Universidade de Aveiro Departamento de Química
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SÓNIA PATRÍCIA MARQUES VENTURA

PRODUCTION OF LIPASE BY EXTRACTIVE FERMENTATION WITH IONIC LIQUIDS

Produção de Lipase por Fermentação Extractiva com Líquidos Iónicos

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Produção de Lipase por Fermentação Extractiva com Líquidos Iónicos

Tese apresentada à Universidade de Aveiro para cumprimento dos requisitos necessários à obtenção do grau de Doutor em Engenharia Química, realizada sob a orientação científica do Professor Doutor João Manuel da Costa Araújo Pereira Coutinho, Professor Associado com Agregação do Departamento de Química da Universidade de Aveiro e da Doutora Isabel Maria Delgado Jana Marrucho Ferreira, Investigadora Auxiliar do Instituto de Tecnologia Química e Biológica da Universidade Nova de Lisboa e Professora auxiliar da Universidade de Aveiro.

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o júri

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palavras-chave

Fermentação extractiva, líquidos iónicos, anião, catião, cadeia alquílica, solubilidade de líquidos iónicos em água, sistemas aquosos de duas fases, biomoléculas, amino-ácido, enzimas, processos de separação/purificação, biocompatibilidade, actividade enzimática, (eco)cotoxicologia, bactéria marinha luminescente, alga verde, cladóceros, EC_{50}, bioacumulação, coeficientes de distribuição octanol-água.

resumo

Novos processos fermentativos, designados por processos de Fermentação Extractiva, são caracterizados por apresentarem etapas de produção e extracção em simultâneo. A extracção líquido-líquido como técnica de separação é amplamente usado na indústria química pela sua simplicidade, baixo custo e facilidade de extrapolação de escala. No entanto o uso de solventes orgânicos nestes processos potencia os riscos ocupacionais e ambientais. Neste contexto, o uso de sistemas de duas fases aquosas baseados em líquidos iónicos, apresenta-se como uma técnica eficaz para a separação e purificação de produtos biológicos.
Este trabalho apresenta um estudo integrado sobre o uso de líquidos iónicos não aromáticos foram determinados. A capacidade para a formação de sistemas de duas fases foi estudada para uma vasta gama de líquidos iónicos hidrofílicos com diferentes aniões, catiões e cadeias alquílicas. A capacidade de separação e purificação de um largo conjunto de líquidos iónicos foi posteriormente investigada, recorrendo-se ao uso de várias biomoléculas modelo de diferentes graus de complexidade, um amino-acido (L-triptofano) e duas enzimas lipolíticas (enzima produzida pela bactéria *Bacillus* sp. e *Candida antarctica* lipase B – CaLB). Esta última foi ainda usada para um estudo de biocompatibilidade, tendo sido determinado o efeito de diferentes LIs hidrofílicos na sua actividade enzimática. Este trabalho mostra um estudo ecotoxicológico duma vasta gama de líquidos iónicos e espécies aquáticas, inseridas em diversos níveis tróficos. A bioacumulação foi investigada através do estudo dos coeficientes de distribuição 1-octanol-água (D_{ow}).

keywords

Extractive fermentation, ionic liquids, anion, cation, alkyl chain, solubility of ionic liquids in water, aqueous two-phase systems, biomolecules, amino-acid, enzymes, separation/purification processes, biocompatibility, enzymatic activity, (eco)toxicology, luminescent marine bacteria, freshwater green algae, cladocerans, EC_{50}, bioaccumulation, 1-octanol-water distribution coefficients.

abstract

Novel fermentation processes characterized by the simultaneously production and separation are known as Extractive Fermentations. Liquid-liquid extraction is a separation technique that can be used for this purpose. These processes are widely used in the chemical industry owing to its simplicity, low costs and ease of scale-up. However, most extraction processes include the use of organic solvents, which have both environmental and occupational risks associated to their use. This makes these extraction systems inappropriate for the development of environmental-friendly technologies. In this context, the use of aqueous two-phase systems (ATPS) based in Ionic Liquids (ILs) appears as an effective and viable method for the separation and purification of biological products.

The present work reports an integrated study on the use of ILs as alternative solvents in Fermentation processes. Original data on the solubility of non-aromatic ILs in water are reported. The ability of several ILs for the formation of aqueous two-phase systems was studied for a wide range of hydrophilic ILs with different anions, cations and alkyl chains. The separation and purification capacity of a large set of ILs was then investigated, using different biomolecules as models with distinct structural complexities, one amino-acid (L-tryptophan) and two lipolytic enzymes (an enzyme produced by the bacterium *Bacillus* sp. and *Candida antarctica* lipase B – CaLB). CaLB was also used on a study of biocompatibility, where the effect of various hydrophilic ILs on its enzymatic activity was addressed.

Finally, this work reports an ecotoxicological study for a large number of ILs and aquatic species, included in different trophic levels. The bioaccumulation data was investigated by the study of the 1-octanol-water distribution coefficients (D_{ow}).

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Notation

List of Symbols

α	hydrogen-bond acidity (solvatochromic parameter)
β	hydrogen-bond basicity (solvatochromic parameter)
ε	Extinction coefficient
π	solvatochromic parameter representing the dispersive forces
σ	standard deviation
μ	dynamic viscosity
$\Delta_{sol}G_m^0$	standard molar Gibbs energy
$\Delta_{sol}S_m^0$	standard molar Entropy
$\Delta_{sol}H_m^0$	standard molar Enthalpy
Δt	time interval
a_w	water activity parameter
A/A_0	residual enzymatic activity
A	Merchuck parameter
B	Merchuck parameter
C	Merchuck parameter
C_B	protein concentration in the bottom phase
C_T	protein concentration in the top phase
C_{oct}	equilibrium concentrations of ionic liquid in the octanol rich-phase
C_w	equilibrium concentrations of ionic liquid in the water rich-phase
D	dilution of the enzymatic solution
D_{ow}	octanol-water distribution coefficient
E	extraction constant
EA	enzyme activity
EA_B	enzyme activity in the bottom phase
EA_T	enzyme activity in the top phase

EC_{50}	Effect concentration estimated to cause a 50 percent negative effect
I^+L^-	ion pair of the ionic liquid
I^+	imidazolium cation
k_1	equilibrium constant
k_2	equilibrium constant
k_3	equilibrium constant
K_{app}	apparent partition coefficient
K_E	partition coefficient of the enzyme CaLB
K_p	partition coefficient of the protein
K_{ow}	octanol-water partition coefficient
K_{Trp}	partition coefficient of L- tryptophan
L^-	IL anion
l_f	final body length
l_i	initial body length
l_x	probability of surviving to age x
M_1	correlation parameter
M_2	correlation parameter
M_3	correlation parameter
m_x	fecundity at age x.
n	number of neonates
PF	purification factor
R_V	volume ratio
R_B^E	recovery of the enzyme in the bottom phase
R_B^P	recovery of the protein in the bottom phase
SA	specific activity after the extraction procedure
SA_i	initial specific activity (before the extraction procedure)
T	temperature

v	volume
V_B	volume of the top phase
V_R	total volume of the reaction system
V_S	total volume of the enzyme
V_T	volume of the top phase
w/v	weight/volume reason
x	age class
X_B	mass fraction composition of salt for the bottom phase
x_{IL}	Mole fraction solubility of ILs in water
X_M	mass fraction composition of salt for the mixture
X_T	mass fraction composition salt for the top phase
Y_B	mass fraction composition of ionic liquid for the bottom phase
Y_M	mass fraction composition of ionic liquid for the mixture
X_T	mass fraction composition ionic liquid for the top phase

List of Abbreviations

ASTM	synthetic hard water medium
ATPS	aqueous two-phase systems
BATIL	Biodegradability and Toxicity of Ionic Liquids conference
BSA	bovine serum albumin
CaCo-2	human carcinoma cells
CaLB	*Candida antarctica* lipase B
CH_3OH	Methanol
CTL	control
C_2	two carbons alkyl chain
C_6	six carbons alkyl chain
C_8	eight carbons alkyl chain
D. magna	*Daphnia magna*
DMSO	dimethyl sulfoxide
ESI-MS	Electrospray ionization mass spectrometry
HF	hydrofluoric acid
H_2O	water
HT-2	human carcinoma cells
i.d.	impossible to determine
IL	ionic liquid
IL-ATPS	aqueous two-phase systems based in ionic liquids
ILs	ionic liquids
IPC-81	*promyelocytic leukaemia* cell
KCl	potassium chloride
K_2CO_3	potassium carbonate
K_2HPO_4	potassium phosphate dibasic
KH_2PO_4	potassium phosphate monobasic

K_3PO_4	potassium phosphate tribasic
LLE	liquid-liquid extraction
MBL	marine biological laboratory medium
$MgSO_4.7H_2O$	magnesium sulphate heptahydrated
min	minutes
Na_2SO_4	sodium sulphate
$NaHCO_3$	sodium bicarbonate
$NaNO_3$	sodium nitrate
$(NH_4)_2SO_4$	ammonium sulphate
NMR	nuclear magnetic resonance
Oct	octanol
o.d.	outer diameter
OECD	guideline for testing of chemicals
-OH	hydroxyl group
-O-	ether group
O-C=O	ester group
PB	phosphate buffer
PEG	poly(ethylene glycol)
PEG 600	poly(ethylene glycol) of average weight 600 $g.mol^{-1}$
PEG 4000	poly(ethylene glycol) of average weight 4000 $g.mol^{-1}$
p*I*	isoelectric point
p-NFL	*para*-nitrophenyl laurate
p-NF	*para*-nitrophenol
P. subcapitata	*Pseudokircheneriella subcapitata*
QSPR	quantitative structure-property relationship
REACH	Registration, Evaluation, Authorization and Restriction of Chemicals
SDS-PAGE	sodium dodecyl sulphate-polyacrylamide gel

std	standard deviation on mass fraction compositions for the tie-lines
T. lanuginosus	*Thermomyces lanuginosus*
TL	tie-line
TLL	tie-line length
TLs	tie-lines
Trp	L- tryptophan
UFT	Centre for Environmental Research and Sustainable Technology
UV	ultraviolet
UV-Vis	ultraviolet visible
V. fischeri	*Vibrio fischeri*
VLE	equilibrium vapor-liquid
$_w$	abbreviation for water in subscript

[C_2mim][NTf_2]: 1-ethyl-3-methylimidazolium bis(trifluoromethylsulfonyl)imide

[C_4mim][NTf_2]: 1-butyl-3-methylimidazolium bis(trifluoromethylsulfonyl)imide

[C_3mpyrr][NTf_2]: 1-methyl-1-propylpyrrolidinium bis(trifluoromethylsulfonyl)imide

[C_4mpyrr][NTf_2]: 1-butyl-1-methylpyrrolidinium bis(trifluoromethylsulfonyl)imide

[C_3mpip][NTf_2]: 1-methyl-1-propylpiperidinium bis(trifluoromethylsulfonyl)imide

[C_3mim][NTf_2]: 1-methyl-3-propylimidazolium bis(trifluoromethylsulfonyl)imide

[C_3mpyr][NTf_2]: 3-methyl-1-propylpyridinium bis(trifluoromethylsulfonyl)imide

[C_3mpip][NTf_2]: 1-methyl-1-propylpiperidinium bis(trifluoromethylsulfonyl)imide

[C_4mim][PF_6]: 1-butyl-3-methylimidazolium hexafluorophosphate

[C_3mim][PF_6]: 1-methyl-3-propylimidazolium hexafluorophosphate

[C_3mpyrr][PF_6]: 1-methyl-1-propylpyrrolidinium hexafluorophosphate

[C_3mpyr][PF_6]: 3-methyl-1-propylpyridinium hexafluorophosphate

[C_3mpip][PF_6]: 1-methyl-1-propylpiperidinium hexafluorophosphate

[C_6mim][PF_6]: 1-hexyl-3-methylimidazolium hexafluorophosphate

[C8mim][PF_6]: 3-methyl-1-octylimidazolium hexafluorophosphate

$[C_4mim][BF_4]$: 1-butyl-3-methylimidazolium tetrafluoroborate

[im]Cl: imidazolium chloride

$[C_1im]Cl$: methylimidazolium chloride

$[C_2im]Cl$: ethylimidazolium chloride

$[C_1mim]Cl$: 1,3-dimethylimidazolium chloride

$[OHC_2mim]Cl$: 1-hydroxyethyl-3-methylimidazolium chloride

$[C_2mim]Cl$: or $[1\text{-}C_2\text{-}3\text{-}C1im]Cl$: 1-ethyl-3-methylimidazolium chloride

$[C_4mim]Cl$ or $[1\text{-}C_4\text{-}3\text{-}C_1im]Cl$:1-butyl-3-methylimidazolium chloride

$[C_6mim]Cl$ or $[1\text{-}C_6\text{-}3\text{-}C_1im]Cl$: 1-hexyl-3-methylimidazolium chloride

$[C_7H_7mim]Cl$ or $[1\text{-}C_7H_7\text{-}3\text{-}C_1im]Cl$: 1-benzyl-3-methylimidazolium chloride

$[1\text{-}C_7\text{-}3\text{-}C_1im]Cl$ or $[C_7mim]Cl$: 1-heptyl-3-methylimidazolium chloride

$[1\text{-}C_8\text{-}3\text{-}C_1im]Cl$ or $[C_8mim]Cl$: 1-octyl-3-methylimidazolium chloride

$[C_2mim]Br$: 1-ethyl-3-methylimidazolium bromide

$[C_2mim][CH_3COO]$: 1-ethyl-3-methylimidazolium acetate

$[C_2mim][MeSO_4]$: 1-ethyl-3-methylimidazolium methylsulfate

$[C_2mim][EtSO_4]$: 1-ethyl-3-methylimidazolium ethylsulfate

$[C_2mim][CH_3SO_3]$: 1-ethyl-3-methylimidazolium methanesulfonate

$[C_2mim][CF_3SO_3]$: 1-ethyl-3-methylimidazolium trifluoromethanesulfonate

$[C_4mim]Br$: 1-butyl-3-methylimidazolium bromide

$[C_4mim][CH_3COO]$: 1-butyl-3-methylimidazolium acetate

$[C_4mim][N(CN)_2]$: 1-butyl-3-methylimidazolium dicyanamide

$[C_4mim][SCN]$: 1-butyl-3-methylimidazolium thiocyanate

$[C_4mim][CH_3SO_3]$: 1-butyl-3-methylimidazolium methanesulfonate

$[C_4mim][HSO_4]$: 1-butyl-3-methylimidazolium hydrogensulfate

$[C_4mim][CF_3SO_3]$: 1-butyl-3-methyl-imidazolium trifluoromethanesulfonate

$[C_4mim][CF_3COO]$: 1-butyl-3-methylimidazolium trifluoroacetate

$[1\text{-}C_4\text{-}2\text{-}C_1pyr]Cl$: 1-butyl-2-methylpyridinium

$[1\text{-}C_4\text{-}3\text{-}C_1pyr]Cl$ or $[C_4mpyr]Cl$: 1-butyl-3-methylpyridinium chloride

$[1\text{-}C_4\text{-}4\text{-}C_1pyr]Cl$: 1-butyl-4-methylpyridinium chloride

$[1\text{-}C_4\text{-}1\text{-}C_1pyr]Cl$: 1-butyl-1-methylpyrrolidinium chloride

$[1\text{-}C_4\text{-}1\text{-}C1pip]Cl$: 1-butyl-1-methylpiperidinium chloride

$[C_8pyr][N(CN)_2]$: 1-octyl-pyridinium dicyanamide

$[TMGC_4]I$: di-butyl-tetramethyl-guanidinium iodide

$[TMGC_7]I$: di-heptyl-tetramethyl-guanidinium iodide

$[TMGC_{12}]I$: di-dodecyl-tetramethyl-guanidinium iodide

$[(C_3O)_4DMG]Cl$: N''N''-dimethyl-N,N,N',N'-tetra-(2-methoxyethyl)-guanidinium chloride

$[(di\text{-}h)_2DMG]Cl$: tetrahexyl-dimethyl-guanidinium chloride

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

$[P_{6,6,6,14}][CH_3SO_3]$: trihexyltetradecylphosphonium methanosulfonate

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

$[P_{i(4,4,4)1}][TOS]$: triisobutyl(methyl)phosphonium tosylate

$[P_{4,4,4,1}][CH_3SO_4]$: tributyl(methyl)phosphonium methylsulfate

$[P_{4,4,4,4}]Br$: tetrabutylphosphonium bromide

$[C_{10}C(O)OEtmim]Br$: 3-methyl-1-(ethoxycarbonyloctyl)imidazolium bromide

$[C_5O_2mim]Cl$: 1-(2-(2-methoxyethoxy)ethyl)-3-methylimidazolium chloride

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1. General Introduction

1.1. EXTRACTIVE FERMENTATION

The most common biotechnological conversions use microorganisms (whole-cells) or enzymes (cell parts) to produce a chemical transformation. Each method has advantages and challenges at both bench and industrial scales [1]. The use of whole cell fermentations benefit from the ability to use low-cost and renewable feedstocks, the large choice of microorganisms occurring either naturally or recombinant, and from the reactions mild conditions, which are conducted at near ambient temperatures [2]. Often, the major step of these biotechnological processes is the development of an efficient technique for the extraction and purification of the target product from the fermentation broth. The product may be sequestered inside the cell or it may be released by the microorganisms and has to be removed by adequate separation methods from the fermentation media [2]. Additionally, the solutions involved are normally complex, which may contain significant levels of impurities such as biosurfactants, polinucleotides, polysaccharides, metabolic products, precursors, unused substrates and cell debris. Moreover, for products like proteins, enzymes, nucleic acids, cell organelles, antibiotics and antigens, the biological activity must be preserved until the end of the purification step [3], as well as the tolerance limits of pH, temperature, osmotic pressure and surface charges. It is thus not surprising that 60 - 90% of the cost of biotechnological processes is expended on the separation/purification steps [3].

The most common fermentation methods produce the target product in one stage that it is then separated “downstream”. A variety of downstream separation processes can be used to remove various products from the fermentation broth, such as evaporation/distillation, precipitation, gas stripping, membrane separations, adsorption, liquid chromatography, centrifugation, sedimentation, electrophorese, and finally, the use of organic solvents [4]. However, due to the diluted media and the production of intermediate inhibitors, the techniques aforementioned may be hard to apply to the separation of the target products. Centrifugation and sedimentation, for example utilize the size and density of the particles and therefore, a high degree of resolution cannot be expected [4]. Chromatographic methods like column chromatography or high-pressure liquid chromatography, although highly specific, can handle only small amounts of feed at a time [4]. Moreover, evaporation and/or distillation processes have a small window of applicability [4]. Membranes processes have improved their selectivities, flux and reduce fouling, but large scale use is still limited [4]. Solvent extraction with organic solvents it is still a widely used separation technique [5, 6]. This process is employed in

the chemical industry owing to its simplicity, low costs and ease of scale-up [7, 8]. Advantages of these systems are their lower viscosities, lower costs and shorter phase separation times. Nevertheless, these conventional separation processes use organic solvents, that are volatile, flammable, deleterious to biomolecules, and environmental and health hazardous [5, 9]. This makes these systems of extraction inappropriate for the development of environmental-friendly technologies and for many biotechnological processes for lack of biocompatibility. Another problem with conventional solvents is that their number is limited, so it may be difficult to find the ideal solvent suited for a particular application, even considering solvent mixtures.

The fermentation processes are normally operated in separated production and separation steps. Recently fermentation processes where the reaction and separation processes take place at the same time have been proposed [10-14]. They can be conducted inside ("*in situ*") or outside ("*ex situ*") the fermentation reactor and are usually known as Extractive Fermentation processes - Figure 1.1.1.

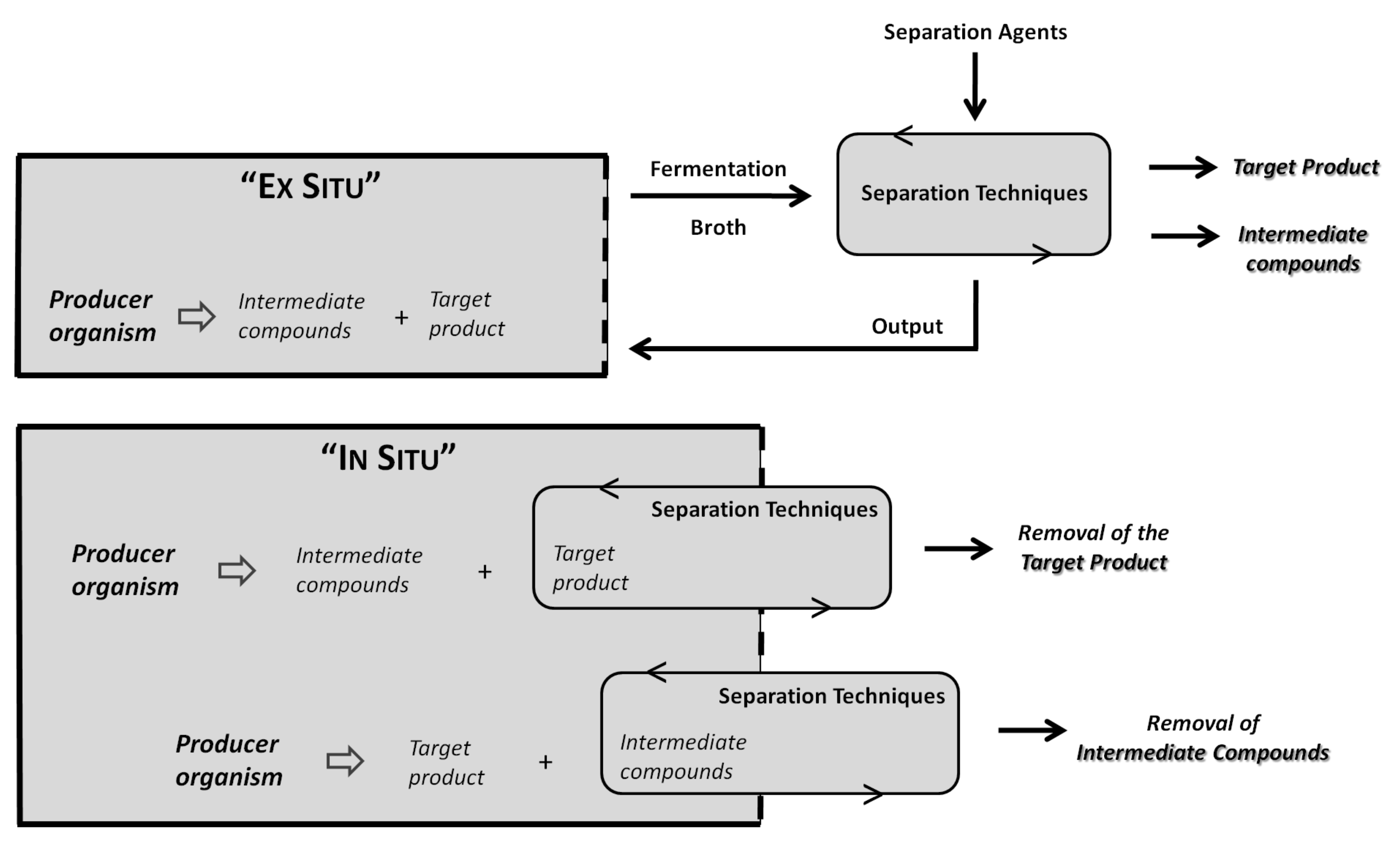

Figure 1.1.1. Schematic figure of the different types of Extractive Fermentation processes.

One of these separation steps can be performed through liquid-liquid extraction involving the use of aqueous two-phase systems, here abbreviated as ATPS. An ATPS is formed by two mutual incompatible polymers, or a polymer and a salt in aqueous solution [15]. Those systems contain about 80 - 90% of water providing an excellent environment for cells, cell organelles and biologically active substances [16]. They have caught the attention of biochemical engineers due to its apparent biocompatibility, low interfacial tension, and easy scale-up [15, 17]. Moreover, this technique is associated with a decrease in the process time, resulting in considerable saving in what concerns the required energy input and manpower [3], better yields and almost no environmental hazards [18]. In extractive fermentation using ATPS, the cells are considered to be immobilized on to one of the ATPS phases and the product partitions into the other phase by proper manipulation of the system. Since the processes of fermentation and product separation are taking place simultaneously, a conceptual understanding of the underlying mechanisms is essential for the design of a successful system (Figure 1.1.1.). ATPS can be successfully used for extractive fermentation as long as the following conditions are satisfied: (i) phase constituents (polymer and/or salts), in the concentration used, should not be toxic or inhibitory to the cells, (ii) phase constituents should not be used by the cells as a substrate, (iii) the physicochemical nature of the phase system like integrity of polymers and rheology, should not be affected by the fermentation process, (iv) cells and limiting substrate must partition wholly or predominantly into one of the phases, (v) feed constituents like mineral salts, substrates like starch, glycerol, must not affect the phase behavior drastically, and finally, (vi) the system must be cost effective in terms of recyclability of polymer and/or salt [4]. There are a huge number of works describing the use of ATPS based in polymer/polymer and polymer/salt systems that have been reviewed in [4, 8]. However, most of polymer-based ATPS display high viscosity [19-21] and normally form opaque aqueous solutions, which could interfere with the quantitative and qualitative analysis of the extracted compounds. During the last few years, a new class of compounds namely Ionic Liquids (ILs) was suggested to replace either the polymers or the salt in ATPS formation and, consequently, in the extractive fermentation as solvents.

1.1.1. IONIC LIQUIDS

Ionic liquids (ILs) are organic salts that are liquid at or near room temperature (by definition they have a melting point below 100 ºC). In contrast to conventional solvents that are constituted by molecules, ILs are constituted exclusively by ions. The crescent interest in ILs has been derived from their unique advantageous properties, such as non-volatility and non-flammability at ambient conditions, versatile solubility, chemical and thermal stabilities [22-25], high ionic conductivity and wide electrochemical potential window [26]. To date, there is a significant number of high-quality papers on IL research activities, from the early ones that generally focused on catalysis [27-34], to current detailed descriptions of specific applications in coordination chemistry [35], analytical chemistry [36], polymer materials [37], nanotechnology [38] and finally as solvents [39, 40]. All these works contribute largely to the rapid rise and stimulation of research interest on ILs. Moreover, the huge number of potential cation and anion structures and their many substitution patterns possibly the design of specific ILs for a specific application with specific properties (tunability of their chemical structures and physical properties) [26, 27, 33]. The estimated number of potential anion/cation combinations available are around to one trillion 10^{12} [41]. The most common cations are imidazolium, pyridinium, piperidinium, pyrrolidinium, ammonium, phosphonium, guanidinium and sulfonium. The anions can be organic or inorganic and the substituent alkyl chains can be represented by small or large chains, simple or functionalized (Figure 1.1.2).

