[154] H. Jia, L. Zheng, One-step synthesis and assembly of gold nanochains using the Langmuir monolayer of long-chain ionic liquids and their applications to SERS, Crystralography A (2012) 2920–2925.

[155] J.A. Pilc, P.A. Sermon, Chromatographic separation of a wide range of ethoxylated non-ionic surfactants and their sulphonates using high-performance liquid chromatography, Journal of Chromatography. 398. 398 (1987) 375–380.

[156] R. Pramanik, S. Sarkar, C. Ghatak, V.G. Rao, S. Mandal, N. Sarkar, Effects of 1-butyl-3-methyl imidazolium tetrafluoroborate ionic liquid on Triton X-100 aqueous micelles: Solvent and rotational relaxation studies, Journal of Physical Chemistry B. 115 (2011) 6957–6963.

[157] X. Qi, X. Zhang, G. Luo, C. Han, C. Liu, S. Zhang, Mixing Behavior of Conventional Cationic Surfactants and Ionic Liquid Surfactant 1-Tetradecyl-3-methylimidazolium Bromide ([C_{14}mim]Br) in Aqueous Medium, Journal of Dispersion Science Technology 34 (2013) 125–133.

[158] Y. Bae, S.M. Lambert, D.S. Soane, J.M. Prausnitz, Cloud-Point Curves of Polymer Solutions from Thermooptical Measurements, Macromolecules. (1991) 4403–4407.

[159] Y. Wang, Y. Li, J. Han, J. Xia, X. Tang, T. Chen, Cloudy behavior and equilibrium phase behavior of triblock copolymer L64 + salt + water two-phase systems, Fluid Phase Equilibrium. 409 (2016) 439–446.

[160] M. Martins, F. Vieira, I. Correia, R. Ferreira, H. Abreu, J. A. P. Coutinho, Recovery of phycobiliproteins from the red macroalga *Gracilaria sp*. using ionic liquid aqueous solutions, Green Chemistry 1 (2016).

[161] R. Bermejo, F.G. Acién, M.J. Ibáñez, J.M. Fernández, E. Molina, J.M. Alvarez-Pez, Preparative purification of B-phycoerythrin from the microalga *Porphyridium cruentum* by expanded-bed adsorption chromatography, Journal of Chromatography B 790 (2003) 317–325.

[162] C. Denis, C. Ledorze, P. Jaouen, J. Fleurence, Comparison of different procedures for the extraction and partial purification of R-phycoerythrin from the red macroalga *Grateloupia turuturu,* Journal of Marine Sciences 52 (2009) 278–281.

[163] E. Cordisco, C.N. Haidar, R. Goñi, B. Nerli, L.P. Malpiedi, Physicochemical characterization of aqueous micellar systems formed by environmentally friendly salts, Fluid Phase Equilibrium 135 (2014) 259–267.

[164] A. F. Jozala, A. M. Lopes, P.G. Mazzola, P.O. Magalhães, T.C. Vessoni Penna, a. Pessoa, Liquid-liquid extraction of commercial and biosynthesized nisin by aqueous two-phase micellar systems, Enzyme Microbial Technology 42 (2008) 107–112.

[165] T. Ingram, S. Storm, P. Glembin, S. Bendt, D. Huber, T. Mehling, Aqueous Surfactant Two-Phase Systems for the Continuous Countercurrent Cloud Point Extraction, Chemie Technologies (2012) 102-124

[166] C.W. Ooi, C.P. Tan, S.L. Hii, A. Ariff, S. Ibrahim, T.C. Ling, Primary recovery of lipase derived from *Burkholderia sp*. ST8 with aqueous micellar two-phase system, Process Biochemistry 46 (2011) 1847–1852.

[167] C. Chaibundit, N.M.P.S. Ricardo, F.D.M.L.L. Costa, S.G. Yeates, C. Booth, Micellization and gelation of mixed copolymers P123 and F127 in aqueous solution, Langmuir 23 (2007) 9229–9236.

[168] R.C. Da Silva, W. Loh, Effect of Additives on the Cloud Points of Aqueous Solutions of Ethylene Oxide–Propylene Oxide–Ethylene Oxide Block Copolymers, Journal of Colloid Interface Sciences 202 (1998) 385–390.