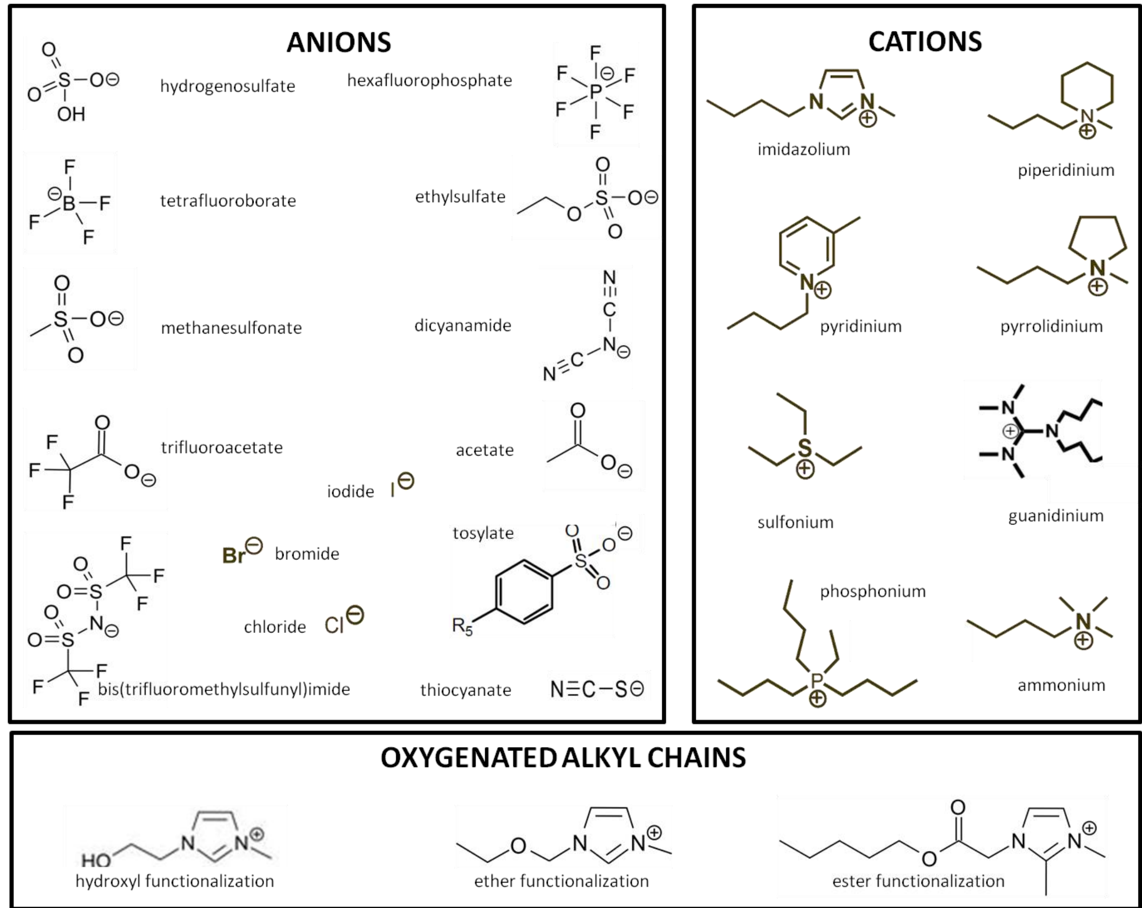

Figure 1.1.2. Illustration of the most common chemical structures of ILs.

One of the emerging research areas using ILs is biocatalysis [2, 20, 42-46]. These ionic compounds were focus of interest in the biocatalysis field due to their capacity to improve the solubility of proteins and enzymes, thus avoiding biocompatibility problems, tailoring the reaction rate and extracting different compounds from the fermentation media [42, 47]. In the extraction field, ILs were used according two different approaches, the direct use of hydrophobic ILs as organic solvent replacements in liquid-liquid separation and the use of IL-based ATPS formed by the addition of salting-out inducing agents to aqueous solutions of hydrophilic ILs. The first exhibits a lower range of possibilities, due to the reduced number of hydrophobic ILs [48, 49]. Moreover, some hydrophobic anions (such as tetrafluoroborate $[BF_4]$, hexafluorophosphate $[PF_6]$, and others) when in aqueous media tend to hydrolyze, causing the formation of acids, which presence in the extraction media is not recommended [50]. The hydrophilic approach on the other hand presents, a huge number of IL possibilities, with different levels of solubility in water [51-56]. These

ATPS separation processes are rich in water, which turns them more environmentally friendly. Nowadays, the hydrophilic IL ATPS approach is one of the most studied both for simple extractions [57-64] as well as to more complex studies of direct extraction of the target products from the fermentation media [65, 66]. A clear example of extractive fermentation using ILs is the production of 2-phenylethanol by *Saccharomyces cerevisiae*, where the use of 1-butyl-3-methylimidazolium bis(trifluoromethylsulfonyl)imide [C_4mim][NTf_2] has allowed the increase of the production of 2-phenylethanol up to five times [67]. Up to now, the imidazolium-based salts are the most widely studied ILs for whole-cell processes, the [PF_6] anion is the most recurrent and *Saccharomyces cerevisiae* the most studied microorganism in extractive fermentations, due to its capacity to synthesize alcohols and ketones of industrial interest, which are important building blocks for the production of flavors, agrochemicals and pharmaceutical products [68]. Beyond these experiments, several other applications followed in this field [69-72]. The reaction systems studied showed that good conversions of up to 80 to 99%, could be achieved using ILs in a biphasic reaction setup. Finally, several authors have suggested the extraction of the target products after fermentation ("*ex situ*" extractive fermentation) by an aqueous phase, aiming to avoid, toxicity [73], biodegradability [74], and biocompatibility [75] issues. For many important organic acids, such as succinic, lactic and acetic acids, which are produced by fermentation, their direct extraction from the fermentation media represents a huge barrier for large scale implementation [76]. However, a process involving these solvents is only possible if the IL is recycled and reused as many times as possible [77]. The number of different approaches around the use of ILs in biocatalysis is increasing [19, 20, 45, 46, 78], leading to the development of studies addressing their performance in biocatalysis [79], their biocompatibility for different macromolecules [80-83] (target products and/or intermediates – Figure 1.1.1.), their extraction/purification performances [84-89], their toxicity for different fermentation producer organisms [66, 90-101] and finally, their environmental and human toxic effects [102-106].

1.1.2. AIMS AND SCOPES

ILs are versatile "green" media for many chemical and engineering processes. ILs can be hydrophobic or hydrophilic depending on the structures of cations and anions. Hydrophilic ILs partially dissociate into ions when mixed with water. These ions are hydrated in aqueous solutions, causing important changes in the properties of the aqueous media. These changes are of great biological importance, due to their influence in the maintenance of intracellular concentrations, the unfolding of proteins, and in the solvation of biomolecules with impacts on the separation processes.

In this context, the importance of a deep study about the performance of different ILs as solvents in specific extractive fermentation processes is crucial. This work reports an integrated study about the influence of the IL' characteristics on the fermentation media. It will be investigated which physicochemical properties are required for these processes, what is their influence in what concerns the recovery of the target and intermediate products, and what damages may these solvents cause when released into the environment, more precisely into aquatic ecosystems. These and other concerns were taken into account in this work that intends to study the use of ILs as alternative solvents for extractive fermentation processes. This document is divided in four major parts, being their interaction sketched in Figure 1.1.3. It is a schematic approach of this thesis and at the same time a simplified representation of the cyclic design for a sustainable extractive fermentation process using ILs as solvents.

The first part of this work - **Chapter 1** addresses the choice for the use of ILs as solvents and also shows the state of art in what concerns the use of those compounds in fermentation processes. It provides an overview about the use of ILs in the various biotechnmological applications including biocatalysis and their use in extractive fermentation processes (so limited until now).

In the second part - **Chapter 2**, some results on the solubility in water of different families of ILs will be reported using a new technique for that quantification. Original data for pyrrolidinium- and piperidinium-based ILs, in the temperature range between 288.15 K and 318.15 K, will be presented. Moreover, the evaluation of this new quantification method and the influence of temperature and IL' cation will be also shown.

In the third part of this work - **Chapter 3**, an extensive evaluation of the aqueous two-phase systems made by a wide range of ILs will be discussed. The ability of different cations, anions and alkyl chain types and lengths for ATPS formation will be addressed. Beyond the evaluation of the effect on the ability to ATPS formation of several IL' features, also different inorganic salts were here addressed. Those ATPS were then used as new alternative separation techniques for the extraction and purification of different biochemicals. These biochemicals comprise the amino-acid L-tryptophan and two enzymes, *Candida antarctica* lipase B here abbreviated as CaLB and an extracellular lipolytic enzyme produced by the bacterium *Bacillus* sp. ITP-001. Those were selected according to their different structural complexity. The enzyme produced by the bacteria *Bacillus* sp. was actually studied concerning the production and purification steps, using a set of different separation techniques, including ILs-based ATPS. In the end, it is also described a set of results concerning the biocompatibility of several ILs towards the lipase CaLB, through several tests on the enzymatic activity of CaLB when it is exposed to various ILs and concentrations.

The fourth part of this thesis - **Chapter 4** will be dedicated to the aquatic (eco)toxicological evaluation of a large range of ILs. **Chapter 4** is divided in two main parts. The first one reports ecotoxicological results for distinct ILs by conjugation of various cations, anions and alkyl chains, using a large range of aquatic species and trophic levels. The second part addresses the bioaccumulation results for some hydrophobic ILs, using the 1-octanol-water distribution coefficient approach.

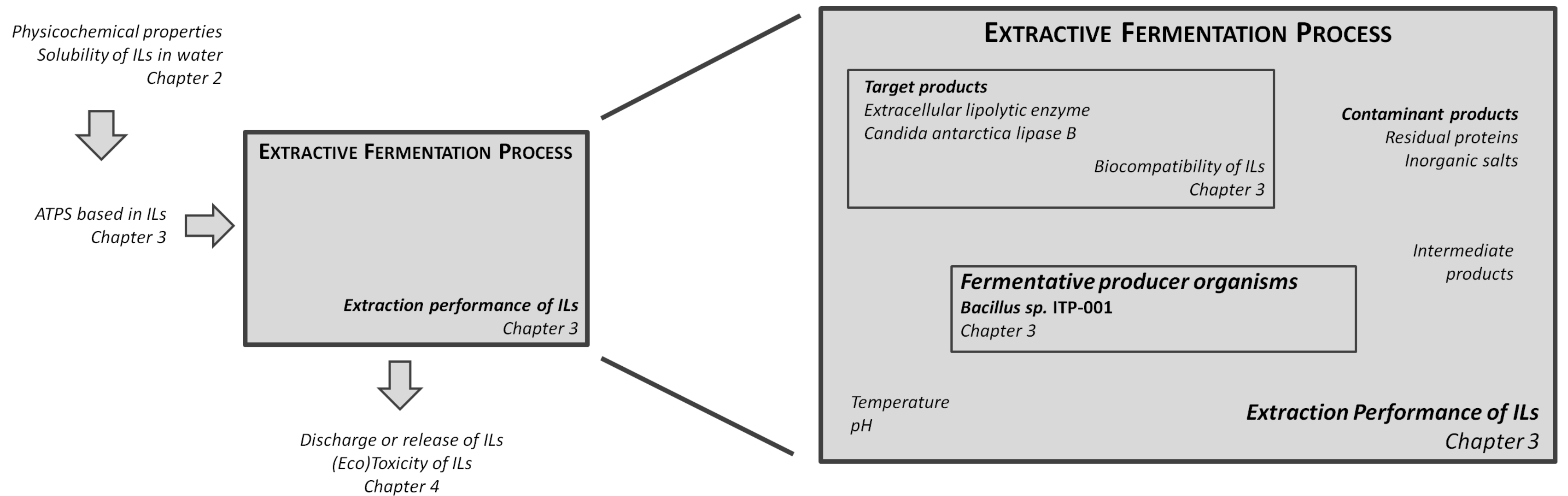

Figure 1.1.3. Illustrative scheme of the main parts of the present thesis.

1.1.3. REFERENCES

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2. SOLUBILITY of ILs IN WATER

The second chapter of this thesis is based on a study addressing the measurement of the solubility of hydrophobic ionic liquids in water using the Electrospray ionization mass spectrometry ESI-MS, a new approach for their quantification. This study appears as a really contribution for this thesis, since the solubility of ILs in water is one of the most important chemical properties for their application in biotechnological processes and in what concerns their environmental impact.

The article here reported was adapted in what concerns the nomenclature of the ILs. Moreover, the QSPR section was removed from the article, since this part was not developed by us.

2.1. PAPER 1

Adapted from

Solubility of Non-Aromatic Ionic Liquids in Water and Correlation Using a QSPR Approach

Fluid Phase Equilibria, 294, 234-240

2.1.1. ABSTRACT

The solubility of ionic liquids (ILs) in water is of significant relevance for both process design and evaluation of their environmental impact. In this work, the solubilities of the non aromatic piperidinium- and pyrrolidinium-based ILs in water, combined with the anion bis(trifluoromethylsulfonyl)imide, were determined in the temperature range from (288.15 to 318.15) K. Electrospray ionization mass spectrometry (ESI-MS) was used as the analytical method after a proper validation of the experimental results obtained. The effect of the ILs structural combinations, such as cation family and alkyl side chain length, in their solubility in water, were analyzed and discussed. From the ILs solubility dependence on temperature, the standard molar thermodynamic functions of solution, namely Gibbs energy, enthalpy and entropy at infinite dilution, were determined. The results indicate that the ILs dissolution in water is an endothermic process and entropically driven.

2.1.2. INTRODUCTION

Ionic liquids (ILs) are low-melting salts that result from the combination of large organic cations with various alkyl substituents and either inorganic or organic anions. Interest in exploring the properties and applications of ILs in a variety of fields, ranging from fundamental science to technology, has been intensified due to their unusual performances and their potential as “green” replacements for the conventional volatile, flammable and toxic organic solvents, as well as the possibility of recycling them, that could lead to a large improvement on processes safety and efficiency [1].

Although ILs are generally referred to as “green” solvents, studies on their toxicity and biodegrability are vital issues. While ILs cannot vaporize leading to air pollution (due to their negligible vapor pressures), all of them present some miscibility with water - even in the limit of infinite dilution - that may be the cause of environmental aquatic concern. The ILs (eco)toxicity can be directly linked to their hydrophilic/lipophilic nature [2-7]. Even though large immiscibility gaps exist between hydrophobic ILs and water, these ILs are indeed more toxic than hydrophilic ones, because of their higher aptitude to accumulate in biological membranes. Therefore, the knowledge of the ILs solubility in water can provide relevant information on the toxicity and bioaccumulation impact of a specific IL in the ecosystem [2-8]. Note, however, that some exceptions to this general relationship exist depending on the aquatic organisms, exposure conditions and

evaluation methods, among others. Recent results from our group show that aromatic ILs are substantially more toxic than non-aromatic ones [9].

Much attention has been dedicated to hydrophilic ILs, yet hydrophobic ILs are gaining considerable relevance in analytical chemistry and separation technology. IL-water two-phase systems have been currently applied in the extraction of low molecular weight neutral compounds, biopolymers and ions, and several reviews on extraction applications using hydrophobic ILs are available [10-14]. In addition, hydrophobic ILs are being explored for potentiometric and voltammetric sensors, and in two-phase organic synthesis [15]. In all approaches the knowledge of the cross contamination between the IL and the second liquid phase is a crucial factor. In spite of the importance of ILs and water liquid-liquid equilibria, only scattered data have been reported [2-4, 16-27]. Moreover, systematic studies for selected ILs, in a wide temperature range, are scarce [2-4]. The solubility in water of the heterocyclic imidazolium- and pyridinium-based ILs has been determined using UV-Vis spectroscopy, since both of these cations present characteristic absorption peaks in the ultraviolet region of the electromagnetic spectrum [2-4]. Nevertheless, for ILs with pyrrolidinium- and piperidinium-based cations the maximum absorption in the UV region occurs at wavelengths lower than 190 nm, due to the absence of π molecular orbitals, requiring a distinct quantification method. Electrospray ionization mass spectrometry (ESI-MS) is a powerful technique for the analysis of positive and negative ions present in solution, and is here proposed as an alternative quantification method for the determination of ILs solubility in water.

The production of gas phase ions from solutions in the electrospray ionization process, involves two main steps: (a) production of charged droplets at a capillary tip and (b) shrinkage of charged droplets due to solvent evaporation and repeated droplet fission leading to gas phase ions (and that can be analyzed in the mass spectrometer). A high voltage (typically 2–3 kV) is applied to a metallic capillary (1mm o.d.) through which the solution is flowing at a flow rate of 10mL.min^{-1}. The strength of the electric field at the capillary tip is typically around 10^6-10^7 V·m^{-1}. In the positive mode, anions migrate in the direction of the capillary, whereas cations migrate in the direction of the counterelectrode. At sufficiently high electric field strengths, a dynamic cone of liquid, a Taylor cone, will form at the tip of the capillary. If the applied field is sufficiently high, the tip becomes unstable and a fine jet emerges from the cone tip. The repulsion between charges causes the jet to break up into smaller droplets. As the solvent evaporates from the droplets with the assistance of warm nitrogen gas, the size of the

droplets decreases until the electrostatic repulsion between the ions becomes equal to the surface tension, and droplet fission occurs. Successive fissions will ultimately lead to the formation of gas phase ions that can be analyzed within the mass spectrometer. A unique feature of this technique is the direct transfer of ions present in solution to the gas phase. Therefore, in this work, the ESI-MS approach was rigorously validated with previous results obtained for the solubility of 1-butyl-3-methylimidazolium hexafluorophosphate in water [3], and subsequently applied in the determination of original solubility data for 1-methyl-1-propylpyrrolidinium bis(trifluoromethylsulfonyl)imide, 1-butyl-1-methylpyrrolidinium bis(trifluoromethylsulfonyl)imide, and 1-methyl-1-propylpiperidinium bis(trifluoromethylsulfonyl)imide, in the temperature range from (288.15 to 318.15) K and at atmospheric pressure. From the results obtained, the impact of the ILs structure, such as the cation family and the alkyl side chain length, were evaluated. Furthermore, from the solubility data dependence on temperature, thermodynamic molar solution properties were determined and discussed.

2.1.3. Experimental Section

The experimental aqueous solubility was performed for the ILs 1-butyl-3-methylimidazolium hexafluorophosphate, $[C_4mim][PF_6]$, 1-methyl-1-propylpyrrolidinium bis(trifluoromethylsulfonyl)imide, $[C_3mpyrr][NTf_2]$, 1-butyl-1-methylpyrrolidinium bis(trifluoromethylsulfonyl)imide, $[C_4mpyrr][NTf_2]$, and 1-methyl-1-propylpiperidinium bis(trifluoromethylsulfonyl)imide, $[C_3mpip][NTf_2]$. The ILs used as internal standards were 1-butyl-3-methylimidazolium tetrafluoroborate, $[C_4mim][BF_4]$, and 1-methyl-3-propylimidazolium bis(trifluoromethylsulfonyl)imide, $[C_3mim][NTf_2]$ - Figure 2.1.1.

1-methyl-3-propylimidazolium bis(trifluoromethylsulfonyl)imide
[C_3mim][NTf_2]

1-butyl-1-methylpyrrolidinium bis(trifluoromethylsulfonyl)imide
[C_4mpyrr][NTf_2]

1-butyl-3-methylimidazolium hexaflurophosphate
[C_4mim][PF_6]

1-butyl-3-methylimidazolium tetrafluoroborate
[C_4mim][BF_4]

1-methyl-1-propylpyridinium bis(trifluoromethylsulfonyl)imide
[C_3mpyrr][NTf_2]

1-methyl-1-propylpiperidinium bis(trifluoromethylsulfonyl)imide
[C_3mpip][NTf_2]

Figure 2.1.1. Chemical structures of ILs studied.

All ILs were acquired at Iolitec within a halides content < 100 ppm. To reduce the water and volatile compounds content to negligible values, ILs individual samples were dried under constant stirring, under vacuum at 0.1 Pa and temperature at 353 K, for a minimum of 48 h. After this procedure, the purity of each IL was additionally checked by ^{1}H, ^{13}C and ^{19}F NMR spectra and found to be >99 wt %. The water used was ultra-pure water, double distilled, passed by a reverse osmosis system and treated with a Milli-Q plus 185 water purification apparatus. The water used presents a resistivity of 18.2 MΩ·cm, a TOC smaller to 5 μg·dm^{-3} and is free of particles >0.22 μm. The methanol used was from Lab-Scan and > 99.9 wt% pure. Solubilities of the ILs in water were carried out at temperatures from (288.15 K to 318.15) K (5 K intervals) and at atmospheric pressure. The IL and water phases (10 cm^{3} of each) were initially vigorously agitated and allowed to reach the mutual saturation by the separation of both phases, in sealed glass vials, for at least 48 h. The temperature was maintained by keeping the glass vials inserted in an aluminium block specially designed for the purpose. The aluminium block was placed in an isolated air bath capable of maintaining the temperature within ± 0.01 K. The temperature control was achieved using a PID temperature controller driven by a calibrated Pt100 (class 1/10) temperature sensor inserted in the aluminium block. In order to reach the temperatures below room temperature a Julabo circulator, model F25-HD, was coupled to the overall oven system

allowing the passage of a thermostatized fluid flux around the aluminium block. The water-rich phases were sampled using glass syringes maintained dry and at the same temperature of the measurements. Samples of ≈ 1.0 g were taken and diluted by a factor of 1:20 (v/v) or 1:50 (v/v) in a 1:1 (v/v) mixture of ultrapure water and methanol containing a previously and fixed amount of internal standard ([C_4mim][BF_4] for [C_4mim][PF_6] quantification and [C_3mim][NTf_2] for [C_3mpyrr][NTf_2], [C_4mpyrr][NTf_2] and [C_3mpip][NTf_2] quantification). The internal standards were also used in the calibration curves determination and at constant concentration. For the calibration curve of [C_4mim][PF_6], a fixed amount (201.6 $mg.dm^{-3}$) of the internal standard [C_4mim][BF_4] was added to each of the six standard solutions of concentrations 50.0, 100.0, 213.6, 320.4, 405.8 and 500.0 $mg.dm^{-3}$ in the solvent mixture CH_3OH:H_2O (1:1, v/v). For the calibration curves of [C_3mpip][NTf_2], [C_3mpyrr][NTf_2] and [C_4mpyrr][NTf_2] a fixed amount (≈250 $mg.dm^{-3}$) of the internal standard [C_3mim][NTf_2] was added to each of the seven standard solutions with concentrations ranging from 50.0 to 800.0 $mg.dm^{-3}$ in the solvent mixture CH_3OH:H_2O (1:1, v/v).

The ILs quantification was determined using a Micromass Quattro LC triple quadrupole mass spectrometer using the calibration curves previously established. The operating conditions of the mass spectrometer were the following: source and desolvation temperatures of 353 K and 423 K, respectively; capillary voltage of 3000 V (or 2600 V for the negative ion mode); and cone voltage of 25 V. N_2 was used as the nebulization gas and the diluted samples (1:1000, v/v) were introduced at a 10 $mL.min^{-1}$ flow rate using the methanol - water (1:1, v/v) mixture as the eluent solvent. For the measurement of peak abundances, an average of 100 scans for each mass spectrum was used. Triplicate independent sampling measurements were performed for both standards and samples.

2.1.4. Results and Discussion

The solubility of [C_4mim][PF_6] in water, using [C_4mim][BF_4] as internal standard, was determined and compared with the results obtained previously using UV-Vis spectroscopy [3] in order to ascertain the reliability of the ESI-MS as an alternative quantification method. The ESI-MS of the ILs solutions in the negative ion mode showed peaks at *m/z* 145 and 87, corresponding to ions [PF_6] (analyte) and [BF_4]

(internal standard), respectively. The relative abundances of ions *m/z* 145 and 87 were measured, in triplicate, and the plot of the abundances ratio as function of the respective concentrations ratio (calibration curve) is provided in *Supporting Information Figure S8.2.1*. The linear dependence, in the concentration range used, is expressed by the fine correlation coefficient of 0.9979.

The water solubility of $[C_4mim][PF_6]$ at the temperatures from (288.15 to 318.15) K, was experimentally determined using $[C_4mim][BF_4]$ as internal standard and ionic abundances measured in three independent aliquots for each temperature. The experimental solubility values thus obtained are presented in Figure 2.1.2, together with the values reported before using the UV-Vis spectroscopic technique [3]. The solubility values obtained by mass spectrometry are, with the exception of the value at 288.15 K, within 5 % of the ones obtained by UV-Vis spectroscopy [3] and no systematic errors were observed. The results obtained by both techniques are in close agreement and reveal the possibility of using an alternative method for the quantification of ILs in aqueous solutions.

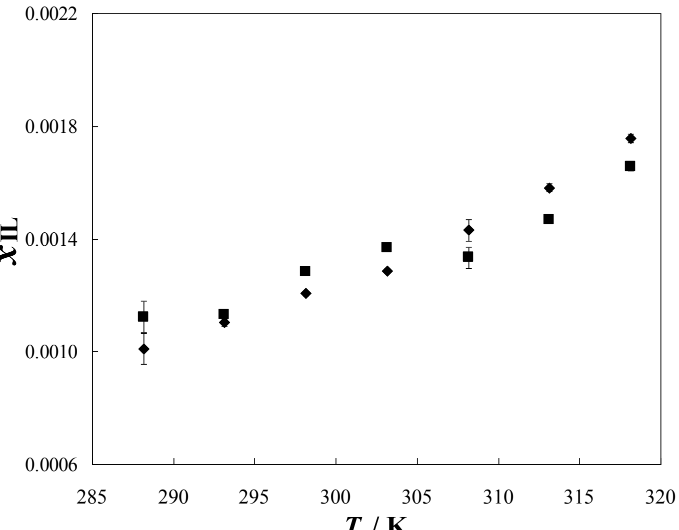

Figure 2.1.2. Mole fraction solubility of $[C_4mim][PF_6]$ in water as a function of temperature determined by: (◆) UV-Vis spectroscopy [3] and (■) ESI-MS (this work).

The experimental procedure and data treatment described previously were further used for the determination of the solubilities of ILs that do not have a significant absorbance in the UV-Vis region, due to lack of aromaticity or insaturation in their cations

(pyrrolidinium and piperidinium). The internal standard chosen was $[C_3mim][NTf_2]$ which shows a peak at *m/z* 125 in the ESI-MS derived from the cation. Note that cluster ions of internal standard and sample ILs, whenever formed, had a relative abundance smaller than 1%, which falls within the standard deviation of the internal standard and sample relative abundances [28]. Calibration curves (plot of the ratio of relative abundances of the cations corresponding to each IL to the internal standard, $[C_3mim]$ with *m/z* 125, as a function of the respective concentrations ratio) for the three ILs studied are presented in *Supporting Information Figure S8.2.2*.

The mole fraction solubilities of studied ILs in water are presented in Table 2.1.1 and Figure 2.1.3. In Figure 2.1.3 experimental data obtained previously [2, 3] for 1-methyl-3-propylimidazolium bis(trifluoromethylsufonyl)imide, $[C_3mim][NTf_2]$, and 3-methyl-1-propylpyridinium bis(trifluoromethylsulfonyl)imide, $[C_3mpyr][NTf_2]$, were also included for comparison purposes. In general, an increase in the solubility of ILs in water was observed with the temperature increase, meaning that these two-phase systems should present an upper critical solution temperature.

Table 2.1.1. Mole fraction solubility of ILs in water, x_{IL}, at different temperatures, and respective standard deviation (σ).

T / K	$[C_4mim][PF_6]$ $10^3 (x_{IL} \pm \sigma)$	$[C_3mpip][NTf_2]$ $10^4 (x_{IL} \pm \sigma)$	$[C_3mpyrr][NTf_2]$ $10^4 (x_{IL} \pm \sigma)$	$[C_4mpyrr][NTf_2]$ $10^4 (x_{IL} \pm \sigma)$
288.15	1.12 ± 0.06	3.42 ± 0.22	4.15 ± 0.03	2.14 ± 0.09
293.15	1.13 ± 0.01	3.59 ± 0.21	4.45 ± 0.08	2.32 ± 0.09
298.15	1.28 ± 0.01	3.68 ± 0.03	4.36 ± 0.04	2.38 ± 0.01
303.15	1.37 ± 0.01	3.86 ± 0.16	4.81 ± 0.20	2.31 ± 0.11
308.15	1.33 ± 0.04	4.18 ± 0.07	4.85 ± 0.03	2.52 ± 0.01
313.15	1.47 ± 0.01	4.51 ± 0.24	5.11 ± 0.05	2.67 ± 0.01
318.15	1.66 ± 0.02	4.63 ± 0.03	5.37 ± 0.03	2.94 ± 0.11

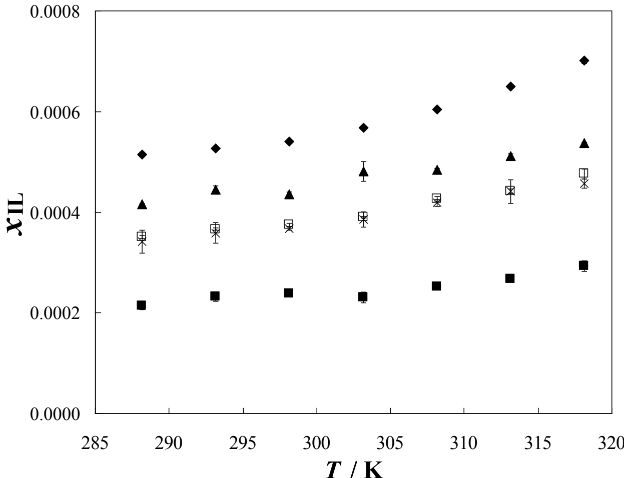

Figure 2.1.3. Mole fraction solubility of ILs in water as a function of temperature: (◆) [C_3mim][NTf_2] [2]; (▲) [C_3mpyrr][NTf_2]; (□) [C_3mpyr][NTf_2] [3]; (×) [C_3mpip][NTf_2]; (■) [C_4mpyrr][NTf_2].

From Figure 2.1.3, the results obtained indicate that the ILs solubility in water at a constant temperature follows the order: [C_4mpyrr] < [C_3mpip] < [C_3mpyr] < [C_3mpyrr] < [C_3mim]. However, the trend observed before for the water solubility in the IL-rich phase [3] was the following: [C_4mpyrr] < [C_3mpip] < [C_3mpyrr] < [C_3mpyr] < [C_3mim]. The differences in solubilities observed in both phases, indicate that the solubility of water in ILs depends largely on the IL availability of electrons for privileged interactions with water. Aromatic ILs present an higher solvation capability for water than aliphatic ones. On the opposite, the solubility of ILs in water seems to be primarily controlled by the cation size, and, to a lower extent, by their aromaticity. Indeed, the sequence obtained for the solubility of ILs in water follows the molar volumes trend of the respective ILs (at 288.15 K) [29]: [C_4mpyrr][NTf_2] with 0.2998 $dm^3 \cdot mol^{-1}$ < [C_3mpip][NTf_2] with 0.2981 $dm^3 \cdot mol^{-1}$ < [C_3mpyr][NTf_2] with 0.2861 $dm^3 \cdot mol^{-1}$ < [C_3mpyrr][NTf_2] with 0.2839 $dm^3 \cdot mol^{-1}$ < [C_3mim][NTf_2] with 0.2729 $dm^3 \cdot mol^{-1}$.

In order to determine the standard molar thermodynamic functions of solution, the experimental solubility of the studied ILs in water was correlated by Eq. (2.1),

$$\ln x_{IL} = M_1 + \frac{M_2}{T/K} + M_3 \ln(T/K) \qquad \text{Eq. 2.1}$$

where M_1, M_2 and M_3 are correlation parameters and x_{IL} is the mole fraction solubility of the IL at each specific temperature, *T*. The correlation parameters and respective standard deviations are presented in Table 2.1.2. The proposed correlation presents a maximum relative deviation from experimental mole fraction solubility data of 4 % and it is useful for determining the ILs solubility in water at temperatures not experimentally available.

Table 2.1.2. Correlation parameters derived from the application of Eq. 2.1 and standard thermodynamic molar properties of solution of ILs in water at 298.15 K (and respective standard deviation, *σ*).

	$[C_3mpip][NTf_2]$	$[C_3mpyrr][NTf_2]$	$[C_3mim][NTf_2]$	$[C_3mpyr][NTf_2]$	$[C_4mpyrr][NTf_2]$
$(A \pm \sigma)$	-127 ± 82	-88 ± 50	-351 ± 20 [2]	-228 ± 77 [3]	-318 ± 189
$(B \pm \sigma)$ (K^{-1})	4604 ± 2990	2990 ± 6174	6365 ± 1011 [2]	9139 ± 3499 [3]	13266 ± 8509
$(C \pm \sigma)$	18 ± 12	12 ± 20	24 ± 3 [2]	33 ± 12 [3]	46 ± 28
$\frac{(\Delta_{sol}H_m^0 \pm \sigma^a)}{kJ \cdot mol^{-1}}$	7.0 ± 1.5	5.8 ± 1.5	5.9 ± 1.5 [2]	6.5 ± 1.5 [3]	5.2 ± 1.5
$\frac{(\Delta_{sol}G_m^0 \pm \sigma^a)}{kJ \cdot mol^{-1}}$	19.60 ± 0.02	19.18 ± 0.02	18.652 ± 0.001 [2]	19.56 ± 0.02 [3]	20.68 ± 0.01
$\frac{(\Delta_{sol}S_m^0 \pm \sigma^a)}{J \cdot K^{-1} \cdot mol^{-1}}$	-42.3 ± 5.1	-44.9 ± 5.1	-42.6 ± 5.0 [2]	-43.9 ± 5.1 [3]	-51.9 ± 5.1

At the equilibrium state the chemical potentials of the IL at the aqueous-rich phase and at the IL-rich phase have to be equivalent, and the electroneutrality of both rich phases must be obeyed. In the current situation, the presence of ILs in the aqueous-rich phase can be considered at infinite dilution, and thus no main solute-solute interactions (electrostatic contribution) and/or ion-pairing subsist, allowing the determination of the standard molar thermodynamic functions of the ILs solution, such as standard molar enthalpy ($\Delta_{sol}H_m^0$ (T)), molar Gibbs energy ($\Delta_{sol}G_m^0$ (T)) and molar entropy ($\Delta_{sol}S_m^0$ (T)) [2, 3]. These associated thermodynamic properties are reported in Table 2.1.2, as well as the respective values for $[C_3mim][NTf_2]$ and $[C_3mpyr][NTf_2]$ for a more global evaluation of the cation influence. In addition, the standard deviations of such properties are provided in Table 2.1.2 and they were determined by the least squares regression

analysis taking into account the standard deviations of the variables involved (*B*, *C* and T). While the molar enthalpies of solution of water in ILs are temperature independent [2, 3] the molar enthalpies of solution of ILs in water are significantly temperature dependent (in the studied temperature range). At 298.15 K, $\Delta_{sol}H_m^0$ indicates that the ILs dissolution is an endothermic process. Moreover, $\Delta_{sol}H_m^0$ shows to be almost independent of both the cation nature and the alkyl side chain length, corroborating thus the results obtained before where it was found that the IL anion nature is the main feature ruling such property [3]. On the other hand, the molar entropies of solution of [C_3mpyrr][NTf_2] and [C_4mpyrr][NTf_2] in water indicate a decrease of approximately – 5 $J \cdot K^{-1} \cdot mol^{-1}$ *per* methylene addition at the cation. Accordingly, the decrease of the ILs solubility in water is driven by the decrease in molar entropy of solution. This decrease in the molar entropy of solution with the cation alkyl side chain length increase was already shown before for imidazolium-based ILs [2, 3] and it is here proven that it is independent of the cation family, occurring also for pyrrolidinium-based ILs. Indeed, the molar entropies of solution are shown to be mainly dependent on the cation alkyl side chain length and on the anion nature [2, 3], while the cation family seems to have no significant influence on such property. Indeed, the negative entropies of solution in combination with the positive Gibbs energies of solution are in agreement with the well known "hydrophobic effect" [30].

2.1.5. CONCLUSIONS

A new pathway for the quantification of ILs solubility in water using ESI-MS was here demonstrated, and original data for pyrrolidinium- and piperidinium-based ILs, in the temperature range between (288.15 and 318.15) K, were presented. The ILs solubility in water decreases with the alkyl side chain length increase at the cation and from piperidinium- to pyridinium- to pyrrolidinium- to imidazolium-based ILs. Contrary to what was observed before for the IL-rich phase [3] where the solubility of water in ILs largely depends on the IL availability of electrons for privileged interactions, the solubility of ILs in water is primarily defined by the cation size, and to a lower extent, on their aromaticity. The standard molar thermodynamic functions of solution, derived from experimental solubility data, indicate that the ILs dissolution in water is an endothermic and entropic driven process.

2.1.6. REFERENCES

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3. AQUEOUS TWO-PHASE SYSTEMS BASED IN ILs AS NEW SEPARATION TECHNIQUES

Advances in biotechnology have opened up numerous possibilities for the large-scale production of many biomolecules that are important for research and industrial applications. The recovery of biomolecules from natural sources and fermentation media is an essential task in biotechnology. The development of new techniques for the separation and purification of biomolecules (amino-acids, steroids, enzymes and proteins, and others) has been of paramount importance in the biotechnological industry. The traditional methods were substituted in so many cases, by liquid-liquid extraction techniques (LLE) with organic solvents due to its simplicity, low costs and easy scale-up. In fact, the purification of biomolecules using LLE has been successfully carried out on a large scale since long. However, these conventional processes consisting in water-organic solvent two-phase systems are generally not suitable for the biomolecules purification due to several issues, such as the biomolecules' inactivation and/or denaturing, and environmental and human health problems, promoted by the use or organic solvents with significant vapor pressures and high levels of toxicology. Since the purification step is one of the most problematic stages in the production of biomolecules, due to their higher costs, and to the environmental and human health problems, and the limited number of solvents available for these processes, the technique has progressed towards the use of ATPS, and recently of ILs and IL-ATPS. The suitability of ILs as "green solvents" for chemical processes has been extensively recognized due to their negligible vapor pressure, which clearly led to their use as replacements for organic solvents and as aqueous two-phase system (ATPS) promoters. The use of ILs in extraction processes follows two different approaches, each with their own advantages and disadvantages. The first is the direct use of hydrophobic ILs as organic replacements in liquid-liquid extraction (LLE). The use of hydrophobic ILs was studied considering mainly the use of fluorinated anions (hexafluorophosphate [PF_6], bis(trifluorosulfonyl)imide [NTf_2] and tetrafluoroborate [BF_4]). Those were already used to the extraction of some biocompounds, such as antibiotics [1] and amino-acids [2, 3]. According to literature [4] and results from our group [5] the use of these extraction systems based in hydrophobic ILs has been discouraged by their high costs and lower partition coefficients. Moreover, it was also pointed out the limited number of hydrophobic ILs, and their high viscosity. Freire and her co-workers [6] also showed that the use of those systems was, from a practical point of view, not really recommended due to the instability of some hydrophobic anions, such as [PF_6], and

$[BF_4]$, particularly in acidic media [5]. So, the direct use of hydrophobic ILs was substituted by the ATPS based in hydrophilic ILs and inorganic salts.

Chapter 3, initially focuses on the study of different hydrophilic ILs and inorganic salts on the formation of ATPS (Papers 2 to 6). As a consequence of the study of the particularities of the ATPS-based in hydrophilic ILs and inorganic salts they have gained particular importance in what concerns their use as new separation/purification techniques for a large set of biochemicals.

The second part of this Chapter shows a number of studies regarding the use of those IL-ATPS on the extraction of different biochemicals, from the structurally less complex compounds amino-acid L-tryptophan (Papers 2 and 3), until structurally more complex macromolecules such as, the lipolytic enzymes (*Candida antarctica* lipase B and the extracellular enzyme produced by the bacterium *Bacillus* sp. ITP-001) - Papers 6 and 7. In the last case, the enzyme was produced and purified using various techniques including IL-ATPS.

Finally, some results concerning the biocompatibility of CaLB through different ILs' features, in particular their effects on the enzymatic activity of the lipase were addressed. Thus, this Chapter reports a compilation of eigth papers produced during this PhD study. Some of those were adapted, in what concerns the abbreviations, to avoid discrepancies and the respective experimental sections, to prevent the duplication of information.

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3.1. PAPER 2

Evaluation of Cation Influence on the Formation and Extraction Capability of Ionic Liquid-based Aqueous Two-Phase Systems

Journal of Physical Chemistry B, 2009, 113, 5194-5199

3.1.1. ABSTRACT

In addition to the large range of applications proposed in literature, ionic liquids (ILs) have been recently reported to be able to form aqueous two-phase systems (ATPS). They could thus be interesting media in biotechnological applications for the separation and purification of vital biomolecules. Therefore, in this work, a systematic study involving a large number of imidazolium-based ILs was conducted to provide new information related to the ILs' ATPS-promoting capability and extraction ability. For that purpose, the influence of the number of alkyl groups present at the cation, the cation side alkyl chain length, and the presence of double bonds, aromatic rings and hydroxyl groups on this alkyl chain were evaluated. Ternary phase diagrams of the ATPS formed by these ILs and K_3PO_4 and the respective tie-lines were measured and presented. The ATPS here investigated were further characterized for the first time, accordingly to their extractive potential for amino-acids, where L-tryptophan was selected as a model biomolecule. The partition coefficients here obtained shown to be substantially larger than those observed in conventional ATPS, demonstrating therefore the fine potential of the IL-based ATPS for biomolecules separation and purification.

3.1.2. INTRODUCTION

The efficiency and viability of any biotechnological process depends largely on downstream processing that ensures the purity and quality of biomolecules and represents about 60 - 90 % of the cost of the final product [1]. Many metabolites and/or bioproducts present narrow tolerance limits of pH, ionic strength, temperature, osmotic pressure, and surface charges; thus, the extraction and isolation techniques must be specific and compatible with the product [2]. Conventional techniques used for product recovery from biotechnological processes are usually expensive and present low yields [3]. There have been, therefore, considerable efforts from the industrial and academic communities for the development of cost-effective separation techniques [4], such as liquid-liquid extraction in aqueous two-phase systems (ATPS). These systems are formed when two mutually incompatible, though both miscible with water, polymer/polymer, polymer/salt, or salt/salt systems are employed. Above a critical concentration of those components, spontaneous phase separation takes place, and the extraction of biomolecules can be achieved by the manipulation of their affinity for each of the aqueous-rich phases.

Water-immiscible organic solvents have long been commonly used in industrial applications. Nevertheless, environmental concerns about the use of volatile organic compounds has increased in the last few years, and there is an emergent interest for the development of "green" solvents for separation processes [5]. In this context, ionic liquids (ILs) have appeared as possible nonhazardous candidates. Their particular characteristics, including high solvation abilities and coordination properties, general inflammability, high thermal and chemical stabilities and negligible vapor pressures [6], make them suitable candidates for a large range of industrial and biotechnological applications. Moreover, the possibility of controlling their inherent physicochemical properties by a wise combination of the cation and/or anion makes possible the manipulation of the extraction phase properties for enhanced yield of product recovery. In addition, ILs-based ATPS offer the opportunity to combine the purification process of active biocatalysts with the improved performance of some enzymes in the presence of ionic media [7].

The first suggestion that ILs could be used to prepare ATPS was reported by Gutowski et al. [8] The potential advantages of factual IL-based ATPS have motivated previous studies on the interactions between water and ILs, and between ILs and salts in aqueous solutions, with the goal of achieving a deeper understanding of the molecular phenomena governing the IL-based ATPS scenario [9-12]. Despite the scattered results that have been reported concerning IL-based ATPS, there are still many gaps in the characterization of ILs, and a general picture of the situation that would allow the finest selection of the IL is still lacking. Research regarding the use of IL-based ATPS has been so far mostly centered on the influence of several inorganic salts on the phase diagrams [4, 5, 8, 13, 14] (where the ion's influence follows the well known Hofmeister series) [10] or in the use of carbohydrates for IL-based ATPS formation [15-19]. Surprinsingly, one of the most interesting practical issues, evaluation of the extraction ability of those IL-based ABS, was seldom studied; only testosterone, epitestosterone, penicillin G, and opium alkaloids were previously used as partitioning solutes [20-22] No reports concerning the extraction of amino-acids or even proteins using IL-based ABS were found in literature.

In this work we evaluate the influence of cations on promoting IL-based ATPS, maintaining the same inorganic salt (K_3PO_4). Ternary phase diagrams (binodal curves and tie-lines) for systems of hydrophilic ILs + K_3PO_4 + water, at 298 K and atmospheric pressure, were determined. The binodal curves were fitted to a three-parameter equation

and the tie-lines were estimated using the Merchuck mathematical approach [23]. The main objective of this study is to establish the impact of different characteristics of the cation's structure on promoting ATPS, such as the alkyl side chain length, the number of substituents at the cation, and the presence of double bonds, benzyl groups, or hydroxyl groups. The ILs selected were hydrophilic imidazolium- and chloride-based ILs. It is known that the anion has a small effect in the ILs toxicity (although chloride-based ILs are non-fluoride candidates and therefore less toxic) [24]. The ILs toxicity is mainly determined by the imidazolium cation and directly correlates with the length of the side alkyl chain and/or with the hydrophobicity of the IL [24]. Thus, the studied ILs are the less toxic at the imidazolium-based family since this study focused essentially in hydrophilic and short side alkyl chain length ILs.

The revised ILs were further analyzed accordingly to their potential biomolecule extractive ability, for which L-tryptophan was selected as a model compound of biotechnological interest. Amino-acids are important compounds in several biotechnological processes, and the development of methods for their separation and purification still is a focal dilemma.

3.1.3. Experimental Section

3.1.3.1. Materials

The ATPS studied in this work were established by using an aqueous solution of K_3PO_4 $\geq$ 98 % w/w pure from Sigma, and different aqueous solutions of hydrophilic ILs. The chloride-based ILs studied were imidazolium chloride, [im]Cl; methylimidazolium chloride, [C_1im]Cl; ethylimidazolium chloride, [C_2im]Cl; 1,3-dimethylimidazolium chloride, [C_1mim]Cl; 1-ethyl-3-methylimidazolium chloride, [C_2mim]Cl; 1-butyl-3-methylimidazolium chloride, [C_4mim]Cl; 1-hexyl-3-methylimidazolium chloride, [C_6mim]Cl; 1-allyl-3-methylimidazolium chloride, [amim]Cl; 1-hydroxyethyl-3-methylimidazolium chloride, [OHC_2mim]Cl; and 1-benzyl-3-methylimidazolium chloride, [C_7H_7mim]Cl. The molecular structures of the ILs are described in Figure 3.1.1.

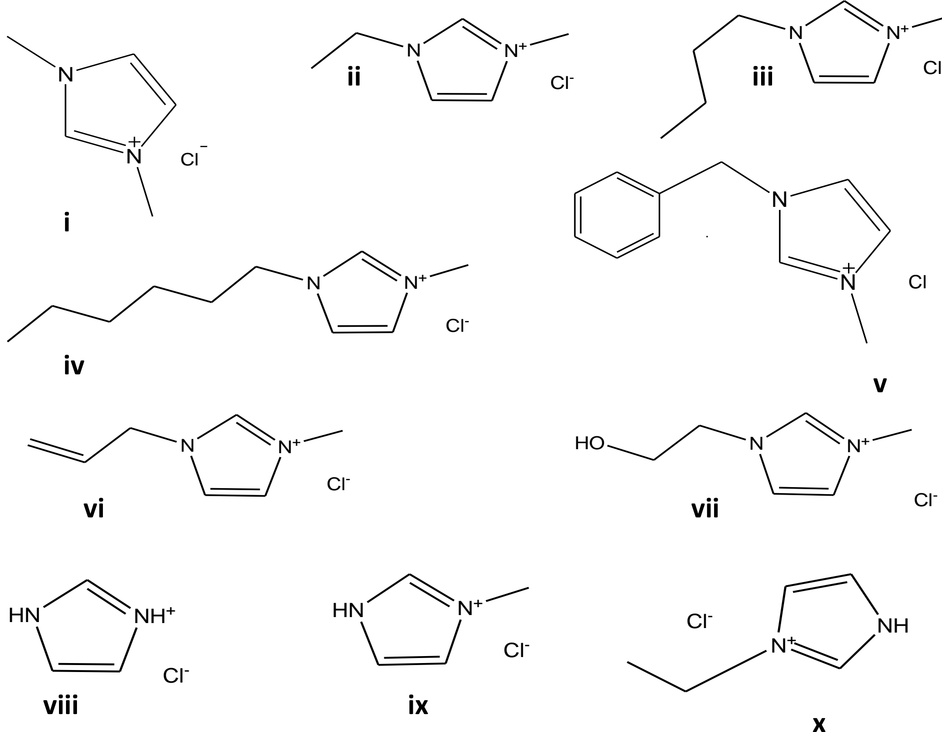

Figure 3.1.1. Chemical structure of the ILs studied: (i) [C_1mim]Cl; (ii) [C_2mim]Cl; (iii) [C_4mim]Cl; (iv) [C_6mim]Cl; (v) [C_7H_7mim]Cl; (vi) [amim]Cl; (vii) [OHC_2mim]Cl; (viii) [im]Cl; (ix) [C_1im]Cl; (x) [C_2im]Cl.

All ILs used in this work were acquired at Iolitec with the exception of [C_1mim]Cl, which was synthesized in our lab (see *Supporting Information Section 8.3.1*). To reduce the water and volatile compound content to negligible values, individual samples of the ILs were dried under constant stirring at moderate vacuum and temperature (≈ 353 K) for a minimum of 48 h. After this procedure, the purity of each IL was further checked by ^{1}H and ^{13}C NMR spectra and found to be > 99.0 % w/w for all samples. The water used was ultrapure, double distilled water, passed through a reverse osmosis system and further treated with a Milli-Q plus 185 water purification apparatus. The L-tryptophan (Figure 3.1.2) with a purity > 99.0 % w/w was from Fluka.

Figure 3.1.2. Molecular structure of L-tryptophan.

3.1.3.2. Phase Diagrams and Tie-Lines

The phase diagram binodals were determined through the cloud point titration method [25, 26] at 298 ± 1 K. The experimental procedure adopted was validated with the phase diagram obtained for the $[C_4mim]Cl$ + water + K_3PO_4 ternary system against literature data [27]. Aqueous solutions of K_3PO_4 at 40 wt % and aqueous solutions of the different hydrophilic ILs at variable concentrations were prepared and used for the phase diagrams binodal determinations. Repetitive dropwise addition of the aqueous inorganic salt solution to the aqueous solution of IL was carried until detection of a cloudy solution, followed by the dropwise addition of ultrapure water until the detection of a monophasic region (clear and limpid solution). Dropwise additions were carried under constant stirring. The ternary system compositions were determined by the weight quantification of all components added within an uncertainty of $\pm 10^{-4}$ g.

The tie-lines (TLs) were determined by a gravimetric method described by Merchuck' group [23]. For the TLs determination a mixture at the biphasic region was prepared, vigorously stirred and allowed to reach the equilibrium by the separation of both phases for 12 h at 298 K using small ampules (ca. 10 mL) especially designed for the purpose. After the separation step, both top and bottom phases were weighed. Each individual TL was determined by application of the lever rule to the relationship between the top mass phase composition and the overall system composition [23]. For that purpose the experimental binodal curves were correlated using Eq. 3.1, [23]

$$Y = A\exp\left[\left(BX^{0.5}\right) - \left(CX^{3}\right)\right] \quad \text{Eq. 3.1}$$

where Y and X are, respectively, the IL and salt weight percentages, and A, B, and C are constants obtained by the regression.

3.1.3.3. Partitioning of L-tryptophan

The partition coefficients of L-tryptophan, K_{Trp}, are defined as the ratio of the concentration of L-tryptophan in the IL and in the K_3PO_4 aqueous-rich phases, described by Eq. 3.2,

$$K_{Trp} = \frac{[Trp]_{IL}}{[Trp]_{K3PO4}} \quad \text{Eq. 3.2}$$

where $[Trp]_{IL}$ and $[Trp]_{K3PO4}$ are the concentration of L-tryptophan in the IL and in the K_3PO_4 aqueous-rich phases, respectively.

A mixture in the biphasic region was selected and used to evaluate the L-tryptophan partitioning at 298 K (the mixture composition is described in Table 4.1.1. for each system). For this purpose aqueous solutions of L-tryptophan with a concentration of approximately 0.78 $g \cdot dm^{-3}$ were used. The biphasic solution was left to equilibrate for 12 h (a time period established in previous optimizing experiments) to achieve a complete L-tryptophan partitioning between the two phases. The amino-acid quantification, in both phases, was carried by UV spectroscopy using a SHIMADZU UV-1700, Pharma-Spec Spectrometer, at a wavelength of 279 nm and using calibration curves previously established. At least, three samples of each individual aqueous phase were quantified. Moreover, both phases were weighed, and the corresponding TLs were obtained as previously described.

3.1.4. RESULTS AND DISCUSSION

3.1.4.1. Phase Diagrams and Tie-Lines

Because ILs are ionic by nature, the IL-based ATPS are more complex than typical poly(ethylene glycol) (PEG)-based ATPS due to the possibility of ion exchange and/or ion-pairing between both salt phases. Bridges et al. [13] have shown that there is ion partition between both phases, yet electroneutrality is maintained. However, the overall deviations observed are small enough to be considered as a source of error in the cloud point titration (as determined by radioanalytical results), thus yielding a satisfactory representation of the ion concentration present at any TL [13].

The experimental phase diagrams at 298 K and at atmospheric pressure for each IL + K_3PO_4 + water system are presented in Figures 3.1.2 and 3.1.3 in molality units, for a

detailed understanding of the ILs impact on the ATPS formation (see *Supporting Information* for experimental weight fraction data – *Tables S8.3.1 to S8.3.7*). Figure 3.1.2 presents the binodal curve for the ILs [amim]Cl, [OHC$_2$mim]Cl, [C$_7$H$_7$mim]Cl, [C$_6$mim]Cl, [C$_4$mim]Cl, [C$_2$mim]Cl and [C$_1$mim]Cl, and Figure 3.1.3 shows the binodal curve data for [C$_2$im]Cl, [C$_1$im]Cl, and [im]Cl.

The observation of Figure 3.1.3 indicates that the larger cation alkyl chain, the greater is the IL's ability for ATPS formation. It is well-known that an increase in the cation alkyl chain length leads to an increase of the IL's hydrophobic nature and therefore to a poorer affinity for water [11].

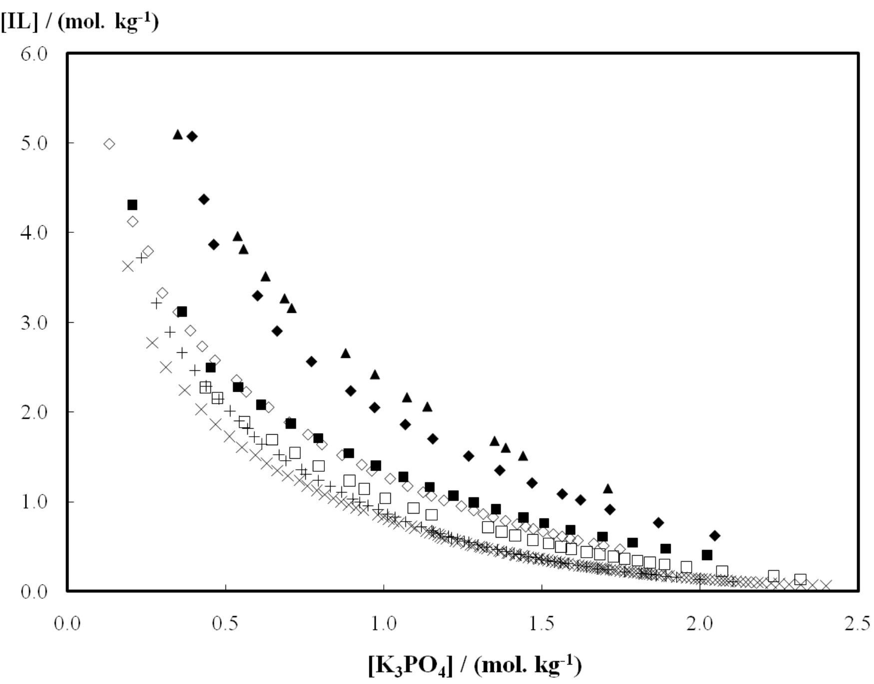

Figure 3.1.3. Phase diagrams for the disubstituted imidazolium-based ternary systems composed of IL + K_3PO_4 + H_2O at 298 K: (♦) [C$_1$mim]Cl; (■) [C$_2$mim]Cl; (□) [C$_4$mim]Cl; (×) [C$_6$mim]Cl; (+) [C$_7$H$_7$mim]Cl; (◊) [amim]Cl; (▲) [OHC$_2$mim]Cl.