[169] L. Willie, Cloud Point Extraction and Preconcentration Procedures for Organic and Related Pollutants of State Concern, Water Resources Research 08 (1992).

[170] J. Nikas, C.L. Liu, T. Srivastava, N.L. Abbott, D. Blankschtein, Protein Partitioning in Two-Phase Aqueous Nonionic Micellar Solutions, Macromolecules 25 (1992) 4797–4806.

[171] J.P. Wilcoxon, Phase Behavior and Metastability in a Nonionic Surfactant-Water-Electrolyte System, Journal of Colloids Interface Sciences (1990).

7. SUPPORTING INFORMATION

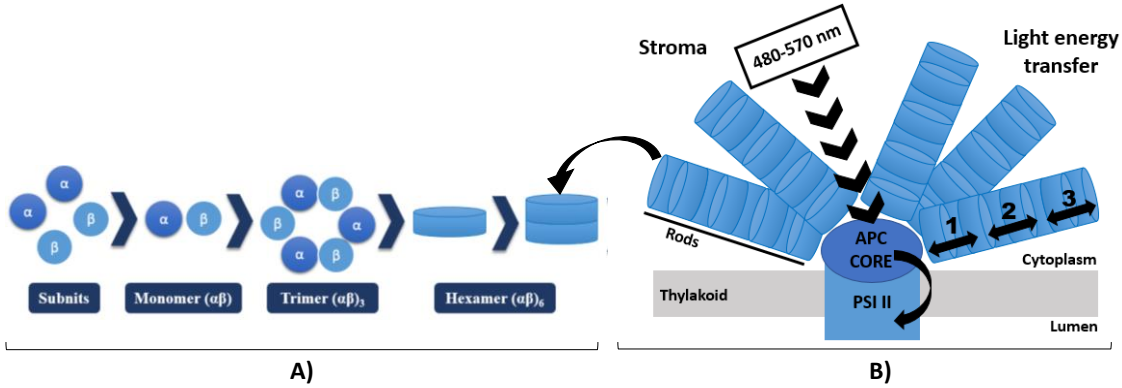


Figure S.I. 1 - A) Schematic representation of phycobiliprotein subunit assemblage, while **B)** represents a common phycobilisome structure in red algae. Region 1 is occupied by phycoerythrin, phycoerythrocyanin or phycocyanin, whereas region 2 has phycoerythrin or phycocyanin and region 3 has phycocyanin. Adapted from [17].

Table S.I. 1 - Absorption and fluorescence spectra of R-phycoerythrin from *Porphyridium cruentum* according to pH, light exposure time and temperature variations. Adapted from [31].