The higher the affinity for water and/or hydrophilic nature of the IL, the less effective is the IL in promoting ATPS. Moreover, the presence of a terminal hydroxyl group at the alkyl chain leads to a large decrease in the ATPS-promoting ability as a result of the higher hydrophilicity of the IL when compared with [C$_2$mim]Cl. Also the presence of a double bond at the allyl group of the imidazolium cation ([amim]Cl) or the presence of a benzyl group ([C$_7$H$_7$mim]Cl) as a substituent group decreases the ability to form ATPS, although in a less pronounced way.

When a second electrolyte is added to a non-electrolyte or electrolyte aqueous solution, the solubility of the solute usually decreases as a consequence of the salting-out effect. In general, ILs with lower water affinity require less salt to promote separation of the two phases, resulting in a binodal curve closer to the axis and in a larger biphasic region. The results here obtained show that the ability of the ILs to form ATPS follows the order: [C_6mim]Cl > [C_7H_7mim]Cl > [C_4mim]Cl > [C_2mim]Cl ≈ [amim]Cl > [C_1mim]Cl > [OHC_2mim]Cl.

Figure 3.1.4 illustrates the experimental binodal curves obtained for the mono- and un-substituted imidazolium chlorides. These systems reveal an "atypical" behaviour for ATPS involving ILs and not previously reported. The asymmetrical behaviour of these aqueous biphasic systems has been previously observed for ATPS of PEG and low weight polysaccharides [28, 29], and they may constitute an interesting approach for product separations. For the same K_3PO_4 molality, two monophasic and one biphasic regions are present, and thus the system can be moved between the various phases by simple variation of the IL concentration. Comparing the three ILs, the ATPS-forming ability ranks from [C_2im]Cl > [C_1im]Cl > [im]Cl.

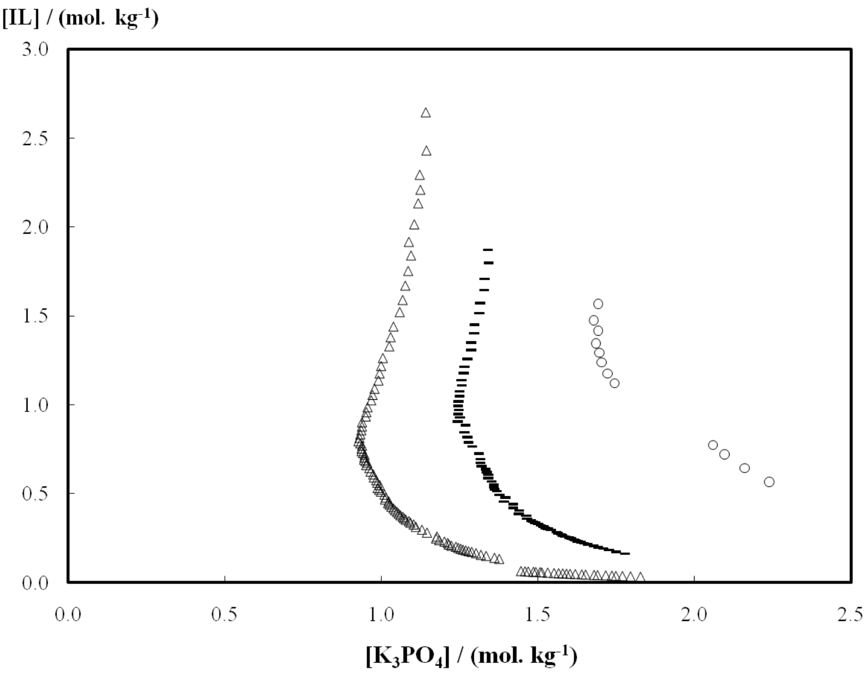

Figure 3.1.4. Phase diagrams for the un- and mono-substituted imidazolium-based ternary systems composed of IL + K_3PO_4 + H_2O at 298 K: (o) [im]Cl; (-) [C_1im]Cl; (Δ)[C_2im]Cl.

The number of alkyl substituents and again the alkyl chain length increase leads to a higher ATPS-inducing capacity. Curiously, comparing the results reported in Figures 3.1.3 and 3.1.4, and depicted in Figure 3.1.5, there are specific ranges of IL concentration where the monosubstituted imidazolium-based ILs are more effective in promoting phase separation than their disubstituted analogues.

In PEG-based ATPS the salt anion has the major impact on the polymer solubility and hence in the ATPS phase diagrams, while the cation has a minor (though sizable) effect [30]. For ILs, it is here shown that the IL cation has a huge influence in the phase diagrams behaviour, allowing the IL-based ATPS to be tailored to meet the specific requirements of a particular separation.

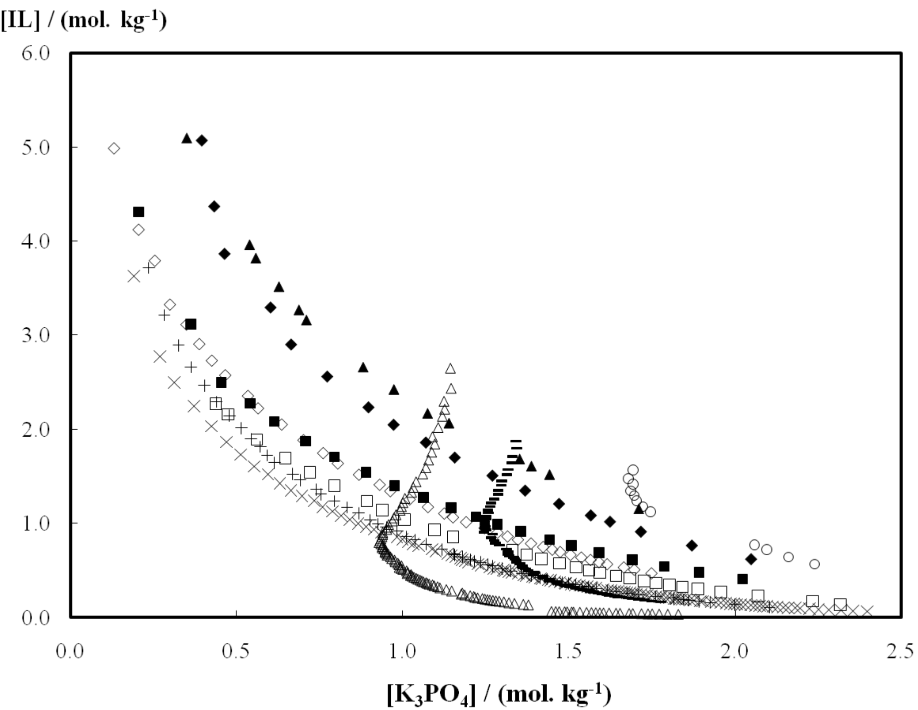

Figure 3.1.5. Phase diagram for all the studied imidazolium-based ternary systems composed of IL + K_3PO_4 + H_2O at 298 K: (o) [im]Cl; (-) [C_1im]Cl; (Δ) [C_2im]Cl; (♦) [C_1mim]Cl; (■) [C_2mim]Cl; (□) [C_4mim]Cl; (×) [C_6mim]Cl; (+) [C_7H_7mim]Cl; (◊) [amim]Cl; (▲) [OHC_2mim]Cl.

The experimental binodal curves were fitted to the empirical relationship described by Eq. 3.1 [23]. The regression parameters and the tie-line equations obtained for each ternary system, as well as the tie-line lengths (TLLs), are reported in Tables 3.1.1 and 3.1.2, respectively.

Table 3.1.1. Correlation parameters used in Eq. 3.1 to describe the binodals.

IL + K_3PO_4 + water	A	B	C
[C_1mim]Cl	109.9	-0.3735	1.991×10^{-5}
[C_2mim]Cl	78.25	-0.3389	2.697×10^{-5}
[C_4mim]Cl	72.64	-0.3185	4.070×10^{-5}
[C_6mim]Cl	84.02	-0.3563	5.462×10^{-5}
[C_7H_7mim]Cl	94.67	-0.3545	6.128×10^{-5}
[amim]Cl	72.00	-0.2924	4.088×10^{-5}
[OHC_2mim]Cl	103.7	-0.3067	1.004×10^{-5}

Table 3.1.2. Experimental data for the Tie Lines (TLs) and respective Tie Line Lengths (TLLs).

Ionic Liquid	Mass fraction / wt %						TLL
	Y_M	X_M	Y_T	X_T	Y_B	X_B	
[C_2mim]Cl	24.88	20.85	47.16	2.23	1.54	40.36	59.46
	26.30	14.93	37.70	4.61	2.84	36.18	47.03
[C_4mim]Cl	15.75	22.67	37.13	4.39	2.19	34.28	45.97
	17.69	21.55	38.18	4.05	1.92	35.01	47.67
	21.36	23.04	49.51	1.45	0.95	38.68	61.20
[C_1mim]Cl	25.20	16.55	35.42	8.96	9.93	27.89	31.75
[C_6mim]Cl	10.78	26.04	39.85	4.33	1.55	32.92	47.79
	15.16	25.33	49.32	2.23	1.00	34.90	58.32
[amim]Cl	26.64	16.60	43.71	2.90	1.63	36.67	53.95
	26.80	21.20	51.18	1.36	0.45	42.63	65.40
[OHC_2mim]Cl	41.17	15.36	22.52	21.62	85.71	0.39	66.66
[C_7H_7mim]Cl	20.17	20.05	47.60	3.73	2.25	30.72	52.78
	17.56	26.09	57.26	2.01	0.58	36.39	66.29

Representation of TLs is shown in Figure 3.1.6 for the ternary system $[C_4mim]Cl$ + K_3PO_4 + water as an example measured in this work. For shorter TLLs the TLs are approximately parallel, whereas for longer TLLs the tie-lines slopes start to deviate. These deviations in the TLs slopes are in agreement with literature [31] and are related with the fact that the K_3PO_4-rich phase is increasingly free of IL at longer TLLs.

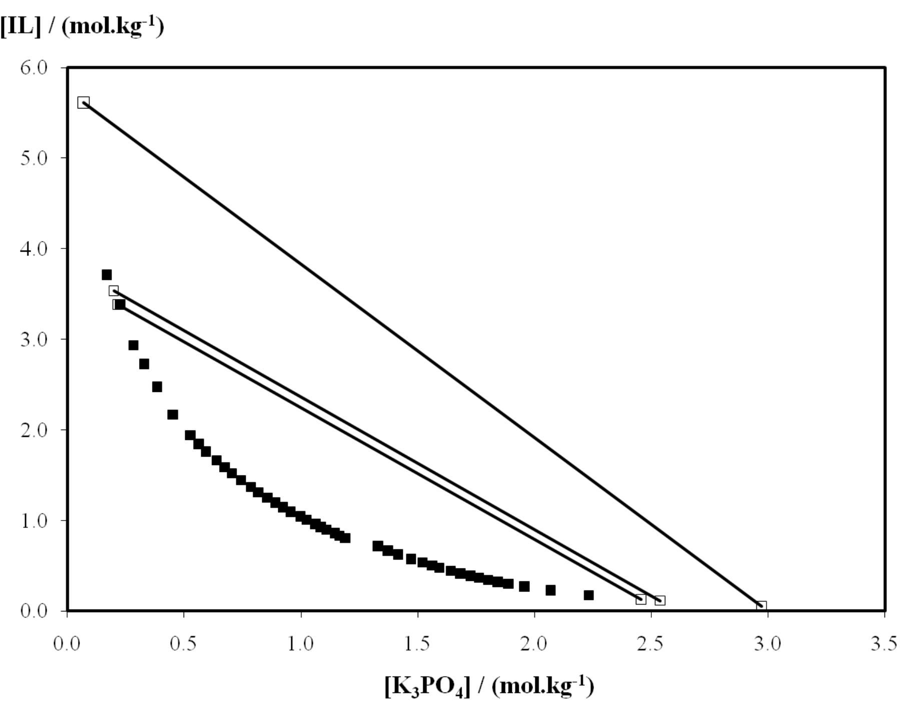

Figure 3.1.6. Phase diagram for the $[C_4mim]Cl$ + K_3PO_4 + water ternary system at 298 K: (■) binodal curve data; (□) TL data.

3.1.4.2. Partitioning of L-tryptophan in ATPS

"Hydrophobic" interactions play a major role in the tertiary structure of proteins and biological membranes and control the partition coefficients in ATPS. The partition coefficients of biomolecules also depend on electrostatic forces, molecular size, solubility, and affinity for both phases, and their magnitudes further depend on the two-phase compositions and on the nature of the biomolecules [32].

As shown in Figure 3.1.7, the partition coefficients of L-tryptophan (K_{Trp}) at 298 K in IL-based ATPS are substantially higher than in usual PEG-polysaccharide systems (with $K_{Trp} \approx 1$) [33] or PEG-inorganic salts systems (with $K_{Trp} \approx$ 1 to 7) [34].

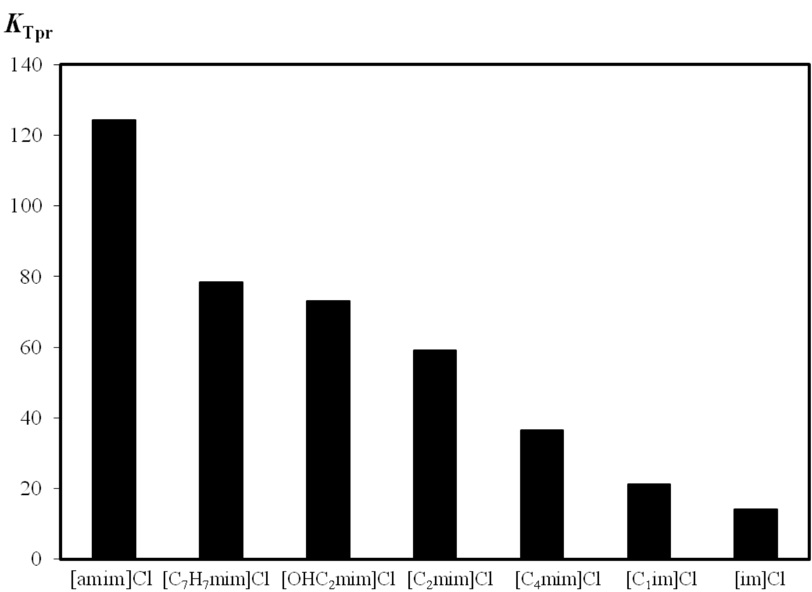

Figure 3.1.7. Partition coefficients of L-tryptophan between the IL and the K_3PO_4-rich aqueous phases at 298 K.

The IL-based ATPS are thus far more efficient for L-tryptophan separation than conventional ATPS. Table 3.1.3 presents the composition of the ternary system employed for the L-tryptophan partition studies, as well as the L- tryptophan oartition coefficient results.

Table 3.1.3. Mass fraction composition and partition coefficients of L-tryptophan in ILs-ATPS systems at 298 K.

Ionic Liquid	Mass fraction / wt %		K_{Trp}
	Y_M	X_M	
[amim]Cl	26.91	15.91	124 ± 5
[OHC$_2$mim]Cl	40.57	16.05	73.1 ± 0.8
[C$_2$mim]Cl	25.90	14.90	59.2 ± 0.4
[C$_4$mim]Cl	25.35	15.97	36.6 ± 0.6
[C$_7$H$_7$mim]Cl	25.11	18.30	78.4 ± 0.5
[C$_1$im]Cl	15.36	30.53	21.3 ± 0.3
[im]Cl	15.24	32.80	14.2 ± 0.4

During the partitioning of L-tryptophan between the two phases there are several competing interactions between the IL, the inorganic salt, the L-tryptophan and water: hydrogen-bonding interactions, $\pi \cdots \pi$ interactions, "hydrophobic" interactions, and electrostatic interactions. In general, the results indicate that K_{Trp} increases with the IL cation's hydrophilic nature and thus the main interactions between the IL and the amino-acid determining L-tryptophan partitioning are "hydrophobic" and hydrogen-bonding type interactions. For the studied ILs the L-tryptophan partition coefficient follows the rank [amim]Cl > [C_7H_7mim]Cl > [OHC_2mim]Cl > [C_2mim]Cl > [C_4mim]Cl > [C_1im]Cl > [im]Cl. The highest partition coefficient occurs for the [amim]Cl followed by [C_7H_7mim]Cl and [OHC_2mim]Cl as a result of the presence of a double bond and a hydroxyl group at one of the alkyl side chains, respectively. Increasing the IL cation alkyl chain decreases the L-tryptophan partitioning in the disubstituted imidazolium-based ILs. The mono- and unsubstituted imidazolium-based ILs present lower partition coefficients for L-tryptophan, which could result from the decrease of the "hydrophobic" interactions existence between them.

Wang and co-workers [35] reported the extraction of amino-acids from an aqueous phase using hydrophobic water-immiscible ILs. The results obtained in this work show that the magnitudes of L-tryptophan partition coefficients using IL-based ATPS can be as much as 1000 times larger compared to the water-immiscible ILs results. Indeed our results show that nearly quantitative extraction of L-tryptophan can be achieved in a single-step extraction procedure. In addition to the higher affinity of hydrophilic ILs for L-tryptophan, another factor should be taken into account. In IL-ATPS the presence of an inorganic salt also leads to the amino-acid salting-out from the aqueous phase, further enhancing the distribution ratio of L-tryptophan. This was previously shown by Fan et al. [36] with the extraction of endocrine-disrupting phenols by the use of hydrophobic ILs in the absence and presence of inorganic salts. However, the effect of the IL on the extraction ability of these systems can be gauged from the results of Salabat et al. [34] where a conventional PEG-based ATPS was used for L-tryptophan extraction with much lower partition coefficients ($K_{Trp} \approx 1$ to 7). The high partition coefficients obtained with IL-based ATPS for the extraction of L-tryptophan show that these systems may be a successful and clean approach for biomolecules separation and purification in biotechnological processes.

3.1.5. CONCLUSIONS

The problems of employing traditional solvents for the separation of biomolecules in biotechnological processes are driving the exploration of ILs as new promising extraction media. The ability of ILs to form salt–salt ATPS allows for hydrophilic ILs to be used in liquid-liquid extractions, and new phase equilibrium data for systems involving hydrophilic imidazolium-based ILs + K_3PO_4 + water have been presented.

The tunability of IL-based ATPS systems was demonstrated by the evaluation of the IL cation influence in promoting ATPS. The results showed that IL-based ATPS can be obtained over a large range of concentrations for both ILs and the inorganic salt. The ability of imidazolium-based ILs for aqueous phase separation was shown to follow the order [C_6mim]Cl > [C_7H_7mim]Cl > [C_4mim]Cl > [C_2mim]Cl ≈ [amim]Cl > [C_1mim]Cl > [OHC_2mim]Cl and [C_2im]Cl > [C_1im]Cl > [im]Cl. The results obtained indicated that the IL cation has a significant influence in the behaviour of the binodal curves and in the promotion of ATPS. Increasing the alkyl chain length (for mono or disubstituted ILs) increases the phase separation ability, whereas the insertion of a double bond, a benzyl group or an hydroxyl group leads to a decrease of ATPS promotion capability.

The capacity of the IL-based ATPS as extraction media was demonstrated with the high L-tryptophan partitioning coefficients obtained. It was shown that depending on the nature of the IL used the partition coefficients can vary between 10 and 120. The ability of ILs to extract L-tryptophan increases with the cation hydrophilicity. These values are substantially higher than those obtained with conventional PEG-based ATPS or with water-immiscible IL two-phase extractions.

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3.2. PAPER 3

Evaluation of Anion Influence on the Formation and Extraction Capacity of Ionic-Liquid-based Aqueous Two-Phase Systems

Journal of Physical Chemistry B, 2009, 113, 9304-9310

3.2.1. ABSTRACT

Extractive fermentation using aqueous two-phase systems (ATPS) is a promising separation process since it provides a nondenaturing environment for biomolecules and improves the stability of cells. Due to environmental concerns and toxicity issues related with common volatile organic solvents, ionic liquids (ILs), a new class of non-volatile alternative solvents, are being currently investigated for extraction purposes. In this work, a wide range of imidazolium-based ILs was studied aiming at obtaining new insights regarding their ability towards the formation of ATPS and their capacity to the extraction of biomolecules. On the basis of the IL cations 1-ethyl-3-methylimidazolium and 1-butyl-3-methylimidazolium, the IL anion influence on ATPS formation was assessed through their combination with chloride, bromide, acetate, hydrogensulfate, methanesulfonate, methylsulfate, ethylsulfate, trifluomethanesulfonate, trifluoroacetate and dicyanamide. Ternary phase diagrams (and respective tie-lines) formed by these hydrophilic ILs, water and the inorganic salt K_3PO_4, were measured and are reported. The results indicate that the ability of an IL to induce ATPS closely follows the decrease in the hydrogen bond accepting strength or the increase in the hydrogen bond acidity of the IL anion. In addition, the extraction capacity of the studied ATPS was evaluated through their application to the extraction of an essential amino acid, L-tryptophan. It is shown that the partition coefficients obtained between the IL and the K_3PO_4-aqueous rich phases were substantially larger than those typically obtained with polymers-inorganic salts or polymers-polysaccharides aqueous systems.

3.2.2. INTRODUCTION

Fermentation processes are often inhibited by the accumulation of products in the bioreactor. The incorporation of a primary step in fermentation for product separation can enhance the product yield and facilitate the downstream processing. Among the different approaches for integrating reaction and product recovery steps in a biological process, the liquid-liquid extraction has shown a great potential [1, 2]. Several aspects of extractive fermentation, as well as general approaches for two-phase bioreactor systems have been discussed in a recent review paper [3]. The liquid-liquid extraction process presents additional advantages over conventional techniques, overcoming the lower efficiency in product recovery and the increased effluent treatment costs as a result of

using a more concentrated feedstock [4]. Liquid-liquid extraction techniques present higher capacity, better selectivity and allow the integration between recovery and purification [4].

Before the Montreal Protocol [5], volatile organic compounds were commonly applied as extractive solvents due to their immiscibility with aqueous medium [6]. After a reconsideration of the environmental impact of these processes, a new class of solvents, ionic liquids (ILs), has emerged in literature showing to be capable of forming aqueous two-phase systems (ATPS) in the presence of an inorganic salt [7]. ILs negligible volatility [8, 9], low flammability [10], thermal stability [11, 12] and, in some cases, biocompatibility contributed to their recognition as ambient-friendly media [13]. In addition, the stability, activity, selectivity and enantioselectivity of a particular enzyme is strongly dependent on the IL nature, and therefore, those properties can be finely tuned by correctly choosing the appropriate cation and/or anion of the IL. Indeed, Park and Kazlauskas [14] correlated the ILs aptitude for enzymes stabilization, activity and selectivity with the ILs' polarity scale of Reichardt and hydrogen-bond basicity. Nevertheless, it should be kept in mind, that the relationship between ILs and enzymes is also dependent on the ions' nucleophilities, impurities, pH, and medium composition, among others. In addition, Ulbert et al. [15] corroborate those results, finding that the higher the IL polarity is, the lower the enzyme thermal stability. Highly hydrophilic ILs denaturate enzymes, either by ionic strength or specific binding to the protein surface. Increasing the opportunity for hydrogen-bonding with ILs increases the stability of enzymes in aqueous solutions.

Most works on ATPS using ILs have been focused on the effect of several inorganic salts [7, 13, 16-18] or in the use of carbohydrates [19-22] often based on the same IL forming system. The inorganic ion's influence seems to be well described by the Hofmeister series, [23] but information on the effect of the IL ions on promoting ATPS is particularly scarce [24, 25]. In a previous work, we studied the IL cation influence on ATPS formation ability [25]. Here, the IL anion influence was studied through their capability of forming ATPS, maintaining the inorganic salt (K_3PO_4). Different phase diagrams for systems of hydrophilic ILs + K_3PO_4 + water, at 298 K and atmospheric pressure, were experimentally determined. The binodal curves were fitted to a three-parameter equation and the tie-lines were estimated using the Merchuck et al. [26] approach.

Although there are a number of reports of IL-based ATPS in the literature, few of these works report the extractive potential of such systems, and only solutes such as testosterone, epitestosterone, penicillin G and opium alkaloids have been considered. [27-29]. Thus, in this work, the investigated ILs for the phase diagrams study were further evaluated concerning their extractive capability for biomolecules, and for that purpose, L-tryptophan, an essential amino acid that could be produced in a fermentative medium, was selected as a model biomolecule.

3.2.3. EXPERIMENTAL SECTION

3.2.3.1. Materials

The ternary phase diagrams determination was performed by using an aqueous solution of K_3PO_4 (≥ 98 wt % pure from Sigma) and different aqueous solutions of ILs. The ILs studied were 1-ethyl-3-methylimidazolium chloride, [C_2mim]Cl, 1-ethyl-3-methylimidazolium bromide, [C_2mim]Br, 1-ethyl-3-methylimidazolium acetate, [C_2mim][CH_3COO], 1-ethyl-3-methylimidazolium methylsulfate, [C_2mim][$MeSO_4$], 1-ethyl-3-methylimidazolium ethylsulfate, [C_2mim][$EtSO_4$], 1-ethyl-3-methylimidazolium methanesulfonate, [C_2mim][CH_3SO_3], 1-ethyl-3-methylimidazolium trifluoromethanesulfonate, [C_2mim][CF_3SO_3], 1-butyl-3-methylimidazolium chloride, [C_4mim]Cl, 1-butyl-3-methylimidazolium bromide, [C_4mim]Br, 1-butyl-3-methylimidazolium acetate, [C_4mim][CH_3COO], 1-butyl-3-methylimidazolium dicyanamide, [C_4mim][$N(CN)_2$], 1-butyl-3-methylimidazolium methanesulfonate, [C_4mim][CH_3SO_3], 1-butyl-3-methylimidazolium hydrogensulfate, [C_4mim][HSO_4], 1-butyl-3-methyl-imidazolium trifluoromethanesulfonate, [C_4mim][CF_3SO_3] and 1-butyl-3-methylimidazolium trifluoroacetate, [C_4mim][CF_3COO]. All the ILs, with the exception of [C_4mim][CF_3COO] that was acquired at Solchemar, were acquired at Iolitec and their structures are represented in Figure 3.2.1.

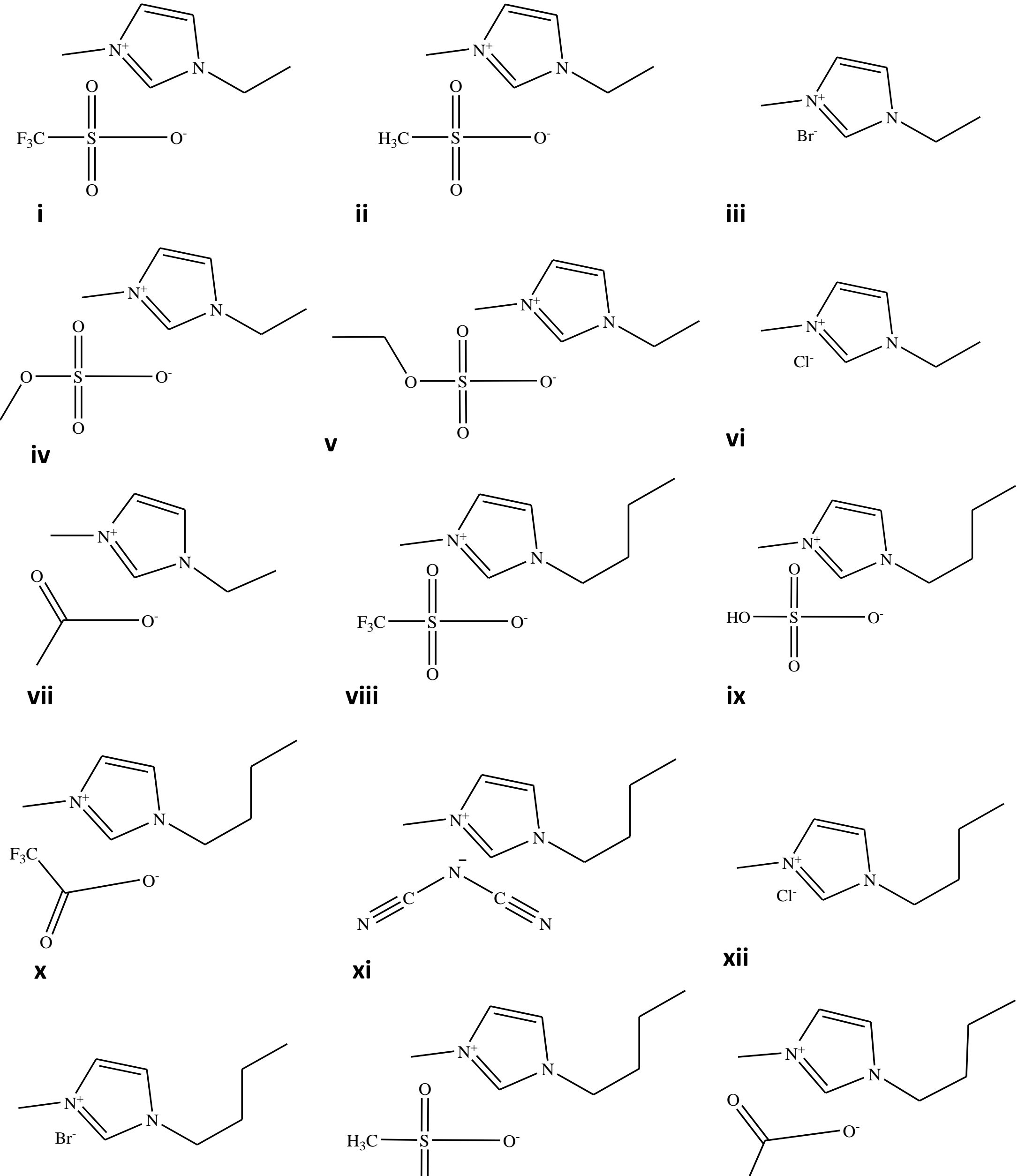


Figure 3.2.1. Chemical structure of the ILs studied: (i) [C_2mim][CF_3SO_3]; (ii) [C_2mim][CH_3SO_3]; (iii) [C_2mim]Br; (iv) [C_2mim][$MeSO_4$]; (v) [C_2mim][$EtSO_4$]; (vi) [C_2mim]Cl; (vii) [C_2mim][CH_3COO]; (viii) [C_4mim][CF_3SO_3]; (ix) [C_4mim][HSO_4]; (x) [C_4mim][CF_3COO]; (xi) [C_4mim][$N(CN)_2$]; (xii) [C_4mim]Cl; (xiii) [C_4mim]Br; (xiv) [C_4mim][CH_3SO_3]; (xv) [C_4mim][CH_3COO].

To reduce the water and volatile compounds content to negligible values, ILs individual samples were dried under constant stirring at moderate vacuum and temperature, for a

minimum of 48 h. After this procedure, the purity of each IL was further checked by ^{1}H, ^{13}C, and ^{19}F and found to be superior to 99 wt % for all samples. The water used was ultra-pure water, double distilled, passed by a reverse osmosis system and further treated with a Milli-Q plus 185 water purification apparatus.

3.2.3.2. Phase Diagrams and Tie-Lines

Aqueous solutions of K_3PO_4 at 40 wt % and aqueous solutions of different ILs at approximately 50 wt % were prepared and used for the determination of the binodal curves. The ternary system compositions were determined by the weight quantification of all components added within an uncertainty of ± 10^{-4} g. The detailed experimental procedure adopted and the respective validation is described in literature [25] and in **Section 3.1.3.2**.

3.2.3.3. Partitioning of L - tryptophan

A ternary mixture composition in the biphasic region was selected and used to evaluate the L-tryptophan partitioning at 298 K. For that reason aqueous solutions of L-tryptophan with a concentration of approximately 0.78 $g \cdot dm^{-3}$ were prepared and used. The biphasic solution was left to equilibrate for 12 h to achieve a complete L-tryptophan partitioning between the two phases. The amino-acid quantification in both phases, was carried by UV spectroscopy using a SHIMADZU UV-1700, Pharma-Spec Spectrometer, at a wavelength of 279 nm. Possible interferences of both the inorganic salt and the IL with the analytical method were taken into account and found to be of no significance at the dilutions carried (the maximum peak of absorbance of imidazolium-based ILs is *circa* 211 nm and, given the dilutions carried in all imidazolium-based IL-rich phases, there was no contribution of those compounds to the absorbance at 279 nm, as experimentally verified). Three samples of each individual aqueous phase were precisely quantified. The partition coefficients of L-tryptophan, K_{Trp}, were determined as the ratio of the concentration of L-tryptophan in the IL and in the K_3PO_4 aqueous-rich phases, and as described by Eq. 3.2 mentioned before. In addition, both phases were weighted and the corresponding TLs obtained as previously described.

3.2.4. RESULTS AND DISCUSSION

3.2.4.1. Phase Diagrams and Tie-lines

The experimental phase diagrams at 298 K and at atmospheric pressure for each IL + K_3PO_4 + H_2O system are presented in Figures 3.2.2 and 3.2.3 (see *Supporting Information* with the experimental weight fraction data *Tables S8.3.7 to S8.3.12*). Figure 3.2.2 shows the binodal curves for [C_2mim]Cl, [C_2mim]Br, [C_2mim[CH_3SO_3], [C_2mim][CH_3COO], [C_2mim][$MeSO_4$], [C_2mim][$EtSO_4$] and [C_2mim][CF_3SO_3].

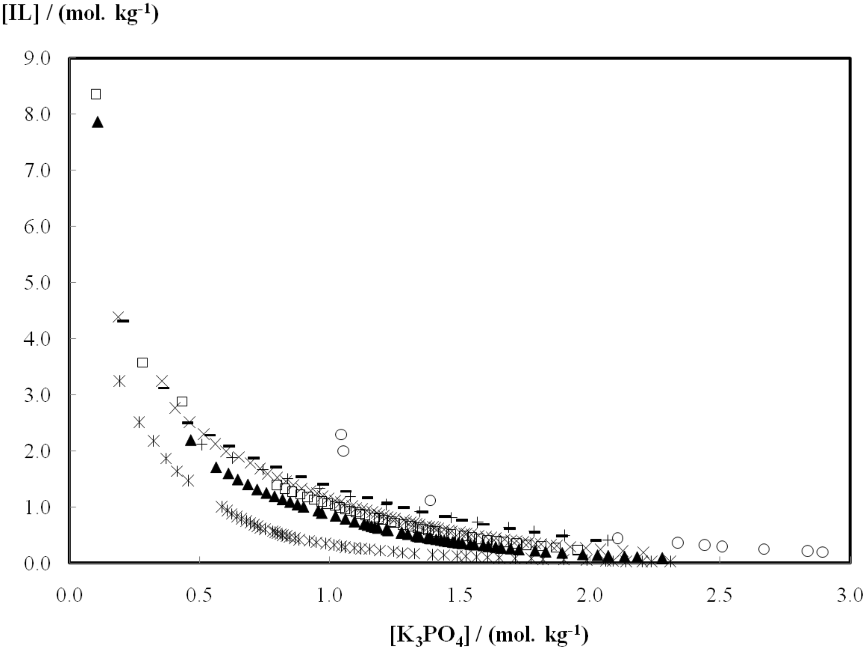


Figure 3.2.2. Phase diagrams for [C_2mim]-based ternary systems composed of IL + K_3PO_4 + H_2O at 298 K: (∗) [C_2mim][CF_3SO_3]; (▲) [C_2mim][$EtSO_4$]; (+) [C_2mim][CH_3COO]; (□) [C_2mim][$MeSO_4$]; (×) [C_2mim]Br; (-) [C_2mim]Cl; (○) [C_2mim][CH_3SO_3].

Figure 3.2.3 shows the binodal curves for [C_4mim]Cl, [C_4mim]Br, [C_4mim][CH_3SO_3], [C_4mim][CH_3COO], [C_4mim][HSO_4], [C_4mim][$N(CN)_2$], [C_4mim][CF_3COO] and [C_4mim][CF_3SO_3] (see *Supporting Information* with the experimental weight fraction data *Table S8.3.7* and *Tables S8.3.13* to *S8.3.18*). The results obtained show that the ability of ILs for ATPS formation follows the order [C_2mim][CF_3SO_3] > [C_2mim][$EtSO_4$] > [C_2mim][$MeSO_4$] > [C_2mim]Br > [C_2mim]Cl ≅ [C_2mim][CH_3COO] > [C_2mim][CH_3SO_3] and [C_4mim][CF_3SO_3] > [C_4mim][$N(CN)_2$] > [C_4mim][HSO_4] > [C_4mim][CF_3COO] > [C_4mim]Br > [C_4mim]Cl ≅ [C_4mim][CH_3COO] ≅ [C_4mim][CH_3SO_3].

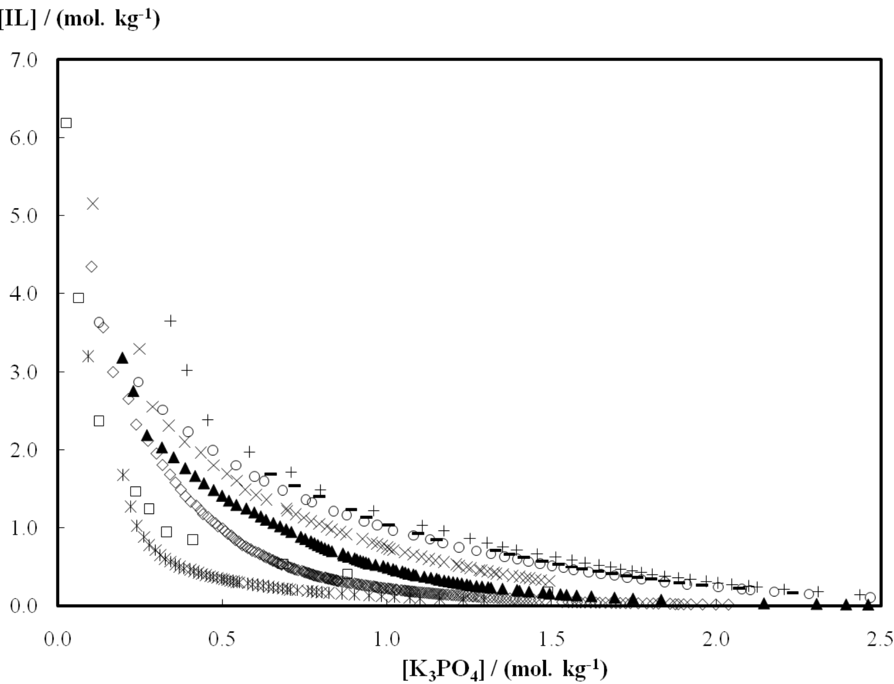


Figure 3.2.3. Phase diagrams for [C$_4$mim]-based ternary systems composed of IL + K_3PO_4 + H_2O at 298 K: (∗) [C$_4$mim][CF$_3$SO$_3$]; (□) [C$_4$mim][HSO$_4$]; (◇) [C$_4$mim][N(CN)$_2$]; (▲) [C$_4$mim][CF$_3$COO]; (×) [C$_4$mim]Br; (○) [C$_4$mim][CH$_3$SO$_3$]; (‾) [C$_4$mim]Cl; (+) [C$_4$mim][CH$_3$COO].

The solubility of a given solute in water is affected by the presence of an electrolyte. The decrease in solubility is known as salting-out effect while the increase is known as salting-in effect. The solubility of an electrolyte is also affected by the presence of a second electrolyte [30]. Hence the water activity plays a dominant role on the phase equilibria and on promoting ATPS. Nevertheless, the addition of an inorganic salt to ionic aqueous systems leads to more complex phase equilibria than typical non-electrolyte systems, and different mechanisms such as ion exchange and ion-pairing may arise. Bridges et al. [17] have shown that there is in fact ion partitioning between both aqueous-rich phases, yet the overall deviations of the ions concentration present at any TL are small enough and can be not considered as a source of error.

Although the salting-out effect is usually explained based on the ordering of bulk water and on the ability of the ions to decrease the water structure by a simple hydration phenomena, recent works are converging upon to the idea that interactions between the IL ions and the inorganic salt ions may be present, and are mainly responsible for the salting-in or salting-out phenomena [31, 32]. Salting-out inducing ions act mainly through an entropic effect resulting from the formation of water-ion hydration

complexes while the salting-in inducing ions directly interact with the hydrophobic moieties of the IL [31, 32]. The ions specific effects seem to be dominated by the ion-ion *versus* ion-water interactions though a complex interplay of factors, and not by the water-structure modification as classically accepted [23]. Keeping in mind that PO_4^{3-} is a high charge density ion (salting-out inducing ion), and that this ion was used in all the ternary systems evaluated, it is possible to assess the IL anions inducing character. Considering the representations in Figures 3.2.2 and 3.2.3, the closer to the axis is located the binodal curve, the larger the IL anion salting-in inducing behaviour - that is the lower the density charge of the IL anions.

Studies regarding the ILs polarity provide useful information on the IL cations and anions solvation at the molecular level. The introduction of empirical solvent polarity scales based on solvent interactions with a reference solute has lead to a rank on the compounds [33]. Frequently used polarity scales, based on solvatochromic probes, are the Reichardt $E_T(30)$ scale and the Kamlet-Taft's solvent parameters [34, 35]. In fact, the polarities of imidazolium-based ILs were found to be similar to that of short chain alcohols [36]. A review regarding the polarity of ILs was presented recently by Reichardt [37]. It should, nevertheless, be kept in mind that the solvent parameters are dependent on the molecule probe employed [35] and are particularly relevant for these nanostructured fluids.

Table 3.2.1 reports the hydrogen-bond basicity (β) and hydrogen-bond acidity (α) of [C_4mim]-based ILs using the solvatochromic probe $[Fe(phen)_2(CN)_2]ClO_4$ [38]. The value of α decreases with increasing hydrogen-bond accepting strength of the IL anion. The ATPS studied concern electrolyte solutions in aqueous medium and reflect the competition between the inorganic salt and the IL ions for the creation of water-ion hydration complexes. Therefore, the ability of a specific anion to be preferentially hydrated largely depends on the anion' hydrogen-bonding accepting ability, and thus the ATPS formation ability strongly depends on anions' hydrogen-bonding basicity. This fact was observed for the ILs anions sequence studied in promoting ATPS (Table 3.2.1). It is here obvious that the compilation of an accurate and extensive polarity scale of ILs is of main connotation, allowing the prediction of their behavior in aqueous systems, before carrying extensive experimental measurements of phase diagrams.

Table 3.2.1. Hydrogen Bond Acidity (α) and Hydrogen Bond Basicity (β) of [C_4mim]-Based ILs with the Solvatochromic Probe [$Fe(phen)_2(CN)_2$]ClO_4 [38].

IL anion	α [38]	β [38]
Cl	0.32	0.95
Br	0.36	0.87
[CH_3COO]	0.36	0.85
[CH_3SO_3]	0.36	0.85
[$MeSO_4$]	0.39	0.75
[CF_3COO]	0.43	0.74
[$N(CN)_2$]	0.44	0.64
[CF_3SO_3]	0.50	0.57
[BF_4]	0.52	0.55
[PF_6]	0.54	0.44
[$(CF_3SO_2)_2N$]	0.55	0.42

The binodal curves were fitted to the empirical correlation of Merchuk et al. [26] (Eq. 3.1). The parameters for this equation were estimated by least-squares regression of the cloud point data and are reported in Table 3.2.2.

Table 3.2.2. Correlation parameters used in Eq. 3.1 (Section 3.1.3.2.) to describe the binodals at 298 K.

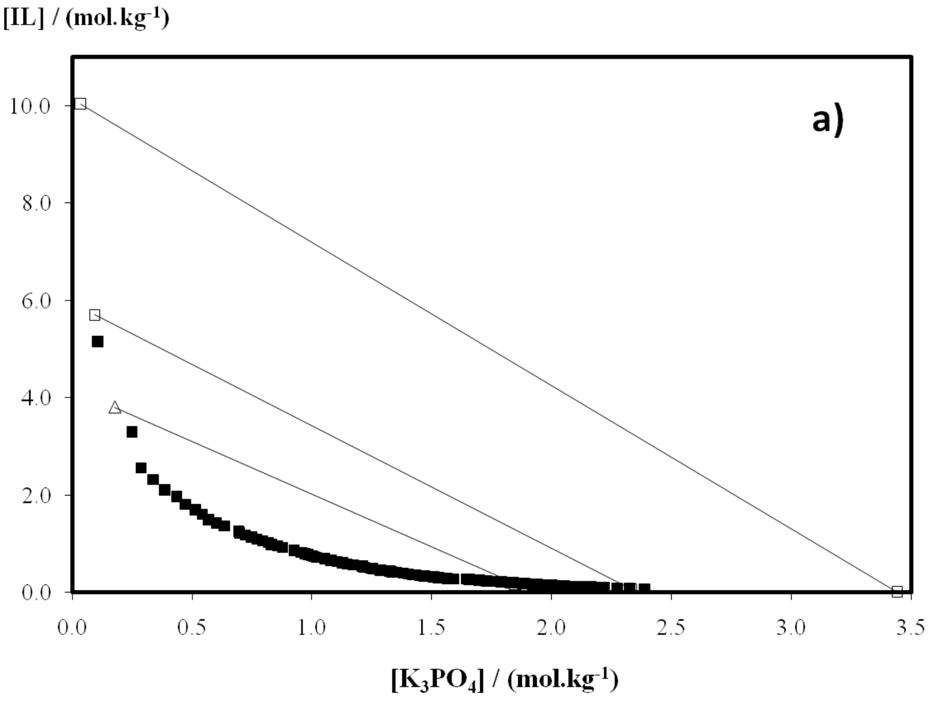
IL anion	A	B	C
$[C_2mim]Cl$	78.25	-0.3389	2.697×10^{-5}
$[C_2mim]Br$	85.60	-0.3134	4.684×10^{-5}
$[C_2mim][CH_3SO_3]$	1476	-0.9004	5.903×10^{-6}
$[C_2mim][CH_3COO]$	56.51	-0.2346	3.022×10^{-5}
$[C_2mim][MeSO_4]$	99.34	-0.3374	4.693×10^{-5}
$[C_2mim][EtSO_4]$	91.81	-0.3320	5.740×10^{-5}
$[C_2mim][CF_3SO_3]$	149.2	-0.5684	9.496×10^{-5}
$[C_4mim]Cl$	72.64	-0.3185	4.070×10^{-5}
$[C_4mim]Br$	95.90	-0.3912	5.197×10^{-5}
$[C_2mim][CH_3SO_3]$	70.24	-0.2539	4.489×10^{-5}
$[C_4mim][CH_3COO]$	109.4	-0.3832	3.126×10^{-5}
$[C_4mim][HSO_4]$	94.08	-0.6021	8.797×10^{-6}
$[C_4mim][N(CN)_2]$	190.7	-0.8344	1.000×10^{-5}
$[C_4mim][CF_3COO]$	92.30	-0.3790	9.897×10^{-5}
$[C_4mim][CF_3SO_3]$	182.8	-0.9676	1.000×10^{-5}

The ternary system composition used at the biphasic region and the TLs equations and respective tie-line lengths (TLLs) are reported in Table 3.2.3.

Table 3.2.3. Experimental data for TLs and respective TLLs at 298 K.

Ionic Liquid	Mass fraction / wt %						TLL
	Y_M	X_M	Y_T	X_T	Y_B	X_B	
[C$_2$mim]Br	15.31	26.21	57.66	1.59	2.27	33.80	64.07
[C$_2$mim]Cl	24.88	20.85	47.16	2.23	1.54	40.36	59.46
	26.30	14.93	37.70	4.61	2.84	36.18	47.03
[C$_2$mim][CH$_3$COO]	30.01	15.16	42.91	1.38	0.53	46.68	62.04
	24.94	14.96	34.31	4.47	1.53	41.14	49.18
[C$_2$mim][MeSO$_4$]	20.31	24.94	57.15	2.68	1.35	36.40	65.19
[C$_2$mim][EtSO$_4$]	14.89	25.16	54.53	3.15	1.71	35.28	61.82
	20.24	21.17	51.41	3.03	2.32	31.60	56.80
	24.21	25.13	59.01	1.77	0.19	41.25	70.84
[C$_2$mim][CF$_3$SO$_3$]	20.00	22.47	74.85	1.47	0.52	29.92	79.59
	25.07	14.93	62.24	2.36	3.65	22.17	61.84
[C$_4$mim]Br	21.09	29.55	68.75	0.72	0.15	42.21	80.17
	24.41	19.90	55.53	1.95	1.53	33.11	62.34
[C$_4$mim]Cl	15.75	22.67	37.13	4.39	2.19	34.28	45.97
	17.69	21.54	38.18	4.05	1.92	35.01	47.67
	21.36	23.04	49.51	1.45	0.95	38.68	61.20
[C$_4$mim][CH$_3$SO$_3$]	30.33	14.86	45.68	2.86	1.40	37.46	56.20
	25.03	14.96	34.19	7.61	3.85	31.97	38.91
[C$_4$mim][N(CN)$_2$]	19.89	22.65	66.45	1.60	1.35	31.03	71.44
	24.86	15.86	60.13	1.91	2.60	24.67	61.84
	15.19	20.12	59.67	1.94	2.43	25.34	61.87
[C$_4$mim][CF$_3$COO]	15.24	20.39	55.74	1.77	2.11	26.43	59.03
	19.90	24.65	69.16	0.58	0.19	34.28	76.77
[C$_4$mim][CF$_3$SO$_3$]	25.00	15.35	66.60	1.09	1.49	23.41	68.83
	25.08	14.96	66.42	1.09	1.59	22.83	68.37

As an example of the TLs measured in this work, Figure 3.2.4 shows TLs for the systems containing the ILs [C$_4$mim]Br and [C$_4$mim][CF$_3$COO].



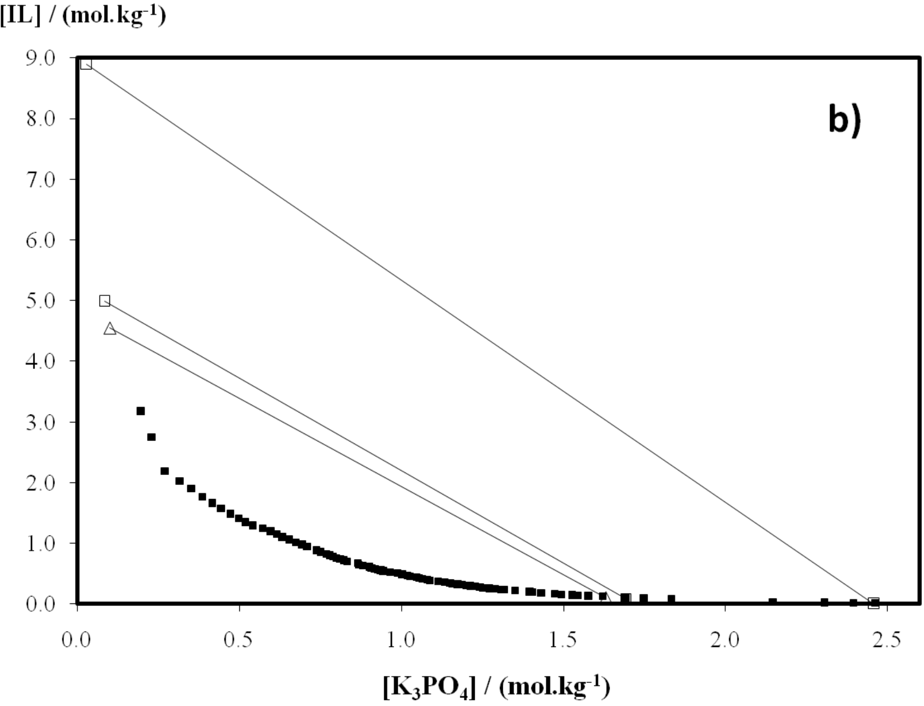


Figure 3.2.4. (a) Phase diagram for the [C_4mim]Br + K_3PO_4 + water ternary system at 298 K and (b) phase diagram for the [C_4mim][CF_3COO] + K_3PO_4 + water ternary system at 298 K: (■) binodal curve data; (□) TL data; (Δ) extraction TL data.

For the majority of the studied systems (and taking into consideration that for all the studied systems the top-rich phase is the IL-rich phase and the bottom phase is the K_3PO_4-rich phase), the IL concentration in the bottom phase is very small, and in some cases the IL is almost completely excluded from that phase. The opposite behaviour is observed at the aqueous K_3PO_4 top phase. Usually, the total composition of the system has no significant effect upon the slope of the TLs, which implies that the TLs are parallel to each other, thus allowing the knowledge of the phase compositions for any given mixture. It was found that for short TLLs, the TLs are approximately parallel while for longer TLLs, the TLs slopes start to deviate. These deviations in the TLs slopes are in agreement with our previous work [25], as well as with literature [39], and it is related with the fact that the K_3PO_4-rich phase is increasingly free of IL at longer TLLs.

3.2.3.4. Partitioning of L-tryptophan

Amino-acids are the constituents of proteins. The partitioning studies of such biomolecules in ATPS have great significance not only regarding their purification, but also aiming at extending them to proteins and enzymes. The success of the extractive potential of ATPS depends on the ability to manipulate phase properties to obtain the appropriate partition coefficients and selectivity for the biomolecule of interest. There are several approaches to manipulate a particular solute partitioning: (i) adjust the system by applying different salts and/or ILs controlling thus the solute' affinity; (ii) change the system composition by changing either the concentration of salt and IL; and (iii) introduction of additional cosolvents, antisolvents, or amphiphilic structures to the overall system.

The mixture compositions selected for the amino acid partitioning, for each particular system, are described in Table 3.2.4. In addition, the respective TLs and TLLs are also included in Table 3.2.4. The graphical representation of the TLs corresponding to the ternary composition used for the L-tryptophan partitioning is presented in Figure 3.2.4 (concomitantly with the TLs obtained when no amino acid is present). It is clear that the addition of amino-acid (at least in the concentrations used) has no significant influence on the TLs and TLLs obtained. The concentration of L-tryptophan is small enough to be irrelevant compared with the total and each individual aqueous phases mass

composition. Thus the TLs and TLLs presented in Table 3.2.4 could be considered as additional phase equilibrium TLs of each individual ternary system.

Table 3.2.4. Mass fraction composition and partition coefficients of L-tryptophan in ILs-based ATPS systems at 298 K.