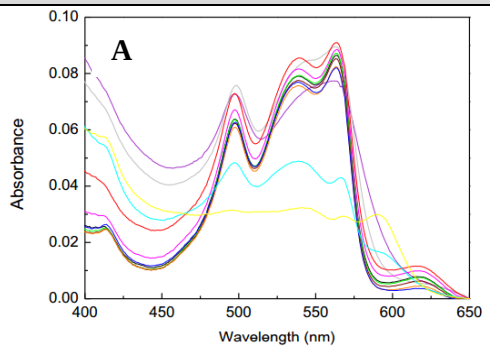
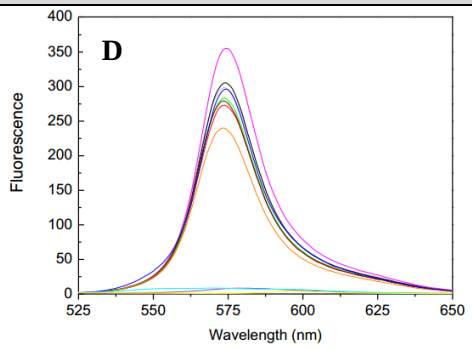
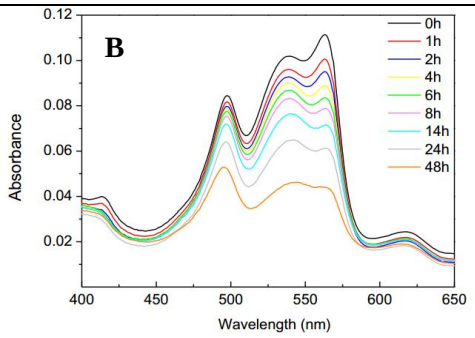
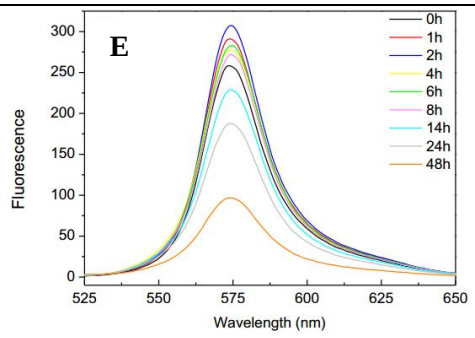
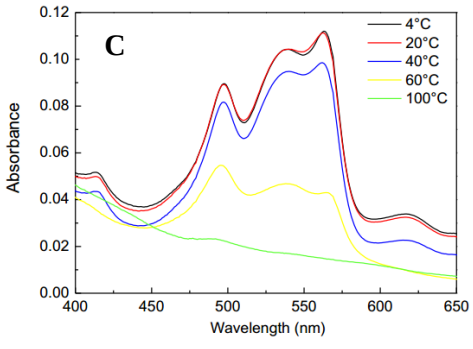
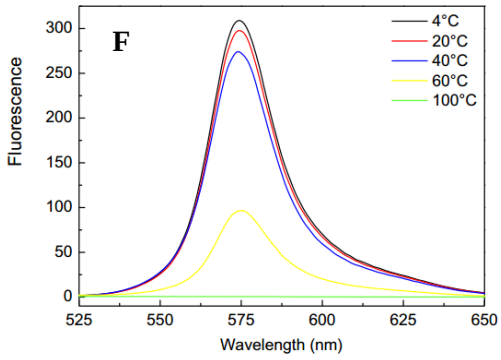
Parameter	Absorption spectra	Fluorescence spectra
pH		
Light exposure time		
Temperature		

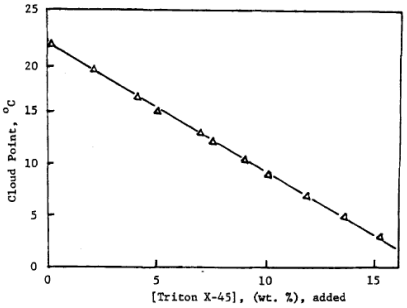
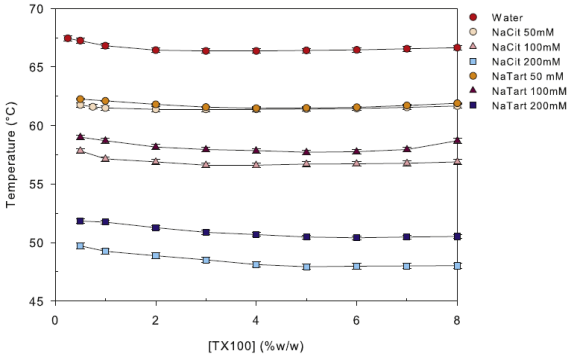
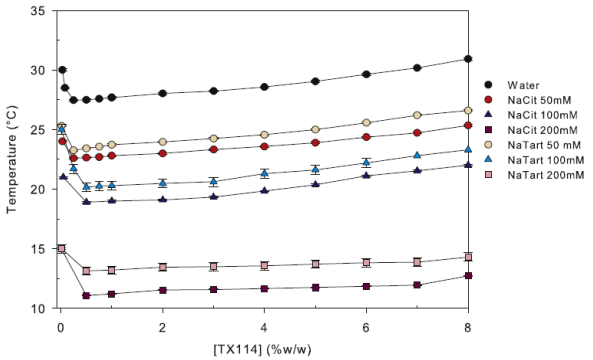
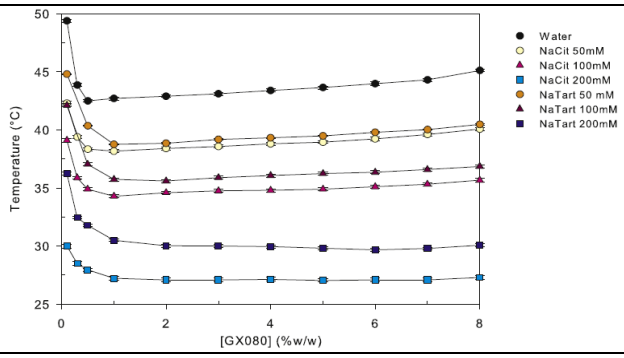
Table S.I. 2 - Comparison of yield and purity index of several extraction and purification methods of phycoerythrin from red algae.

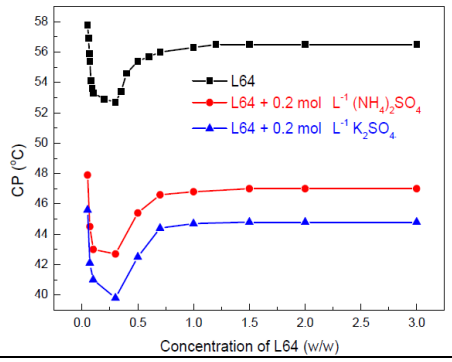
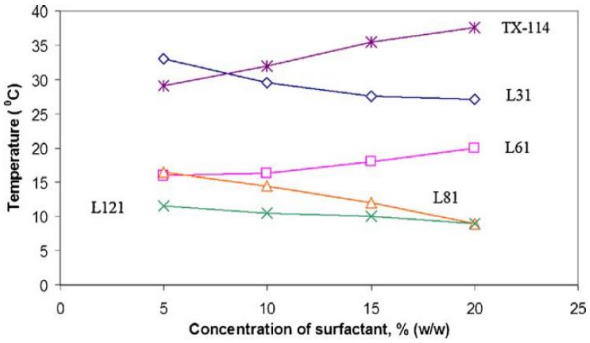
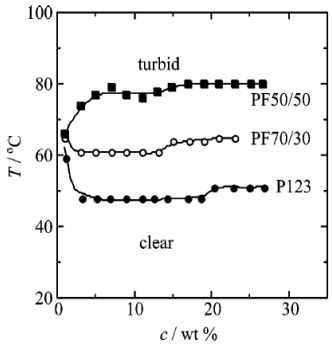
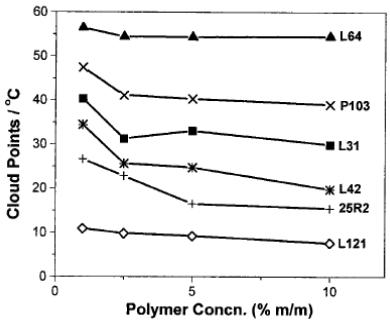
Solid-liquid extraction method		Purification method	Species	Purity index	Yield (mg/g DW algae)	Extraction conditions			Ref.
						Temperature (ºC)	Time	Solid-liquid ratio (m/v)	
Classic Processes	**Soaking seaweeds in water** (extraction by osmotic shock)	Expanded-bed adsorption column Ion-exchange chromatography	*Polysiphonia urceolata*	3.26	1.570 (frozen algae)	4	24h	1:3	[64]
			Gracilaria verrucosa	4.40	1.500 (frozen algae)	Room temperature	Overnight	1:1	[65]
	Diluted phosphate buffer (5-50 mM, pH 7.0) (Macroalgae are ground in liquid nitrogen and the product is homogenized in sodium phosphate buffer)	Ammonium sulphate precipitation Ion-exchange chromatography	*Portieria hornemannii*	5.20	1.230 (fresh algae)	Freeze-thaw (x3)	Overnight	10:25	[58]
		Expanded-bed chromatography Packed-bed chromatography	*Porphyridium cruentum*	4.60	0.166 (frozen algae)	4	24h	n.d.	[161]
		Ammonium sulphate precipitation Gel permeation chromatography	*Gracilaria longa*	4.50	0.100 (fresh algae)	4	24h	n.d.	[59]
		Hydroxyapatite column Gel filtration	*Corallina elongata*	6.67	0.600 (freeze-dryed algae)	n.d.	n.d.	1:3 (referring to DW)	[34]
		Ammonium sulphate precipitation Ion-exchange chromatography	*Gracilaria lemaneiformis*	n.d.	1.730 (fresh algae)	Freeze–thaw cycles of -25 and 4°C	Overnight	10:25	[61]