Ionic Liquid	Mass fraction / wt %						TLL	K_{Trp}
	Y_M	X_M	Y_T	X_T	Y_B	X_B		
[C$_2$mim]Cl	25.90	14.90	37.01	4.84	3.10	35.52	45.73	59.2 ± 0.4
[C$_2$mim][CH$_3$COO]	24.94	14.96	34.31	4.47	1.53	41.14	49.18	16.4 ± 0.8
[C$_2$mim][MeSO$_4$]	24.98	14.94	34.74	9.06	9.99	23.96	28.89	4.47 ± 0.9
[C$_2$mim][EtSO$_4$]	24.97	15.06	38.31	6.66	4.58	27.89	39.86	5.96 ± 0.5
[C$_2$mim][CF$_3$SO$_3$]	24.87	16.35	70.01	1.77	2.77	23.50	70.66	17.5 ± 0.8
[C$_4$mim]Cl	25.35	15.97	39.18	3.73	1.44	36.58	50.03	36.6 ± 0.6
[C$_4$mim]Br	25.82	15.17	45.45	3.62	3.77	28.15	48.36	35.6 ± 0.8
[C$_4$mim][CH$_3$SO$_3$]	25.03	14.96	34.19	7.61	3.85	31.97	38.91	10.4 ± 0.4
[C$_4$mim][N(CN)$_2$]	24.40	16.82	62.83	1.77	2.40	25.44	64.90	45.1 ± 0.9
[C$_4$mim][CF$_3$COO]	25.13	15.22	53.44	2.07	2.50	25.72	56.16	36.1 ± 0.7
[C$_4$mim][CF$_3$SO$_3$]	25.08	16.18	73.31	0.89	1.44	23.68	75.39	16.6 ± 0.6

To successfully apply ILs as extraction media in ATPS, the factor governing the partitioning of biomolecules between the two equilibrium aqueous-rich phases must be understood. The partition coefficients give the partition behaviour of L-tryptophan between the IL and the K_3PO_4-rich phases. The higher the K_{Trp} the higher the tendency for the solute to migrate to the IL-rich phase. Among the different interactions between molecules, "hydrophobic" interactions play a key role in the behaviour of proteins in solution [40]. Therefore, the relative hydrophobicity of ILs can provide some insights regarding their ability to extract amino-acids and proteins. Moreover, the solute-IL interactions are likely to involve van der Waals forces, electrostatic interactions, hydrogen-bonding and $\pi \cdots \pi$ stacking between the imidazolium ring and the aromatic ring of L-tryptophan. Indeed, $\pi \cdots \pi$ stacking between imidazolium and benzene molecules has been reported previously [41].

For the ternary system compositions described in Table 3.2.4. (approximately 25 wt % of IL and 15 wt % of K_3PO_4), the partition coefficients of L-tryptophan follow the rank: [C$_2$mim]Cl > [C$_2$mim][CF$_3$SO$_3$] ≅ [C$_2$mim][CH$_3$COO] > [C$_2$mim][EtSO$_4$] ≅

$[C_2mim][MeSO_4]$ and $[C_4mim][N(CN)_2]$ > $[C_4mim]Cl$ ≅ $[C_4mim][CF_3COO]$ ≅ $[C_4mim]Br$ > $[C_4mim][CF_3SO_3]$ > $[C_4mim][CH_3SO_3]$. L-tryptophan partitions preferentially for IL-rich phases composed by halogenated ions such as Cl or Br or to the most hydrophobic anions, i.e., anions with lower hydrogen bonding accepting strength (β), such as $[N(CN)_2]$ and fluoride-based anions. Indeed, these partition coefficients seem to closely follow the Hofmeister series [42]. Salting-in inducing IL ions tend to increase the solute partition coefficient while the salting-out inducing IL ions tend to decrease it.

Attending to the results described in **Section 3.1.4.2**, the influence of the IL imidazolium-based cation on the extraction capacity of ATPS seems to be more important than the influence of the anion. Indeed, in the study of the cation influence the K_{Trp} was found to vary between 10 and 120 (at approximately the same mass fraction compositions of IL plus inorganic salt). The presence of benzyl groups and double bounds at the imidazolium side alkyl chain increased substantially such partition coefficients. Besides the $\pi \cdots \pi$ stacking, the possibility of solute-IL hydrogen-bonding seems to rule the partition behaviour. Nonetheless, it should be kept in mind that solutes others than L-tryptophan should be explored in order to develop a full picture of the extractive potential of IL-based ATPS.

The partition coefficients obtained in this work for L-tryptophan are substantially higher than those observed in conventional polymer-polysaccharide ($K_{Trp} \cong 1$) or polymer-inorganic salts ($K_{Trp} \cong$ 1-7) reported in literature [43, 44]. These results indicate that ILs are a novel option for the purification and separation of biomolecules with much larger partition coefficients than conventional ATPS. Moreover, ILs can be fine tuned in order to manipulate such partition coefficients. At this stage, further academic and industrial investigations on the topic of the ILs potential as extraction media, as well as their toxicity and biodegradability issues, are vital requisites.

3.2.4. CONCLUSIONS

Ionic liquids have shown to be able to induce aqueous phases separation in the presence of inorganic salts, and thus to form ATPS. Therefore, new experimental equilibrium data for the compositions of coexisting phases of ATPS involving hydrophilic imidazolium-based ILs + K_3PO_4 + H_2O, at the same conditions of temperature and pressure (298 K and atmospheric pressure), were studied and reported.

The ability of imidazolium-based ILs for aqueous phase separation was shown to closely follow the hydrogen bond accepting strength decrease of the anions composing the IL. The results indicate that IL-based ATPS can be obtained over a large range of concentration of both the inorganic salt and the IL, and such systems can be finely tuned by the adjustment of the IL anion.

The capacity of the IL-based ATPS as prospective extraction media in biotechnological processes was demonstrated by the high partitioning coefficients obtained for one essential amino-acid: L-tryptophan. The values obtained are substantially larger than those observed with conventional polymer-based ATPS and further studies regarding ILs aiming at obtaining a complete perspective of the molecular interactions controlling the solutes partition behavior is of utmost importance.

3.2.5. REFERENCES

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3.3. PAPER 4

Ionic-Liquid-Based Aqueous Two-Phase Systems With Controlled pH: The Ionic Liquid Cation Effect

Journal of Chemical Engineering Data, 2011,
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3.3.1. ABSTRACT

This work addresses the evaluation of the ability of ionic liquid cations on the formation of aqueous two-phase systems (ATPS), with K_2HPO_4 or a mixture of inorganic salts, K_2HPO_4/KH_2PO_4, aiming at controlling the pH values of the co-existing aqueous phases. Using chloride-based ionic liquids, the effect of the cation core, the length of the alkyl side chain, and the positional isomerism on the ATPS formation ability, were investigated. All binodal curves were determined by the cloud point titration method at 298 K. From the obtained phase diagrams it is shown that the biphasic area increases with the cation side chain length, from ethyl to hexyl chains, although for longer chains, an inversion on the binodal curves sequence appears due to the self-aggregation of the longer chain ionic liquids in aqueous solutions. The influence of the cation core and the positional isomerism of the ionic liquids on their ability to form ATPS closely correlates with the ionic fluids affinity for water.

3.3.2. INTRODUCTION

Aqueous two-phase systems (ATPS) are formed when polymer/polymer, polymer/salt, and salt/salt mixtures are dissolved in water above critical concentrations. [1-3] Because both phases in ATPS are essentially composed of water, they offer a gentle and more biocompatible environment, better suited for the biomolecules' extraction than conventional approaches that make use of molecular organic solvents. The application of polymer-based ATPS is already well documented. [1-4] However, most of the aqueous solutions of the phase-forming polymers present high viscosities, form opaque systems, and present a limited range of polarities at their co-existing phases. [5] For that reason, there has been a large effort to find new alternatives to polymers in the composition of ATPS.

Ionic liquids (ILs) are salts with a melting temperature below 100 ºC [6, 7] and that have shown to be particularly valuable as extractive solvents for the most distinct solutes. They possess a large array of fascinating properties, such as a negligible vapor pressure, a high chemical and thermal stability, a large liquidus temperature range, and widely tunable thermophysical properties by the appropriate pairing of different cations with any of a growing number of anions. [8] Therefore, in recent years, ILs have received an extensive attention as "designer solvents". [9] IL-based ATPS (IL-ATPS)

were reported, for the first time, by Rogers and his co-workers in 2003. [10] After this first impulse, the number of works describing different IL-ATPS has been growing in the past few years. Those works have addressed not only a wide variety of ionic liquids [11, 12] but also the use of various salting-out inducing salts (mostly phosphates, [11-19] hydrogenophosphates, [13, 15, 19-22] citrates, [23-27] and carbonates [13-15, 20, 22, 28-31], as well as carbohydrates, [32, 33] polymers, [34-36] amino-acids, [37, 38] and more recently, anionic surfactants. [39] Since ILs are used in aqueous solution they are not constrained by the need of low melting temperatures and even compounds that do not fit the low melting point definition of ILs can be used for preparing IL-ATPS. These systems have been extensively studied as successfully separation/extraction methods of different compounds and molecules, such as alkaloids, [11, 37, 38] phenolic compounds [40], antibiotics, [21, 30, 41-45] amino-acids, [12, 18, 27, 34, 40, 46] proteins, [16, 47-51] enzymes, [16, 28, 52-54] and drugs. [55]

This work is focused on the study of the ability of IL cations to form ATPS with two salts, K_2HPO_4, and the phosphate buffer solution composed of K_2HPO_4/KH_2PO_4. These salts were chosen to obtain ATPS with controlled pH values. Aiming at evaluating the IL cation influence towards the formation of ATPS, with the two inorganic salts and/or inorganic salt mixtures, this work will be divided in three major sections: the first part addresses the impact of the cation side alkyl chain length; the second part describes the influence of different cation cores (imidazolium, pyridinium, pyrrolidinium and piperidinium); and the last part addresses the influence of the cation positional isomerism.

3.3.3. Material and Methods

3.3.3.1. Materials

The K_2HPO_4 and KH_2PO_4, with mass fraction purities higher than 98 %, were purchased from Sigma-Aldrich. The ILs used are as described: 1-ethyl-3-methylimidazolium chloride, [1-C_2-3-C_1im]Cl (> 99 wt %); 1-butyl-3-methylimidazolium chloride, [1-C_4-3-C_1im]Cl (> 99 wt %); 1-hexyl-3-methylimidazolium chloride, [1-C_6-3-C_1im]Cl (> 98 wt %); 1-heptyl-3-methylimidazolium chloride, [1-C_7-3-C_1im]Cl (> 99 wt %); 1-benzyl-3-methylimidazolium chloride, [1-C_7H_7-3-C_1im]Cl (> 99 wt %), 1-octyl-3-methylimidazolium chloride, [1-C_8-3-C_1im]Cl (> 99 wt %); 1-butyl-2-

methylpyridinium, $[1\text{-}C_4\text{-}2\text{-}C_1pyr]Cl$ (> 98 wt %); 1-butyl-3-methylpyridinium chloride, $[1\text{-}C_4\text{-}3\text{-}C_1pyr]Cl$ (> 98 wt %); 1-butyl-4-methylpyridinium chloride, $[1\text{-}C_4\text{-}4\text{-}C_1pyr]Cl$ (> 98 wt %); 1-butyl-1-methylpyrrolidinium chloride, $[1\text{-}C_4\text{-}1\text{-}C_1pyr]Cl$ (> 99 wt %); 1-butyl-1-methylpiperidinium chloride $[1\text{-}C_4\text{-}1\text{-}C_1pip]Cl$ (> 99 wt %). All ILs were purchased at IoLiTec (Ionic Liquid Technologies, Germany) and their mass fraction purities were further confirmed by ^1H NMR and ^{13}C NMR. Their ionic structures with their respective abbreviations are reported in Figure 3.3.1. Ultrapure water, double distilled, passed by a reverse osmosis system and further treated with a Milli-Q plus 185 water purification apparatus, was used.

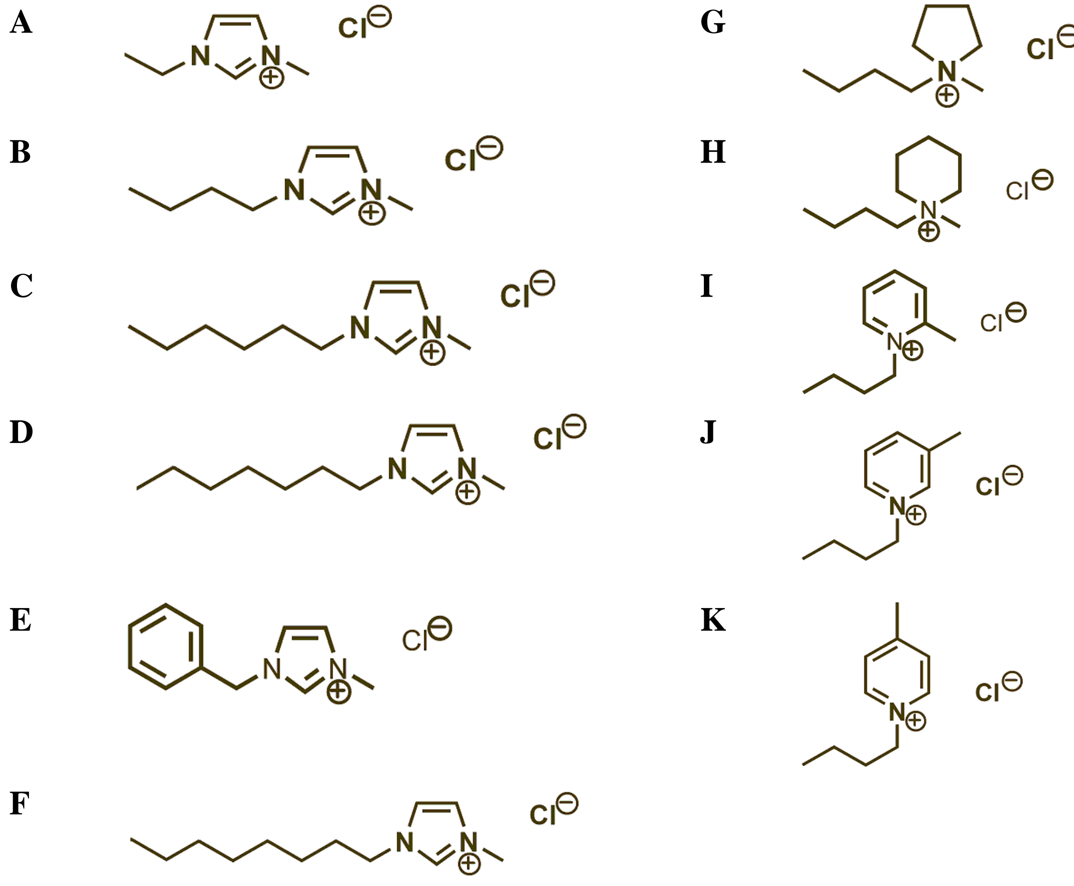

Figure 3.3.1. Chemical structure of the ILs studied: $[1\text{-}C_2\text{-}3\text{-}C_1im]Cl$ (A), $[1\text{-}C_4\text{-}3\text{-}C_1im]Cl$ (B), $[1\text{-}C_6\text{-}3\text{-}C_1im]Cl$ (C); $[1\text{-}C_7\text{-}3\text{-}C_1im]Cl$ (D); $[1\text{-}C_7H_7\text{-}3\text{-}C_1im]Cl$ (E), $[1\text{-}C_8\text{-}3\text{-}C_1im]Cl$ (F), $[1\text{-}C_4\text{-}1\text{-}C_1pyrr]Cl$ (G), $[1\text{-}C_4\text{-}1\text{-}C_1pip]Cl$ (H), $[1\text{-}C_4\text{-}2\text{-}C_1pyr]Cl$ (I), $[1\text{-}C_4\text{-}3\text{-}C_1pyr]Cl$ (J), $[1\text{-}C_4\text{-}4\text{-}C_1pyr]Cl$ (K).

3.3.3.2. Phase Diagrams and Tie-Lines

The binodal data were determined for all the ILs studied using the cloud point titration method previously described by us [12, 18] at 298 K (± 1 K). Aqueous solutions of the inorganic salts at *circa* 40 wt % of K_2HPO_4 (with a pH value of 9.1) and K_2HPO_4/KH_2PO_4 (phosphate buffer solution with a pH value of 7.0), and aqueous solutions of each IL (≈ 60 wt %) were individually prepared. Repetitive drop-wise addition of the aqueous inorganic salt solution to the aqueous solution of IL was carried out until the detection of a cloudy solution, followed by the drop-wise addition of ultrapure water until the detection of a monophasic region. The whole procedure was performed under constant stirring. The ternary system compositions were determined by the weight quantification of all components added within ± 10^{-4} g.

The tie-lines (TLs) were determined by a gravimetric method previously described by us, [12, 18, 54] and originally proposed by Merchuck et al. [56] A mixture at the biphasic region was prepared, vigorously stirred, and allowed to reach equilibrium by the phase separation for 24 h at 298 K, using small ampules (10 cm^3) especially designed for this task. After this period, both phases were carefully separated and weighed. Each individual TL was determined by the application of the level arm-rule. [56] For that purpose, the experimental binodal curves were correlated by the already mentioned Eq 3.1. [56]

3.3.4. RESULTS AND DISCUSSION

The phase diagrams, presented in Figures 3.3.2 and 3.3.3, depict the effect of different alkyl side chains at the IL cation in the formation of ATPS. The binodal data of these systems (in mass fraction units) is presented at *Supporting Information Tables S8.3.19* to *S.8.3.27*.

Figure 3.3.2 reports the ATPS composed of three different imidazolium-based ILs and the inorganic salt K_2HPO_4. It is observed that the increase of the cation aliphatic chain from C_2 to C_6 leads to an increase of the biphasic region envelope due to the increasing hydrophobicity of the IL. [12, 18, 57] This behavior has been previously described for IL-ATPS containing other salts as a characteristic of ILs with alkyl chains up to 6 carbon atoms. [12, 28]

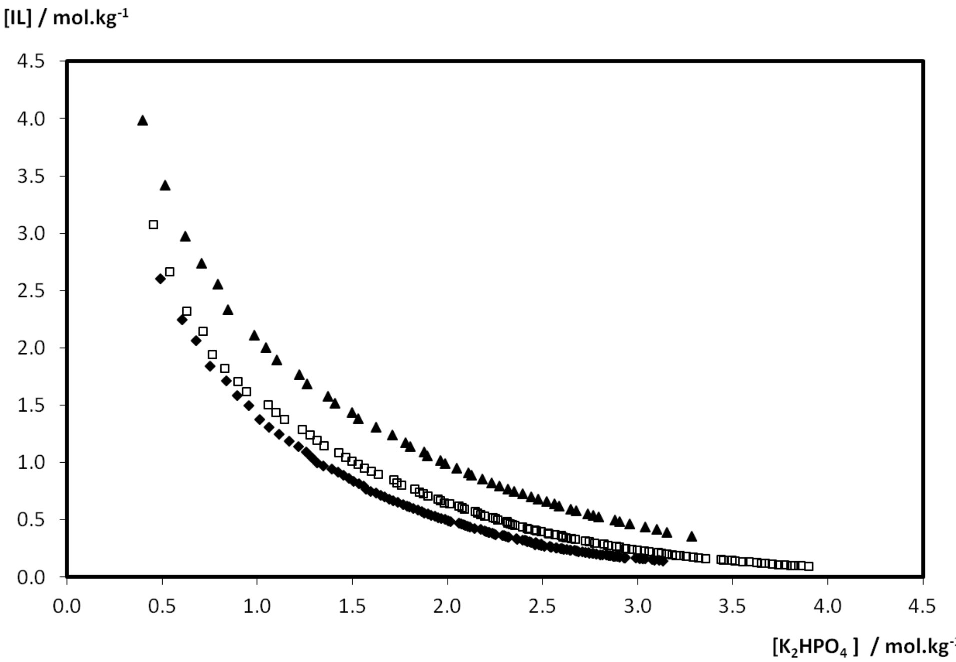

Figure 3.3.2. Phase diagrams for the ILs [1-C_n-3-C_1im]Cl and the salt K_2HPO_4 at 298 K: (▲) [1-C_2-3-C_1im]Cl; (□) [1-C_4-3-C_1im]Cl; (◆) [1-C_6-3-C_1im]Cl.

The study of the influence of the IL alkyl chain length through the ATPS formation ability, conjugated with the phosphate buffer (PB) solution containing the salts K_2HPO_4/KH_2PO_4, is reported in Figure 3.3.3 for a larger number of ILs with a wider range of aliphatic chain lengths. The binodal curves for the systems with the PB, and with different imidazolium-based ILs, have an increasing tendency of phase separation that can be described by the series: [1-C_2-3-C_1im]Cl < [1-C_7-3-C_1im]Cl ≈ [1-C_7H_7-3-C_1im]Cl < [1-C_8-3-C_1im]Cl ≈ [1-C_4-3-C_1im]Cl < [1-C_6-3-C_1im]Cl.

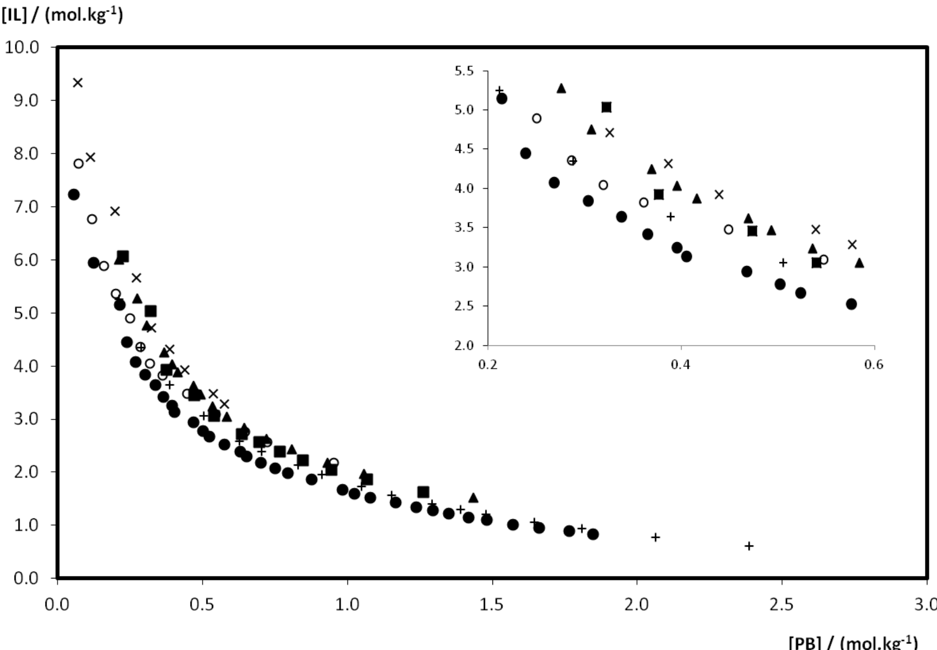

Figure 3.3.3. Phase diagrams for the ILs [1-C_n-3-C_1im]Cl and the phosphate buffer solution at 298 K: (×) [1-C_2-3-C_1im]Cl; (○) [1-C_4-3-C_1im]Cl; (●) [1-C_6-3-C_1im]Cl; (▲) [1-C_7-3-C_1im]Cl; (+) [1-C_8-3-C_1im]Cl; (■) [1-C_7H_7-3-C_1im]Cl.

The same tendency in the increasing ability of ATPS formation with the cation side chain length, between C_2 and C_6, is observed in both salt solutions. However, as the number of carbons in the alkyl chain increases, above C_6, the ability for ATPS formation changes and no longer follows the hydrophobicity of the IL. This trend was previously observed by us [17] using the salt K_3PO_4, and has also been described by others, [12, 20, 22, 50, 58-60] for other inorganic salts (K_2HPO_4 and K_2CO_3). Najdanovic-Visak et al., [60] suggested that when the number of carbons in the longer alkyl side chain of the imidazolium cation is superior to 6, the capacity of the ATPS formation is altered due to the possibility of micelle formation in aqueous solutions. While the salting-out effect of the inorganic salt remains, the tendency of the IL to self aggregate in aqueous solution reduces the ability of the liquid-liquid demixing, and thus, decreases the aptitude for ATPS formation. Finally, the presence of a benzyl group ([C_7H_7]) in the imidazolium cation core has no major impact through the ATPS formation capability. Only a marginal increase in the ability to form ATPS was observed when compared with [C_7mim]Cl.

Figure 3.3.4 shows the influence of the IL cation core towards the ATPS formation capacity using the phosphate buffer solution. The mass fraction data for each binodal

curve are reported in *Supporting Information Tables S8.3.28* to *S8.3.30*. According to these results, the sequence for the ability of phase formation for the various families is [1-C_4-1-C_1pyrr]Cl ≈ [1-C_4-3-C_1im]Cl < [1-C_4-1-C_1pip]Cl < [1-C_4-3-C_1pyr]Cl. This order is a reflection of the IL ability to be solvated by water.

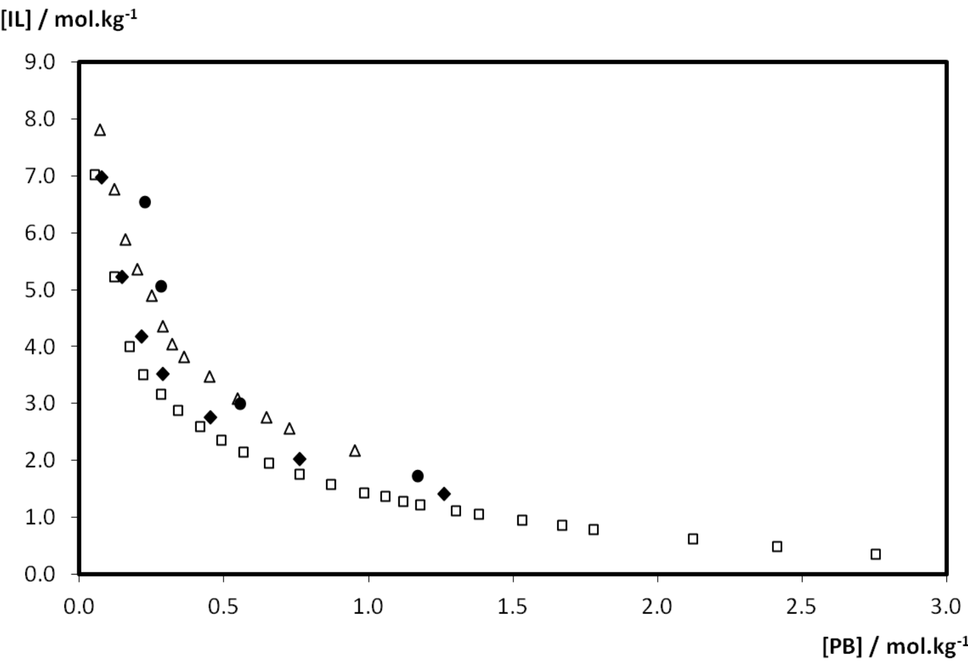

Figure 3.3.4. Phase diagrams for ILs constituted by different cation cores and the potassium phosphate buffer solution at 298 K: (●) [1-C_4-1-C_1pyrr]Cl; (Δ) [1-C_4-3-C_1im]Cl; (◆) [1-C_4-1-C_1pip]Cl; (□), [1-C_4-3-C_1pyr]Cl.

Although we have previously shown that the solubility of water in ILs largely depends on the availability of electrons at the aromatic cores for privileged hydrogen-bonding interactions, on the other hand, the solubility of ILs in water is mainly ruled by steric and entropic contributions. [57, 61] As a result, the solubility of ILs in water follows the IL cation molar volume, [62] and the trend obtained here closely agrees with our previous data. [61] Larger cations such as pyridinium and piperidinium are more able to induce ATPS when compared with the smaller 5-sided rings of imidazolium and pyrrolidinium. Albeit the pyridinium-based IL (with an aromatic core) is sligthly more able to induce ATPS than the piperidinium-based IL (with an aliphatic core), we must be aware of steric effects that result from the position of their alkyl chains. The presence of a second aliphatic chain in pyridinium in a non nitrogenated atom leads to a higher entropic contribution, and thus, to a higher aptitute of this IL to produce ATPS.

Figure 3.3.5 shows the influence of positional isomerism of the methyl moiety in a pyridinium cation through the formation of ATPS. For such a purpose, three pyridinium-based ILs were investigated, namely [1-C_4-4-C_1pyr]Cl, [1-C_4-3-C_1pyr]Cl, and [1-C_4-2-C_1pyr]Cl. The mass fraction data of each binodal curve are reported in *Supporting Information Tables S8.3.30 - S8.3.32*.