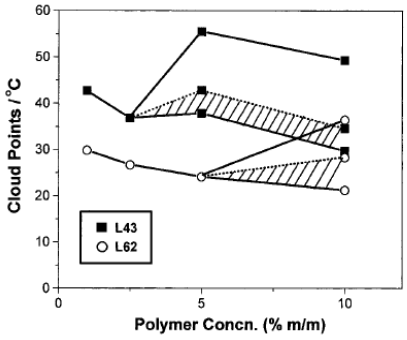
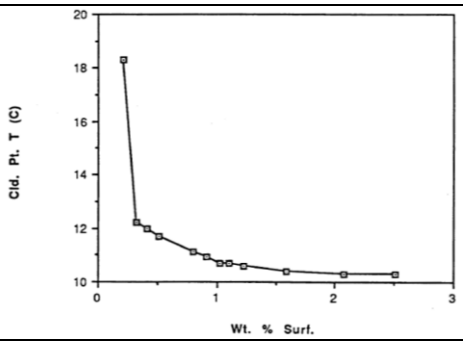
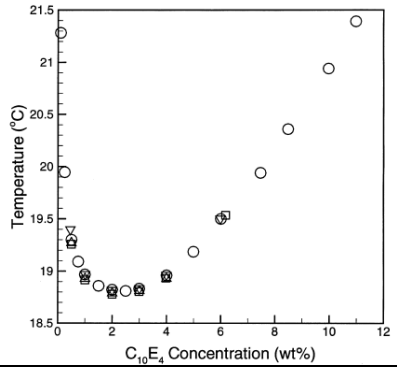
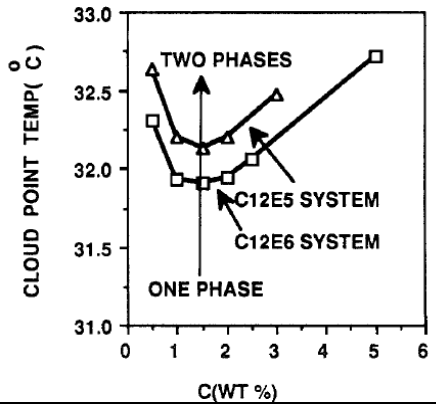
		Ammonium sulphate precipitation Ultrafiltration	*Gracilaria turuturu*	1.51	2.750 (freeze-dryed algae)	n.d.	n.d.	n.d.	[162]
		Ammonium sulphate precipitation Expanded-bed adsorption column	*Porphyra yezoensis*	n.d.	6.060 (fresh algae)	Freeze–thaw cycles of -25 and 4°C (x3)	n.d.	7:75	[60]
		Continuous ammonium sulphate precipitation Hydroxyapatite column	*Porphyra yezoensis*	3.92	2.000 (dry algae)	n.d.	n.d.	1:5	[62]
		Adsorption by activated charcoal Ammonium sulphate precipitation Ion-exchange chromatography	*Ceramium isogonum*	2.10	0.038	Freeze-thaw cycles of -20ºC and 4ºC	n.d.	n.d.	[63]
Enzymatic Processes	Enzymatic process as a pre-treatment step	-	*Palmaria palmata*	0.40	4.360	Room temperature	n.d.	n.d.	[50]
	Cellulase + xylanase enzymatic digestion (pre-treatment)	-	*Palmaria palmata*	n.d.	0.300	Room temperature	n.d.	n.d.	[51]

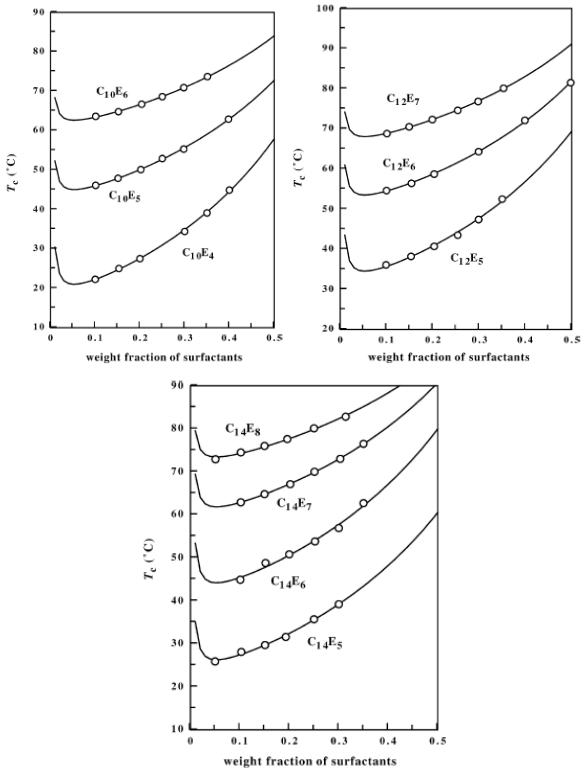
NOTE: n.d. = no data available

Table S.I. 3 - Binodal curves of conventional AMTPS using nonionic surfactants currently available in literature.

Surfactant	Binodal curve	Ref.
Triton X-45		[107]
Triton X-100		[115,160]
Triton X-114		[114,138, 142,160–163]
Genapol X080		[163]

Pluronic L-64		[159]
Triton X-114 **Pluronic L-31, L-61, L-81 and L-121**		[166]
Pluronic P123 and F127		[167]
Pluronic L-64, L-31, L-42, 25R2, L-121 and P103		[168]

Pluronic L-43 and L-62		[168]
C_8E_3		[169]
$C_{10}E_4$		[136,169]
$C_{12}E_5$ **$C_{12}E_6$**		[171]

$C_{10}E_6$ **$C_{10}E_5$** **$C_{10}E_4$** **$C_{12}E_7$** **$C_{12}E_6$** **$C_{12}E_5$** **$C_{14}E_5$** **$C_{14}E_6$** **$C_{14}E_7$** **$C_{14}E_8$**		[149]

Note: Reading the papers is recommended in order to know clearly the composition of the aqueous systems.

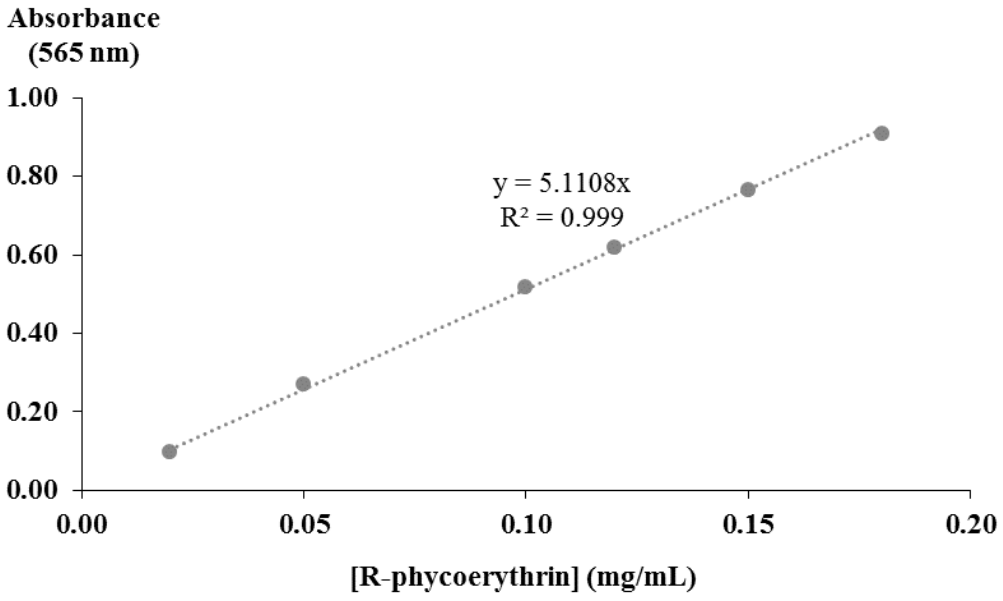


Figure S.I. 2 - Calibration curve for R-phycoerythrin quantification in aqueous solution at 565 nm made with purified R-phycoerythrin solutions using a UV-Vis microplate reader (Synergy HT microplate reader – BioTek).