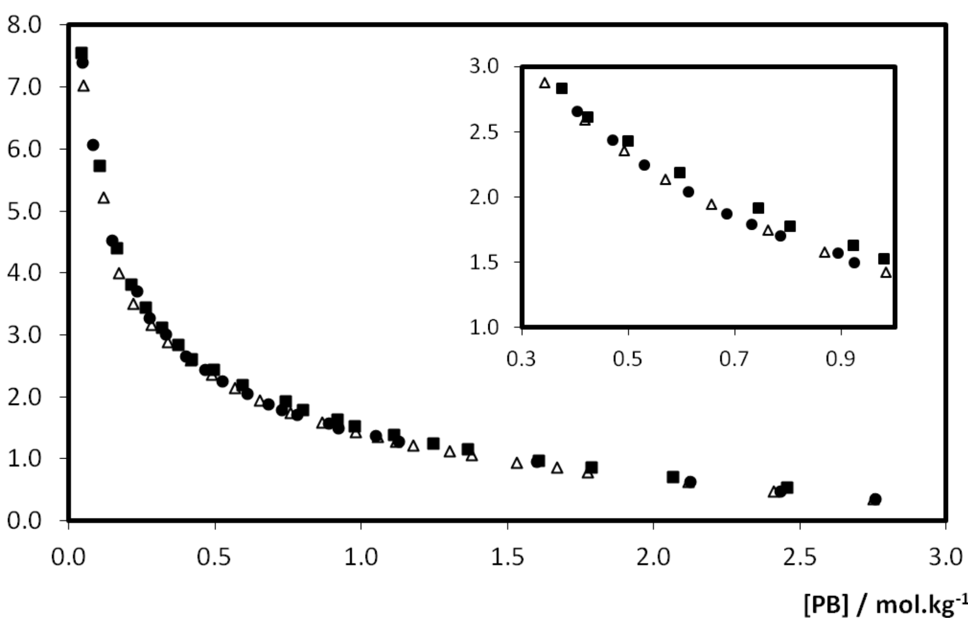

Figure 3.3.5. Phase diagrams for pyridinium-based ILs and the potassium phosphate buffer solution at 298 K: (■), [1-C_4-2-C_1pyr]Cl; (∆) [1-C_4-3-C_1pyr]Cl; (●) [1-C_4-4-C_1pyr]Cl.

From the inspection of Figure 3.3.5, the sequence for the crescent ability of phase separation follows the rank: [1-C_4-2-C_1pyr]Cl < [1-C_4-3-C_1pyr]Cl ≈ [1-C_4-4-C_1pyr]Cl. Still, no major differences in the phase diagrams are observed among the three positional isomers. To the best of our knowledge, there are no references in literature regarding the formation of IL-ATPS using positional isomers, making thus a comparison with other systems impracticable.

The influence of the positional/structural isomerism on the mutual solubilities of ILs with water was discussed in a recent paper by Freire et al. [63] The authors [63] observed that a methyl substitution at the *para* position in a pyridinium ring contributes to a higher increment on the solubility of water in the IL when compared with the respective isomer with a methyl substitution at the *meta* position. This trend was

explained based on the delocalization of the positive charge at the aromatic ring. [63] However, at the water-rich phase, the solubility of the corresponding isomers in water is less dependent on the ability of the aromatic protons to hydrogen bond with the oxygen of water. [63] In this context, both isomers present similar values of solubility in water with only a slightly higher ability of the *para* isomer to be solvated in aqueous media. This slight dependence on the ILs isomerism, and thus on the cation ability to hydrogen-bond with water, in the solubility of the diverse ILs in water, closely correlates with the trends obtained here where no major divergences were observed. Nevertheless, we should be aware that the phosphate buffer is also composed of an inorganic salt (KH_2PO_4) tending for the salting-in regime, and not only by a strong salting-out inducing species (K_2HPO_4). Previously we have shown that low charge density salts lead to the salting-in of an IL in aqueous solution by the occurrence of specific interactions between the ions and the hydrophobic tails of the IL, while the salting-out phenomenon was a result of entropic contributions associated to the preferential formation of the ions' hydration complexes. [64, 65] Therefore, in this work, we believe that a delicate mechanism on the salting-in/-out balance associated to the steric hindrance of the IL is taking place, leading thus to a more complex scenario of dominant interactions.

The experimental binodal curves for the three IL-ATPS formed with K_2HPO_4 and the eleven systems composed of phosphate buffer were correlated using Eq 3.1. The adjusted parameters are reported in Table 3.3.1.

Table 3.3.1. Adjusted parameters (± 10^{-4} g) obtained from the regression of Eq. 3.1 at 298 K and atmospheric pressure.

Ternary System		Regression Parameters		
Salt	**Ionic liquid**	**A**	**B**	**C**
K_2HPO_4	$[1\text{-}C_2\text{-}3\text{-}C_1im]Cl$	76.8955	-0.2896	1.9880×10^{-5}
	$[1\text{-}C_4\text{-}3\text{-}C_1im]Cl$	71.3577	-0.2840	2.9722×10^{-5}
	$[1\text{-}C_6\text{-}3\text{-}C_1im]Cl$	88.9897	-0.3298	3.4743×10^{-5}
K_2HPO_4/KH_2PO_4	$[1\text{-}C_2\text{-}3\text{-}C_1im]Cl$	76.3424	-0.2604	2.1623×10^{-4}
	$[1\text{-}C_4\text{-}3\text{-}C_1im]Cl$	79.4000	-0.2913	8.4900×10^{-6}
	$[1\text{-}C_6\text{-}3\text{-}C_1im]Cl$	80.5229	-0.2934	3.3300×10^{-5}
	$[1\text{-}C_7\text{-}3\text{-}C_1im]Cl$	100.4882	-0.3183	6.8700×10^{-6}
	$[1\text{-}C_8\text{-}3\text{-}C_1im]Cl$	93.6696	-0.3003	2.4360×10^{-5}
	$[1\text{-}C_7H_7\text{-}3\text{-}C_1im]Cl$	109.9598	-0.3722	-1.1490×10^{-5}
	$[1\text{-}C_4\text{-}1\text{-}C_1pyrr]Cl$	125.9078	-0.4682	-4.3490×10^{-5}
	$[1\text{-}C_4\text{-}1\text{-}C_1pip]Cl$	82.6342	-0.3416	8.1200×10^{-6}
	$[1\text{-}C_4\text{-}2\text{-}C_1pyr]Cl$	76.9970	-0.3348	1.5875×10^{-5}
	$[1\text{-}C_4\text{-}3\text{-}C_1pyr]Cl$	76.1169	-0.3428	1.9877×10^{-5}
	$[1\text{-}C_4\text{-}4\text{-}C_1pyr]Cl$	78.2432	-0.3495	1.9715×10^{-5}

The tie-lines, and respective tie-line lengths (TLLs), determined for each system are presented in Table 3.3.2, while Figure 3.3.6 depicts an example on the TLs obtained for the system $[1\text{-}C_8\text{-}3\text{-}C_1im]Cl$ + H_2O + PB. In Figure 3.3.6 it can be observed that higher differences in the mass fraction of both solutes lead to higher TLLs.

Table 3.3.2. Mass fraction compositions for the TLs, and respective TLLs, at the top (*T*) and bottom (*B*) phases, and at the initial biphasic composition of the mixture (*M*), composed of IL (*Y*) and inorganic salt (*X*) at 298 K and atmospheric pressure.

ATPS		Mass fraction / wt %						
Salt	Ionic Liquid	Y_M ± std	X_M ± std	Y_T ± std	X_T ± std	Y_B ± std	X_B ± std	TLL
K_2HPO_4	[1-C_2-3-C_1im]Cl	19.79±0.05	25.08±0.04	2.30±0.44	43.13±0.02	38.53±0.03	5.64±0.18	52.21
	[1-C_4-3-C_1im]Cl	19.97±0.05	24.88±0.04	0.73±1.37	44.83±0.02	39.98±0.03	4.13±0.24	56.54
	[1-C_6-3-C_1im]Cl	20.11±0.05	24.96±0.04	43.26±0.02	4.73±0.21	0.83±0.01	41.80±0.02	56.34
K_2HPO_4 /KH_2PO_4	[1-C_2-3-C_1im]Cl	25.06±0.04	29.98±0.03	46.21±0.02	3.70±0.27	0.04±0.01	61.06±0.02	73.64
	[1-C_4-3-C_1im]Cl	25.11±0.04	29.95±0.03	46.79±0.02	3.29±0.30	1.48±0.68	59.03±0.02	71.83
	[1-C_6-3-C_1im]Cl	25.08±0.04	29.97±0.03	55.31±0.02	1.65±0.61	0.06±0.01	53.48±0.02	75.68
	[1-C_7-3-C_1im]Cl	25.03±0.04	29.99±0.03	45.10±0.02	6.31±0.16	2.70±0.37	56.33±0.02	65.57
	[1-C_8-3-C_1im]Cl	30.11±0.03	20.05±0.05	49.01±0.02	4.62±0.22	1.84±0.54	43.14±0.02	60.91
		34.92±0.03	20.08±0.05	56.95±0.02	2.74±0.36	0.99±1.01	46.78±0.02	71.21
	[1-C_7H_7-3-C_1im]Cl	24.97±0.04	30.01±0.03	28.26±0.04	13.95±0.07	22.65±0.04	41.37±0.02	27.99
	[1-C_4-1-C_1pyrr]Cl	30.07±0.03	15.00±0.07	38.81±0.03	7.42±0.13	23.33±0.04	24.06±0.04	20.87
	[1-C_4-1-C_1pip]Cl	25.09±0.04	29.97±0.03	26.98±0.04	10.94±0.09	22.81±0.02	52.79±0.02	42.06
	[1-C_4-2-C_1pyr]Cl	24.99±0.04	23.02±0.04	44.14±0.02	2.76±0.36	1.29±0.01	48.10±0.02	62.39
	[1-C_4-3-C_1pyr]Cl	24.98±0.04	23.06±0.04	45.23±0.02	2.30±0.43	0.81±0.01	47.84±0.02	63.62
	[1-C_4-4-C_1pyr]Cl	24.97±0.04	23.05±0.04	46.26±0.02	2.26±0.44	1.00±0.01	46.45±0.02	63.26

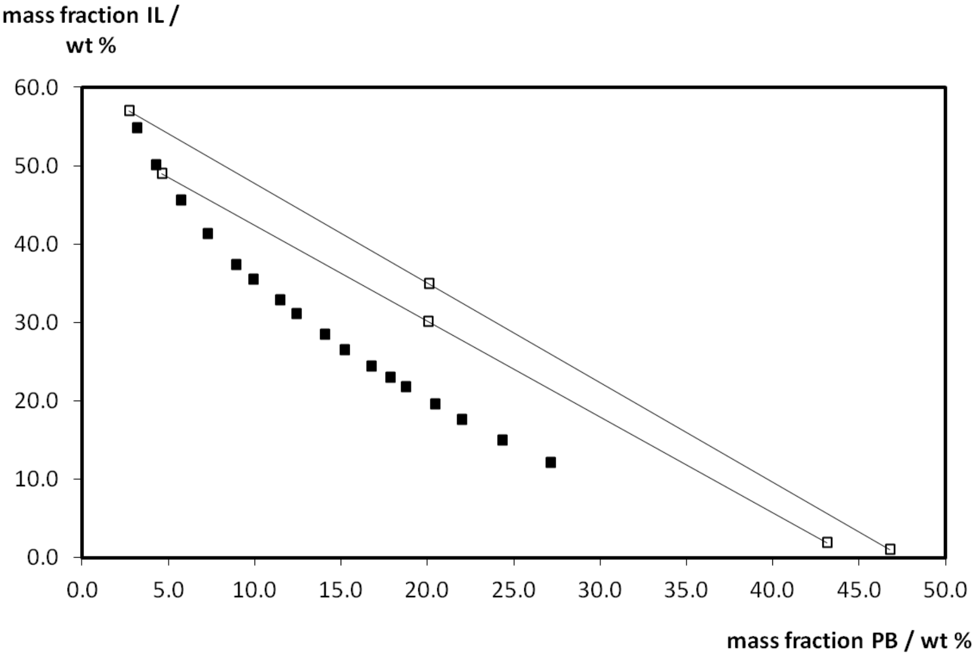

Figure 3.3.6. Phase diagram for the IL [1-C_8-3-C_1im]Cl and the phosphate buffer solution at 298 K: (■) binodal curve data; (□) TL data.

3.3.5. CONCLUSIONS

In this work novel ATPS phase diagrams for eleven ILs, based in piperidinium, pyridinium, pyrrolidinium and imidazolium cores, were determined. Taking into account the phase diagrams behaviour, the effect of the IL cation core, the cation side alkyl chain length, and the positional isomerism at the cation towards the formation ability of ATPS, was presented and discussed. An increase in the length of the aliphatic chain at the cation leads to an increase on the IL ability to form ATPS, when C_2 to C_6 alkyl chains are considered. However, when the number of carbons at the alkyl side chain is higher than 6, there is an inversion on the ILs sequence, due to the self-aggregation of ILs in aqueous phases. Finally, the influence of the cation core and the positional isomerism at the cation were also considered, and in general, the ability of ILs to induce ATPS is more dependent on the size or molecular volume of the ions, and thus more dependent on steric contributions, than on the hydrogen-bonding ability of the diverse cations to interact with water.

3.3.6. REFERENCES

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3.4. PAPER 5

Ionic-Liquid-Based Aqueous Two-Phase Systems With Controlled pH: The Ionic Liquid Anion Effect

3.4.1. ABSTRACT

This work addresses the effect of different anions in imidazolium-based ionic liquids (ILs) on the formation of aqueous two-phase systems (ATPS) using a phosphate buffer solution (KH_2PO_4/K_2HPO_4 mixture of salts) aiming at controlling the pH value of the co-existing aqueous phases. Several ATPS were investigated and the corresponding binodal curves were determined by the cloud point titration method at 298 K. In general, the IL anions aptitude to induce the formation of ATPS increases with the decrease of the ability of the ions to form hydration complexes, *i.e.*, with the decrease on the hydrogen-bond basicity of individual ILs. This trend is in close agreement to that observed in ATPS with different inorganic salts, showing that the ILs rank is not dependent on the salting-out species used.

3.4.2. INTRODUCTION

Liquid-liquid extraction approaches that make use of aqueous two-phase systems (ATPS) are advantageous choices for the development of more environmentally benign and biocompatible separation processes, due to the use of non-volatile compounds and a dominant aqueous media. [1-3] ATPS are typically formed by the concomitant addition of aqueous solutions of two-water soluble polymers or by the addition of a salting-out inorganic salt to a polymer aqueous solution. However, polymer-polymer ATPS have a relatively high cost, are highly viscous, and require longer periods for phase separation. [4] For electrolyte-polymer systems, the high ionic strength of the aqueous media strongly limits their applications in the biotechnological field.

In the past decade, ionic liquids (ILs) have shown great potential as possible replacements for conventional organic solvents, and also as phase tunable additives. The large number of potential anion/cation combinations allows the fine-tuning of the ILs' physical properties, [5] and the design of ILs with an adjustable solubility in water and controllable biocompatibility. [6-8] Those factors are crucial in the ATPS formation ability and on the use of ILs in the biotechnological field as separation/purification agents [9-11].

Since the first work reporting ATPS composed of ILs and inorganic salts, by Rogers and co-workers, [12] the number of publications dealing with IL-based ATPS (IL-ATPS) has been rising in the past few years. In most of these works, the number of ILs

studied is indeed quite limited, with the study of the inorganic salt effect as the major source of interest. Concerning the employed ILs, most works have focused on imidazolium-based ILs, [4, 13-17] and reported the effect of the cation side alkyl chain length [11, 18-21], with the chloride [13, 21-24] and bromide counterions [13, 21, 24-28]. We have been the only authors to address a wide range of IL cations and anions in our previous works, conjugated with the kosmotropic salt K_3PO_4 [19-22, 29-32] and more recently Na_2SO_4. [33, 34]

The K_3PO_4 is the inorganic salt most used as IL-ATPS promoter, followed by hydrogenophosphates, [13, 18, 21, 22, 25, 35] carbonates [9, 13, 18, 21-23, 29, 35, 36], citrates [24, 26-28, 37, 38], or other compounds (weaker salting-out species), such as carbohydrates, [16, 38] and aminoacids. [39, 40] Nevertheless, in most of these systems there is not an adequate control of the pH value of the aqueous phases which is a crucial issue regarding the separation/extraction of particular biomolecules. Only recently, the inorganic phosphate buffer solution composed of K_2HPO_4/KH_2PO_4 has found some interest among researchers within the topic of biomolecules purification. [8, 41] This work, and its companion article, [34] is focused on the capability of forming IL-ATPS making use of a phosphate buffer (PB) - K_2HPO_4/KH_2PO_4 mixture. The major advantage of this salt, over other salting-out inducing salts, relays on the possibility of controlling the pH values of the aqueous phases, what is essential when dealing with biomolecules. Imidazolium-based ILs combined with a wide series of hydrophilic anions were investigated, and the effect of the IL anion on the formation of ATPS is here presented and discussed.

3.4.3. EXPERIMENTAL SECTION

3.4.3.1. Materials

The present study was carried out using aqueous solutions of K_2HPO_4 and KH_2PO_4. The salts have purities higher than 98 % (w/w) and were purchased from Sigma-Aldrich. The ILs used were: 1-ethyl-3-methylimidazolium chloride, [C_2mim]Cl; 1-butyl-3-methylimidazolium chloride, [C_4mim]Cl; 1-butyl-3-methylimidazolium dicyanamide, [C_4mim][$N(CN)_2$]; 1-butyl-3-methylimidazolium methanesulfonate, [C_4mim][CH_3SO_3]; 1-butyl-3-methylimidazolium triflate, [C_4mim][CF_3SO_3]; 1-butyl-3-methylimidazolium acetate, [C_4mim][CH_3COO]; 1-butyl-3-methylimidazolium ethylsulfate [C_4mim][$EtSO_4$]; 1-butyl-3-methylimidazolium tosylate, [C_4mim][TOS]; 1-butyl-3-

methylimidazolium thiocyanate, $[C_4mim][SCN]$; 1-ethyl-3-methylimidazolium methylsulfate, $[C_2mim][MeSO_4]$; 1-ethyl-3-methylimidazolium ethylsulfate, $[C_2mim][EtSO_4]$, 1-ethyl-3-methylimidazolium acetate, $[C_2mim][CH_3COO]$. All ILs were purchased at IoLiTec (Ionic Liquid Technologies, Germany), and presented mass fraction purities higher than 99 %, latter confirmed by us by ^{1}H NMR and ^{13}C NMR. Their ionic structures and respective abbreviations are presented in Figure 3.4.1.

Ultrapure water, double distilled, passed by a reverse osmosis system and further treated with a Milli-Q plus 185 water purification apparatus, was used.

Figure 3.4.1. Ionic structure of the ILs studied.

3.4.3.2. Phase Diagrams and Tie-Lines

The binodal data of the systems composed of IL, water and the phosphate buffer were determined using the cloud point titration method at 298 K (± 1 K). The experimental procedure adopted in this work follows the method already validated by us for ATPS constituted by other ILs and inorganic salts. [20, 32] Aqueous solutions of a phosphate buffer solution (PB) composed with the two inorganic salts K_2HPO_4 and KH_2PO_4 (pH = 7.0; Henderson-Hasselbalch equation equivalents = 1.087) at 40 wt % and of each IL, at *circa* 60 wt %, were previously prepared. Repetitive drop-wise addition of the aqueous

inorganic salt solution to the aqueous solution of IL was carried out until the detection of a cloudy solution, followed by the drop-wise addition of ultrapure water until the detection of a limpid and monophasic phase. The whole procedure was performed under constant stirring. The ternary system compositions were determined by the weight quantification of all components added within ± 10^{-4} g.

The tie-lines (TLs) associated to each phase diagram were determined by a gravimetric method previously described by us [20, 32, 42] and originally proposed by Merchuck et al. [43]. A mixture at the biphasic region was prepared, vigorously stirred, and allowed to reach the equilibrium by the phases separation for at least 24 h and at 298 K, using small ampules (10 cm^3) especially designed for this step. After the separation of the co-existing phases, they were carefully and individually weighed. Each TL was determined by application of the level arm rule. [43] For that purpose, the experimental binodal curves were correlated using the already mentioned Merchuck' equation - Eq 3.1 [43].

3.4.4. RESULTS AND DISCUSSION

The present study addresses the capacity of twelve ILs for the formation of IL-ATPS using a phosphate buffer solution aiming at controlling a neutral pH at the co-existing phases. All phase diagrams were determined at 298 K (± 1 K) and at atmospheric pressure. The binodal curves obtained are depicted in Figure 3.4.2 (for [C_2mim]-based ILs) and Figure 3.4.3 (for [C_4mim]-based ILs). The mass fraction binodal data for all the investigated systems are presented in *Supporting Information Tables S8.3.22, S8.3.27* and *Tables S8.3.33 - S8.2.42*. The experimental binodal curves for the investigated systems were correlated using Eq. 3.1. The obtained regression parameters are reported in Table 3.4.1. The respective tie-lines, and corresponding tie-line lengths (TLLs), are presented in Table 3.4.2.

Table 3.4.1. Adjusted parameters (± 10^{-4} g) obtained from the regression of Eq. 3.1 at 298 K and atmospheric pressure.

Ionic liquid	A	B	C
$[C_2mim]Cl$	76.3424	-0.2604	2.1623×10^{-4}
$[C_4mim]Cl$	79.4000	-0.2913	8.4900×10^{-6}
$[C_2mim][MeSO_4]$	77.2459	-0.1965	2.3104×10^{-5}
$[C_2mim][EtSO_4]$	117.1339	-0.3659	2.1570×10^{-5}
$[C_2mim][CH_3COO]$	113.8498	-0.2700	3.4745×10^{-5}
$[C_4mim][CH_3COO]$	91.3711	-0.3199	9.0045×10^{-5}
$[C_4mim][N(CN)_2]$	83.8177	-0.3589	7.1500×10^{-5}
$[C_4mim][CF_3SO_3]$	167.8747	-0.7390	1.8035×10^{-5}
$[C_4mim][CH_3SO_3]$	178.6973	-0.4364	-5.8154×10^{-6}
$[C_4mim][TOS]$	96.5243	-0.3822	1.0000×10^{-5}
$[C_4mim][SCN]$	89.9972	-0.4288	2.0000×10^{-5}
$[C_4mim][EtSO_4]$	82.2535	-0.2914	3.4903×10^{-5}

Table 3.4.2. Mass fraction compositions for the initial mixture (*M*) and TL data, and respective TLLs, for the top (*T*) and bottom (*B*) phases, composed of IL (*Y*) and inorganic salt (*X*) at 298 K and atmospheric pressure.

Ionic Liquid	Mass fraction composition / wt %						TLL
	Y_M ± std	X_M ± std	Y_T ± std	X_T ± std	Y_B ± std	X_B ± std	
$[C_2mim]Cl$	25.06±0.04	29.98±0.03	46.21±0.02	3.70±0.27	0.04±0.01	61.06±0.02	73.64
$[C_2mim][CH_3COO]$	25.01±0.04	23.00±0.04	36.76±0.03	14.44±0.07	2.49±0.40	39.42±0.03	42.41
$[C_2mim][MeSO_4]$	24.99±0.04	22.99±0.04	41.66±0.02	9.29±0.11	6.10±0.16	38.52±0.03	46.03
$[C_2mim][EtSO_4]$	25.04±0.04	22.99±0.04	45.66±0.02	6.54±0.15	2.58±0.39	40.89±0.03	55.10
$[C_4mim]Cl$	25.11±0.04	29.95±0.03	46.79±0.02	3.29±0.30	1.48±0.68	59.03±0.02	71.83
$[C_4mim][CH_3COO]$	25.22±0.04	22.92±0.04	35.73±0.03	7.84±0.13	0.00±0.01	59.12±0.02	65.50
$[C_4mim][N(CN)_2]$	25.12±0.04	29.95±0.03	69.23±0.02	0.28±3.52	0.00±0.01	46.84±0.02	83.43
$[C_4mim][CF_3SO_3]$	25.08±0.04	29.97±0.03	82.88±0.01	0.91±1.10	0.00±0.01	42.57±0.02	92.77
$[C_4mim][CH_3SO_3]$	25.00±0.04	30.00±0.03	16.25±0.06	45.05±0.02	33.77±0.03	14.92±0.07	34.85
$[C_4mim][TOS]$	29.58±0.03	15.01±0.07	55.51±0.02	2.01±0.50	0.96±0.01	29.36±0.03	61.03
$[C_4mim][SCN]$	19.89±0.05	15.37±0.07	57.76±0.02	1.07±0.94	1.24±0.80	22.41±0.05	60.41
$[C_4mim][EtSO_4]$	17.84±0.06	25.03±0.04	37.27±0.03	7.15±0.14	1.34±0.75	40.21±0.03	48.82

The effect of different IL anions towards the ATPS formation was evaluated making use of the common cation $[C_2mim]^+$ and is presented in Figure 3.4.2. The ability of the ILs anions to induce ATPS formation follows the order: $[C_2mim][MeSO_4]$ ≈ $[C_2mim]Cl$ < $[C_2mim][CH_3COO]$ ≈ $[C_2mim][EtSO_4]$.

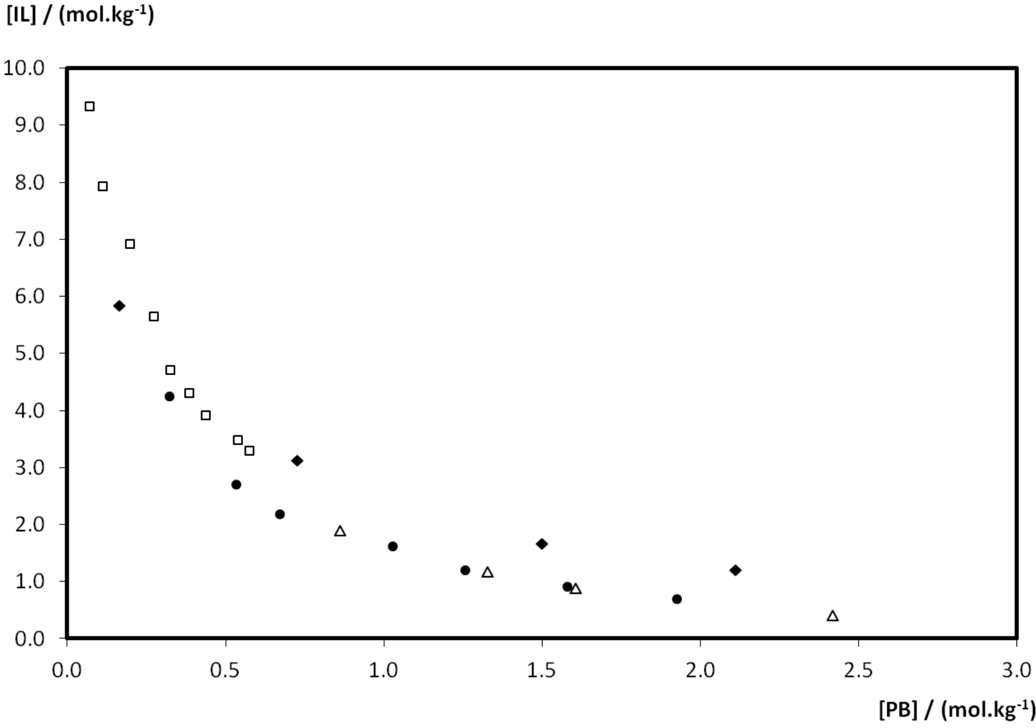

Figure 3.4.2. Phase diagrams for $[C_2mim]$-based ILs and the potassium phosphate buffer solution at 298 K: (◆) $[C_2mim][MeSO_4]$; (●) $[C_2mim][EtSO_4]$; (∆) $[C_2mim][CH_3COO]$; (□) $[C_2mim]Cl$.