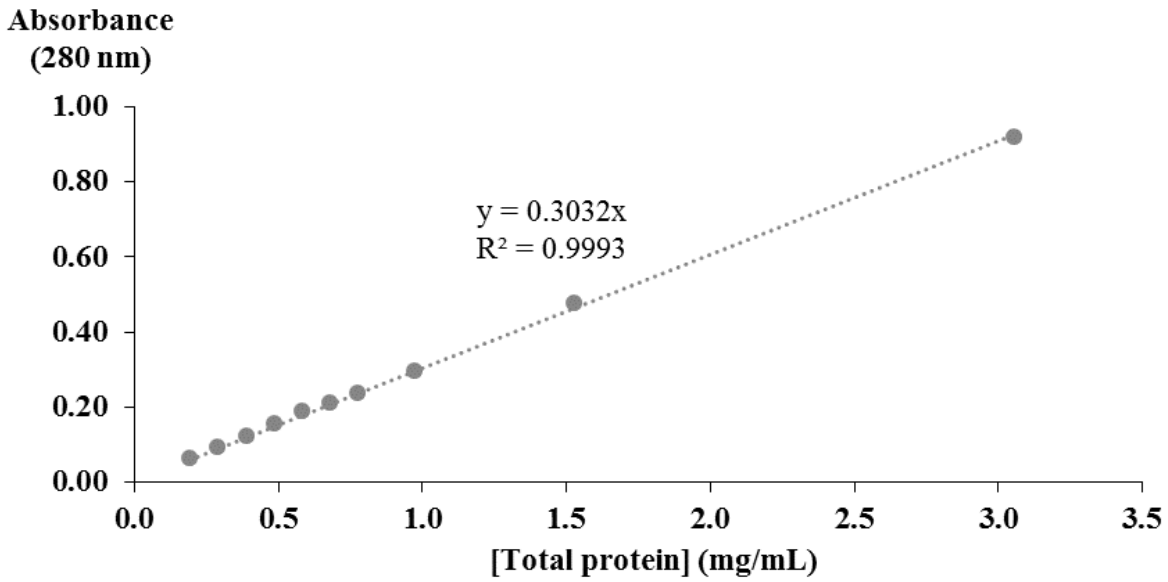


Figure SI. 3 - Calibration curve for total protein quantification in aqueous solution at 280 nm made with Bovine Serum Albumin using a UV-Vis microplate reader (Synergy HT microplate reader – BioTek).

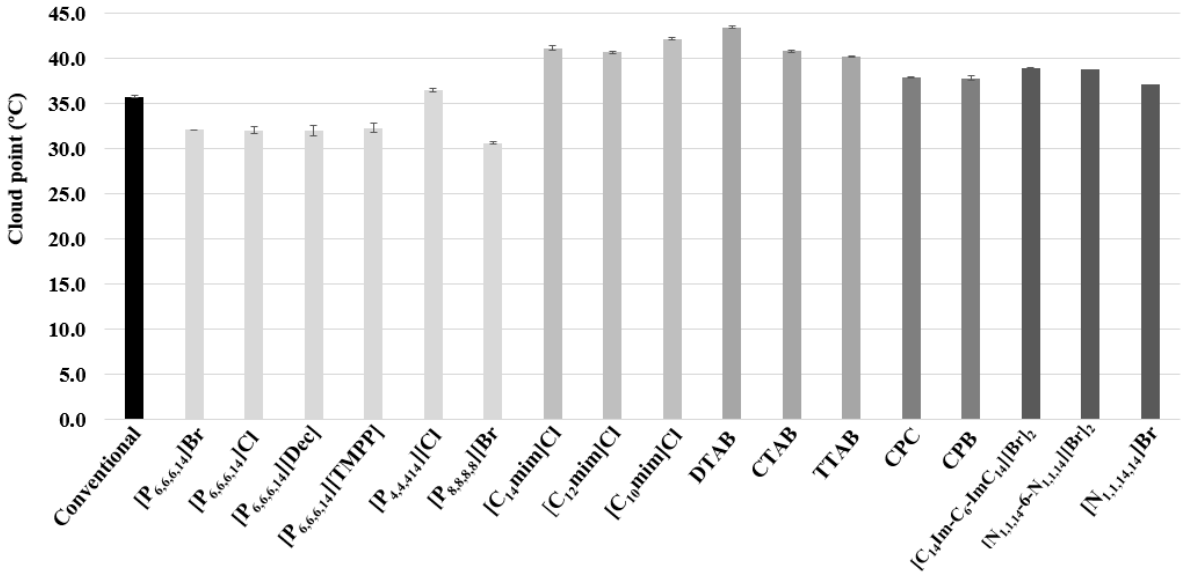


Figure S.I. 4 - Cloud points of AMTPS composed of 10 wt% of Tergitol 15-S-7, 0.3 wt% of IL and 89.7 wt % of McIlvaine buffer at pH 7.0.

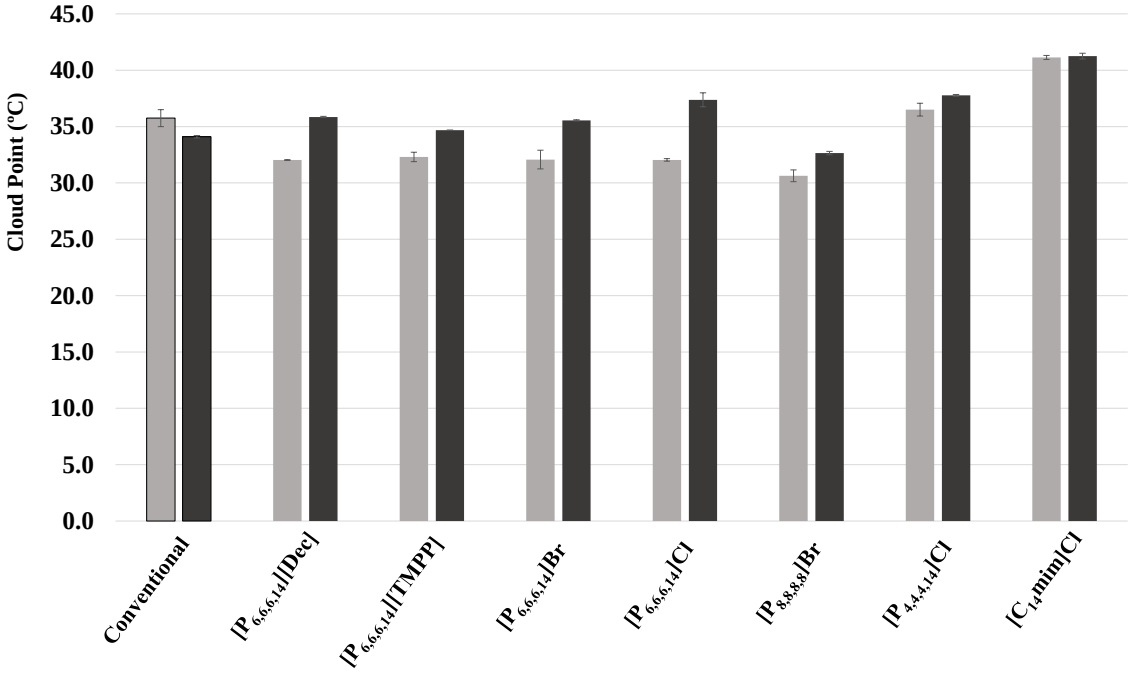


Figure S.I. 5 - Cloud points of AMTPS composed of 10 wt% of Tergitol 15-S-7, 0.3 wt% of IL and 89.7 wt% of McIlvaine buffer at pH 7.0 and 8.0 .