In addition, Figure 3.4.3 depicts the binodal curves for $[C_4mim]$-based ILs. Accordingly to the obtained trends, the sequence for the ability of the various ILs for phase separation, at 0.8 $mol.kg^{-1}$, is as follows: $[C_4mim][CH_3SO_3]$ < $[C_4mim]Cl$ < $[C_4mim][CH_3COO]$ < $[C_4mim][EtSO_4]$ < $[C_4mim][N(CN)_2]$ < $[C_4mim][SCN]$ < $[C_4mim][TOS]$ < $[C_4mim][CF_3SO_3]$.

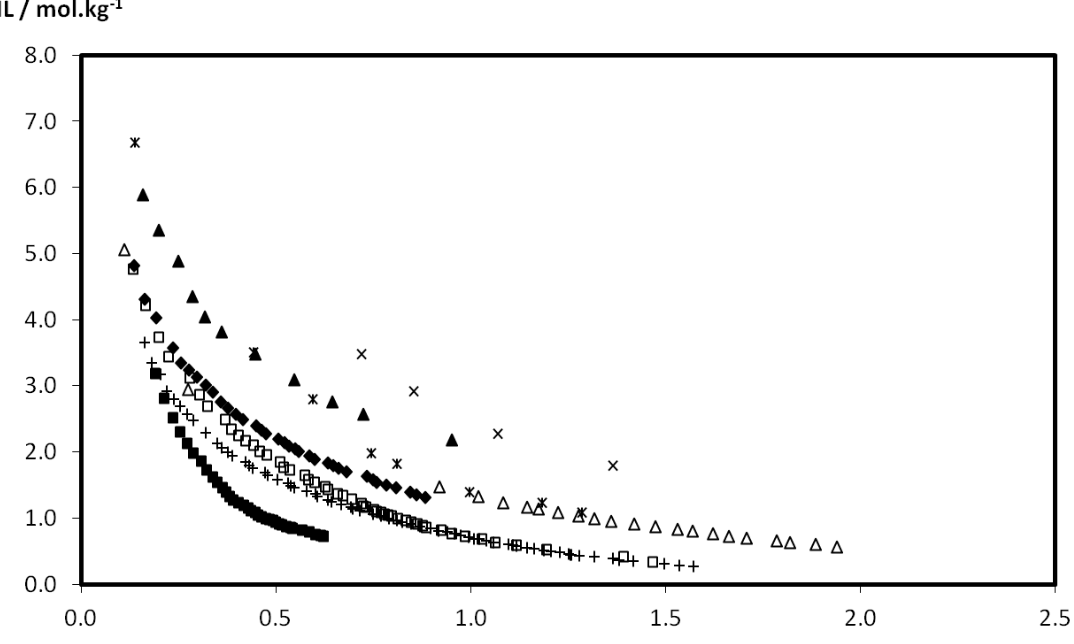

Figure 3.4.3. Phase diagrams for the [C_4mim]-based ILs and the potassium phosphate buffer solution (PB) at 298 K: (×) [C_4mim][CH_3SO_3]; (■) [C_4mim][CF_3SO_3]; (▲) [C_4mim]Cl; (*) [C_4mim][CH_3COO]; (Δ) [C_4mim][$EtSO_4$]; (+) [C_4mim][TOS]; (□) [C_4mim][SCN]; (◆) [C_4mim][$N(CN)_2$].

The anions rank observed for the [C_2mim]-based and the [C_4mim]-based series is in close agreement with the results previously obtained by our group regarding the aptitude of [C_4mim]-based ILs in inducing ATPS with K_3PO_4 and Na_2SO_4 as the main salting-out species [33, 34]. Hence, the cation alkyl chain length does not affect the anion ability in promoting ATPS, and the inorganic salt used does not changes the ILs trend concerning their phase diagrams behavior. Indeed, comparing with the previous results, and although the sequence of the ILs is not altered, the main differences among different inorganic salts relays on their ability to salt-out the IL and on the pH of the aqueous media. Both K_3PO_4 [20, 32] and the K_2HPO_4/KH_2PO_4 mixture are able to salt-out all the ILs analyzed from aqueous solutions. On the contrary, this effect is more pertinent when dealing with Na_2SO_4, that being a neutral salt in aqueous solutions, leads to acidic solutions for most of the imidazolium-based ILs investigated. [33] As a result, this salt was found to be less efficient in the promotion of IL-ATPS indicating that the pH of the aqueous solution has a significant impact on the liquid-liquid demixing.

Indeed, only imidazolium-based ILs with longer alkyl chains and/or with anions with low hydrogen bond basicity are able to undergo the formation of ATPS. [33]

In general, [33, 34] in complex mixtures, as those containing high charge density salts with an enhanced capacity for creating ion-water hydration complexes (such as K_3PO_4, K_2HPO_4 and Na_2SO_4), the capability of the inorganic salt to induce ATPS increases with the decreasing on the ionic liquid affinity for water – because the ionic liquid is salted-out by the inorganic ions. Therefore, more hydrophobic ILs are more easily salted-out due to their poorer affinity for water. These ILs are those composed of long aliphatic side chains at the cation (or the anion) and/or composed of anions with lower hydrogen-bond basicity values (anions with a low hydrogen bond acceptor strength). In this context, the ability of the IL anions to induce ATPS closely correlates with the decrease on the hydrogen-bond basicity (β) values of ionic fluids with common cations, [44-47] and as previously discussed by us. [33, 34] Therefore, IL anions that tend to preferentially interact with the protons of water (and thus, to preferentially create hydration complexes) require more quantities of inorganic salt capable of inducing the liquid-liquid demixing.

Previously we have demonstrated that the salting-out effect of ILs is ruled by entropic contributions that are a direct result of the formation of water-ion hydration complexes and the increase of the surface tension of water. [48, 49] This tendency was previously confirmed with the cation side alkyl chain length and with the cation core influence on the ATPS formation [34, 50]. In general, the larger the molar volume of the cations, the higher is the ability of the IL to form ATPS. In fact, when comparing ILs with aromatic and aliphatic cores no major differences were observed demonstrating that the hydrogen-bonding of the cations with water is not the major driving force in creating ATPS. [50] On the other hand, when analyzing the effect of the IL anion a more complex scenario is at work. Anions are typically more polarizable than cations, due to their more diffuse valence electronic configuration. Hence the anions' hydration is usually stronger than that of cations. [48] In this context, the facility of the anions to hydrogen-bond with water, and hence to form hydration complexes, plays a significant role and is more relevant than that observed with previous works were the effect of the IL cation was investigated [20, 34]. Indeed, this is the main reason behind the strong correlation between the ILs ability to induce ATPS and that of the hydrogen-bond basicity values. When comparing different anions both enthalpic and entropic effects occur and cannot be discarded.

All the interpretations made with the somewhat more complex ternary systems (IL+ water + inorganic salt) are in close agreement with our previous results on binary systems (IL + water). [51-54] Although the increase on the solubilization of an IL cation in water is a direct result of a decrease on the molar entropy of solution (and where no major changes on the molar enthalpy of solution are observed), [51] on the contrary, the solubility of an IL anion in water leads to significant changes in both the molar enthalpy and entropy of solution. [52] In fact, the lower the change in the molar enthalpy of solution, the larger is the IL solubility in water, indicating that it is the solvation of the IL at the IL-rich phase that rules the overall liquid-liquid phase behavior in ATPS. [52]

3.4.5. CONCLUSIONS

Novel phase diagrams of twelve ATPS with a controlled pH were determined by the cloud point titration method at 298 K. The ability of different IL anions on the formation of IL-ATPS in the presence of a phosphate buffer was presented and discussed. Based on the phase diagrams behavior it was observed that the sequence of the ILs, regarding their aptitude for creating ATPS, is in close agreement to that obtained previously with the K_3PO_4 and Na_2SO_4 inorganic salts, and demonstrating that the ILs rank is not dependent on the salting-out species used.

In general, the lower the hydrogen bond basicity of the IL (*i.e.*, less affinity of the IL anion to interact with the protons of water), the higher is the ability of the ionic fluid to undergo liquid-liquid demixing in presence of the phosphate buffer. Contrarily to the IL cation effect on the formation of ATPS, which is mostly governed by steric and entropic contributions, the IL anion influence towards the formation of biphasic systems is a direct result of a delicate balance between enthalpic and entropic contributions.

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3.5. PAPER 6

Design of Ionic Liquids for Lipase Purification

Journal of Chromatography B, 2011, 879, 2679-2687

3.5.1. ABSTRACT

Aqueous two-phase systems (ATPS) are considered as efficient downstream processing techniques in the production and purification of enzymes, since they can be considered harmless to biomolecules due to their high water content and due to the possibility of maintaining a neutral pH value in the medium. A recent type of alternative ATPS is based on hydrophilic ionic liquids (ILs) and salting-out inducing salts.

The aim of this work was to study the lipase (*Candida antarctica* lipase B - CaLB) partitioning in several ATPS composed of ionic liquids (ILs) and inorganic salts, and to identify the best IL for the enzyme purification. For that purpose a wide range of IL cations and anions, and some of their combinations were studied. For each system the enzyme partitioning between the two phases was measured and the purification factors and enzyme recoveries were determined. The results indicate that the lipase maximum purification and recovery were obtained for cations with a C_8 side alkyl chain, the dicyanamide $[N(CN)_2]$ anion and ILs belonging to the pyridinium family. However, the highest purification parameters were observed for 1-methyl-3-octylimidazolium chloride $[C_8mim]Cl$, suggesting that the IL extraction capability does not result from a cumulative character of the individual characteristics of ILs. The results indicate that the IL based ATPS have an improved performance in the lipase purification and recovery.

3.5.2. INTRODUCTION

Enzymes are classified according to the type of reaction they catalyse. Lipases (EC 3.1.1.3) are a sub-class of enzymes within the esterase family whose natural function is to hydrolyse long chain triacylglycerols, such as oils or fats [1]. Lipases are widely available in nature where their main function is to digest lipids in order to make these available as an energy source for cells [2]. Fungi and bacteria secrete lipases to their surroundings to facilitate nutrient absorption from the external medium. There are many potential applications of lipases, as in leather and cosmetics processing, animal feed, pulp and paper processing and textile [3]. However, the most significant industrial applications of lipases are mainly found in the pharmaceutical sector, in food and detergents [4]. The number of enzymes commercially available and their range of applications are gradually increasing. There are many reasons for the growing interest in enzyme-mediated reactions compared to chemical processes, including the high degree of specificity, the mild reaction conditions and a lower probability of occurring side

reactions. Furthermore, enzyme-mediated processes are energy saving and reduce the extent of thermal degradation. A very useful trait of lipases is their enantio-selectivity, a very useful characteristic in the pharmaceutical area [5]. Some other attributes are also highlighted in literature, such as, their regio-selectivity and broad substrate specificity [6].

The microbial lipases are produced through a fermentation process, in which secondary or intermediate products are also generated. These frequently prevent the use of the fermented broth in industrial procedures or are deleterious to the enzyme. For the success of commercial production of enzymes and proteins, efficient downstream processing techniques are essential. Moreover, when these processes are applied to biological materials, rigorous purification steps, delicate enough to preserve the biological activity, are required. The most common techniques used are the ammonium sulphate precipitation [7], ionic and affinity chromatography [8, 9], dialysis, filtration, electrophoresis [10-13] and reverse micelles approaches [14, 15]. Nevertheless, some of these separation processes are costly and time consuming, and are not easily scalable [7]. Among the various separation techniques, liquid-liquid (solvent) extraction is one of the best-known, well-established, versatile and easy to use. Moreover, liquid-liquid extraction allows for a good resolution and high yield, a relatively high capacity, easy scale-up and requires low material cost. However, most of the extraction techniques employ organic solvents, which are immiscible with water, volatile, flammable and health hazardous. This makes these systems of extraction inappropriate for the development of environmental-friendly technologies. Another problem with conventional solvents is their limited number, making difficult to find the ideal solvent suited for a particular application, even considering solvent mixtures.

In times of improved molecular engineering and protein design, the development of new and biocompatible extraction methods, for the separation and purification of enzymes and proteins, is gaining increasing importance. An effective and economically viable method for the separation and purification of biomolecules is their partition in an aqueous two-phase system (ATPS). ATPS phase separation occurs on mixing two mutual incompatible solutes in water by which two aqueous phases are formed with different compositions. Solutes distribute themselves between the two phases depending on their relative affinity for each one of the individual phases. This technique is widely used in biomolecules purification where its advantages include favourable selectivity, low cost and adaptability to continuous sample processing and retention of biological

activity [16, 17]. Nonetheless, most of polymer-based ATPS display high viscosity [18, 19]. Finally, these polymer ATPS normally form opaque aqueous solutions, which could interfere with the quantitative and qualitative analysis of extracted compounds.

During the last few years, ILs have been applied for bio-purification and bio-extraction processes, and advantages, such as the improvement of enzyme stability, substrate and/or product selectivity, and suppression of side reactions were observed [20-25]. Moreover, IL-based ATPS are substantially less viscous than typical polymer-based ATPS [26, 27]. Recently we reported viscosity data for various imidazolium-based ATPS where it was observed that the viscosities of the IL-rich phases (3.23 - 8.05 $mPa.s^{-1}$) are similar to those of the salt-rich phases (2.00 - 4.76 $mPa.s^{-1}$) and much lower than those of polymer based ATPS [26].

Most water-soluble room temperature ILs are salting-in inducing electrolytes (usually known as “chaotropic” salts) that can form a second aqueous phase in the presence of aqueous solutions of salting-out inorganic salts (“kosmotropic” salts) resulting in the formation of ATPS [28-33]. In aqueous systems composed of IL, inorganic salt and water, the driving force for phase separation is the competition between the IL and the salt for water molecules. The higher affinity of the inorganic salt for water induces a migration of water away from the IL ions decreasing their hydration and reducing the ILs solubility in water [34, 35]. The inorganic salts commonly employed are ammonium-, potassium- or sodium-based salts of multiply charged anions, such as phosphate [21, 36, 37], sulphate [36], carbonate [36, 37], or citrate [38] (strong salting-out inducing anions). In the past few years, the [38] applications of IL-based ATPS included extraction studies with a significant number of low molecular mass compounds, such as L-tryptophan [31, 39], vanillin [26], alkaloids [27, 37, 40] and steroids, such as testosterone and epitestosterone [41]. The partition coefficients for these systems range from 10 to 120 with recoveries up to complete extraction in a single step extraction[40]. Moreover, the IL-rich aqueous phase is compatible with liquid chromatography and detection limits of spectroscopic techniques, and thus suitable for analyzing biological fluids, as human urine samples [40]. Besides the studies involving ILs and inorganic salts, recently an imidazolium-derivatized poly(ethylene glycol) (PEG) was used in the ATPS formation aiming the extraction of penicillin (recovery around 96%) [42]. Recently, the use of ILs as adjuvant promoters of polymer-based ATPS has been suggested [43].

The extraction of biomacromolecules, such as enzymes and proteins, by water-insoluble ILs is limited by their low solubility in ILs and by the possibility of extracting them without activity loss. ATPS containing water-soluble ILs are likely to be more suitable for the isolation of proteins [7, 36, 44-52]. ATPS formed by ILs and inorganic salts showed higher recoveries of model proteins than methods employing PEG and salt systems [7, 36]. Pei et al. [7, 51] studied the partitioning behaviour of bovine serum albumin (BSA) at different temperatures in the systems 1-butyl-3-methylimidazolium bromide $[C_4mim]Br/K_2HPO_4$ and 1-butyl-3-methylimidazolium dicyanamide $[C_4mim][N(CN)_2]/K_2HPO_4$ obtaining a purification factor of 6.96 and an extraction efficiency of 82.7 - 100.7%, respectively, although the simultaneous extraction of BSA and saccharides was described [51]. Du et al. [36] and Deive et al. [46] reported some studies with ATPS applied to the extraction of proteins such as BSA [36] and *Thermomyces lanuginosus* [46], respectively. Du et al. [36] showed that the enrichment factor of BSA in 1-butyl-3-methylimidazolium chloride $[C_4mim]Cl$ + K_2HPO_4 ATPS was found between 5 and 20 with extraction efficiencies higher than 90%. Deive et al. [46] found recuperation values for the lipase *T. lanuginosus* at the IL-rich phase between 20% and 80% using the 1-etyl-3-methylimidazolium ethylsulfate $[C_2mimEtSO_4]$ + K_2CO_3 system. Dreyer et al. [47, 48] reported the partition coefficients for trypsin (23.48), lysozym (17.98), myoglobin (3.17) and an alcohol dehydrogenase from *Lactobacillus brevis* (3.0) for Ammoeng^{TM} 110 + K_2HPO_4/KH_2PO_4 based ATPS (pH 7.0). The protein penicillin G was used by Liu et al. [49, 50], in two different reports, with extraction yields in the range of 90.8-93.7% using 1-butyl-3-methylimidazolium tetrafluoroborate $[C_4mim][BF_4]$ and $NaH_2PO_4.2H_2O$ ATPS (pH 4-5). Finally, Cao et al. [44] described the efficiency of extraction for horseradish peroxidase using the system $[C_4mim]Cl$ + K_2HPO_4 (more than 80%). Through literature it was observed that the study of different systems was scarce and mainly focused in $[C_4mim]$ as the cation core and Cl as the principal anion. The IL-based ATPS also displayed better process properties, such as little emulsion formation, fast phase separation [7, 26, 36] and low toxicities. IL-based ATPS are formed using hydrophilic ILs. Since the toxicity of ILs is directly related with their hydrophobicity [53-55], this means that these ILs have, at least, lower toxicities than their more hydrophobic counterparts [56].

The results obtained so far for the extraction of both low and high molecular mass compounds by IL-based ATPS are very promising and indicate a general potential for

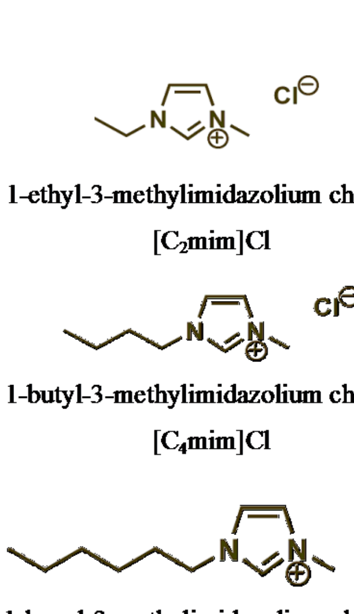
wider use. In the present work, the partition coefficients of *Candida antarctica* lipase B (CaLB) in various IL-based ATPS were investigated. The study of the optimization of the IL structural features for the lipase purification was carried out employing imidazolium-, pyrrolidinium-, pyridinium- and piperidinium-based ILs combined with several anions (chloride, dicyanamide, methanesulfonate and triflate). Moreover, the cation alkyl chain length and the addition of functional groups to the cation were also evaluated.

3.5.3. MATERIAL AND METHODS

3.5.3.1. Material

The present study was carried out using aqueous solutions of K_3PO_4, K_2HPO_4 and KH_2PO_4 with purities higher than 98 % (w/w). All salts were purchased at Sigma-Aldrich. All ILs were acquired at IoLiTec (Ionic Liquid Technologies, Germany) with mass fraction purities higher than 98 %, confirmed by us using ^{1}H-NMR, ^{13}C-NMR and ^{19}F-NMR. Their molecular structures and respective names and abbreviations are reported in Figure 3.5.1.

The enzyme used throughout this work was lipase B of *Candida antarctica* (CaLB) which was a courtesy from Novozymes. The arabic gum (acacia tree) and the reagent dye coomassie brilliant blue G-250 were acquired from Fluka (http://www.fluka.org/fluka.php). Potassium hydroxide and the protein bovine serum albumin (BSA, purity = 97 %) were obtained from Merck. Finally, the molecular mass standard (LMW-SDS Marker Kit 14-97 kDa) was acquired from GE Healthcare.



1-ethyl-3-methylimidazolium chloride

$[C_2mim]Cl$

1-butyl-3-methylimidazolium triflate

$[C_4mim][CF_3SO_3]$

1-butyl-3-methylimidazolium chloride

$[C_4mim]Cl$

1-butyl-3-methylimidazolium methanesulfonate

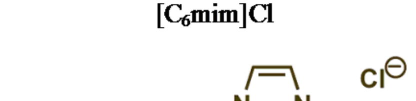
$[C_4mim][CH_3SO_3]$

1-hexyl-3-methylimidazolium chloride

$[C_6mim]Cl$

1-butyl-1-methylpyrrolidinium chloride

$[C_4mpyrr]Cl$



1-heptyl-3-methylimidazolium chloride

$[C_7mim]Cl$

1-butyl-3-methylpyridinium chloride

$[C_4mpyr]Cl$

1-benzyl-3-methylimidazolium chloride

$[C_7H_7mim]Cl$

1-butyl-1-methylpiperidinium chloride

$[C_4mpip]Cl$

1-methyl-3- octylimidazolium chloride

$[C_8mim]Cl$

1-octylpyridinium dicyanamide

$[C_8pyr][N(CN)_2]$

1-butyl-3-methylimidazolium dicyanamide $[C_4mim][N(CN)_2]$

Figure 3.5.1. Chemical structure of the ILs studied.

3.5.3.2. Phase diagrams and Tie-lines

The binodal data were determined for all the ILs studied using the cloud point titration method [31, 39] at 298 (± 1 K). Those ATPS used were all described in detail in the **Sections 3.3.3.2** and **3.4.3.2**.

3.5.3.3. Partitioning of the lipolytic enzyme

All the partitioning systems were prepared from ATPS composed of 25 wt% of IL + 30 wt% of the phosphate buffer aqueous solution + 0.4 wt% of CaLB (the total mass of the extraction systems prepared is 5.0 g). All systems were prepared in graduated glass centrifuge vials of *circa* 10 cm^3. After the ATPS preparation, the mixture was gently stirred and centrifuged at 2500 rpm. The graduated tubes of centrifuge were placed at 298.00 (± 0.01) K, for at least 12 h to reach equilibrium, using a Julabo F25 water bath. After reaching equilibrium, both phases were carefully separated. The two aqueous phases were then cautiously collected for the determination of their volume and weight.

In this study, the partition coefficient was defined as the ratio of protein concentration or enzyme activity in the bottom and top phases, as described by Eqs. (3.3) and (3.4),

$$K_P = \frac{C_T}{C_B} \qquad \text{Eq. 3.3}$$

$$K_E = \frac{EA_T}{EA_B} \qquad \text{Eq. 3.4}$$

where C_T and C_B are, respectively, the total protein concentration ($mg.mL^{-1}$) in the top and bottom phases, and EA_T and EA_B are the enzyme activity ($U.mL^{-1}$) of the top and bottom phases, respectively. These partition experiments were carried out in triplicate, being the results reported in this work the average of the three assays. It should be remarked that for all studied ATPS, the top phase is the IL-rich phase while the bottom phase is the phosphate-buffer-rich phase. To further evaluate this process as a purification technique, the enzyme specific activity (SA, expressed in $U.mg^{-1}$ of protein), the enzyme (R_B^E) and protein (R_B^P) recovery in bottom phase, and the purification factor (PF) were calculated for all systems as,

$$SA = \frac{EA}{C} \qquad \text{Eq. 3.5}$$

$$R_B^E = \frac{100}{1 + R_V K_E} \qquad \text{Eq. 3.6}$$

$$R_B^P = \frac{100}{1 + R_V K_P}$$ Eq. 3.7

$$PF = \frac{SA}{SA_i}$$ Eq. 3.8

where R_V represents the volume ratio between V_T and V_B that are, respectively, the volumes of top and bottom phases. The enzyme specific activity (SA) (Eq. 3.5) can be evaluated for both phases through the ratio of the enzyme activity (EA) and the protein concentration (C) in each one of the phases. The purification factor (PF) was calculated by the ratio between the SA after and before (SA_i) the extraction procedure.

3.5.3.4. Enzymatic Activity

Lipolytic activities were assayed using the oil emulsion method according to a modification proposed by Soares et al. [57]. The substrate was prepared by mixing 50 mL of olive oil with 50 mL of an aqueous solution of arabic gum (7 % (w/v)). The reaction mixture containing 5 mL of substrate, 2 mL of sodium phosphate buffer (100 mM and pH 7.0) and enzyme extract (1 mL) was incubated in a thermostatic batch reactor operating at 310.0 K, at 100 rpm, and during 5 min. After 5 minutes of reaction, an aliquot of ≈0.33 g was taken and added to 2 mL of a solution composed of acetone-ethanol-water (1:1:1). The exact weight of each aliquot was determined before at the end of the addition procedure. The fatty acids produced were titrated with a potassium hydroxide solution (40 mM) using a phenolphthalein solution. A blank titration was done on a sample where the enzyme was replaced by distilled water. One unit (U) of enzyme activity was defined as the amount of enzyme that produces 1 μmol of free fatty acid *per* minute ($\mu mol.min^{-1}$) under the assays established conditions (310 K, pH 7.0, 100 rpm). The mentioned experimental techniques followed the protocols previously described by Carvalho et al. [58]. The possibility of the hydrolysis of olive oil by the ILs was tested in control essays at the measurement conditions in absence of enzyme and verified to be negligible.

3.5.3.5. Protein Determination

Total protein concentration was determined by the Bradford's method [59], using a SHIMADZU UV-1700, Pharma-Spec Spectrometer, of 595 nm, and a calibration curve

previously established for the standard protein bovine serum albumin - BSA. To cancel the influence of the ILs presence on the protein concentration analysis, a control system for each IL-based ATPS without enzyme was prepared under the same conditions. Equilibrium conditions (24 h, 298 K) and the phase separation procedure were those previously described.

3.5.3.6. Polyacrylamide Gel Electrophoresis

Equivalent amounts of CaLB from each sample were subjected to electrophoresis on a 12.5 % sodium dodecyl sulphate-polyacrylamide gel (SDS-PAGE) and overlaid with a 4 % stacking gel as described by Laemmli [60] on a Bio-Rad mini-PROTEAN electrophoresis system. Gels were then stained with colloidal comassie blue. Digital gel images were acquired in a GelDoc system. Those assays were performed at 160 V for 1 h. The molecular mass standard (LMW-SDS Marker Kit 14-97 kDa) was composed of phosphorylase b (97 kDa), albumin (66 kDa), ovalbumin (45 kDa), carbonic anhydrase (30 kDa), trypsin inhibitor (20.1 kDa) and α-lactalbumin (14.4 kDa).

3.5.4. RESULTS AND DISCUSSION

3.5.4.1. Phase Diagrams

Phase diagrams are required for the design of aqueous two-phase extraction processes and for the development of models able to predict the partitioning of solutes between the two phases. Aiming at a better definition of the biphasic region compositions, adequate for enzyme partitioning and purification, the experimental phase diagrams at 298 K and atmospheric pressure, for all the systems used to extract the enzyme (inorganic salts + ILs + H_2O) were determined and are presented in **Sections 3.3.4** and 3.4.4 and in literature [31, 39, 61, 62]. These diagrams allow for a comprehensive understanding of the impact of the salts and ILs nature on the formation of ATPS. The mass fraction data for binodal curves and respective tie-lines are provided in literature [31, 39, 61, 62].

The binodal curves of the ATPS formed by the various phosphate-based salts, K_3PO_4, K_2HPO_4 and phosphate buffer solution (PB or KH_2PO_4/K_2HPO_4) with the IL [C_4mim]Cl are presented in Figure 3.5.2 (see *Supporting Information* with the experimental weight fraction data – *Tables S8.3.7, S8.3.20 - S8.3.27*).

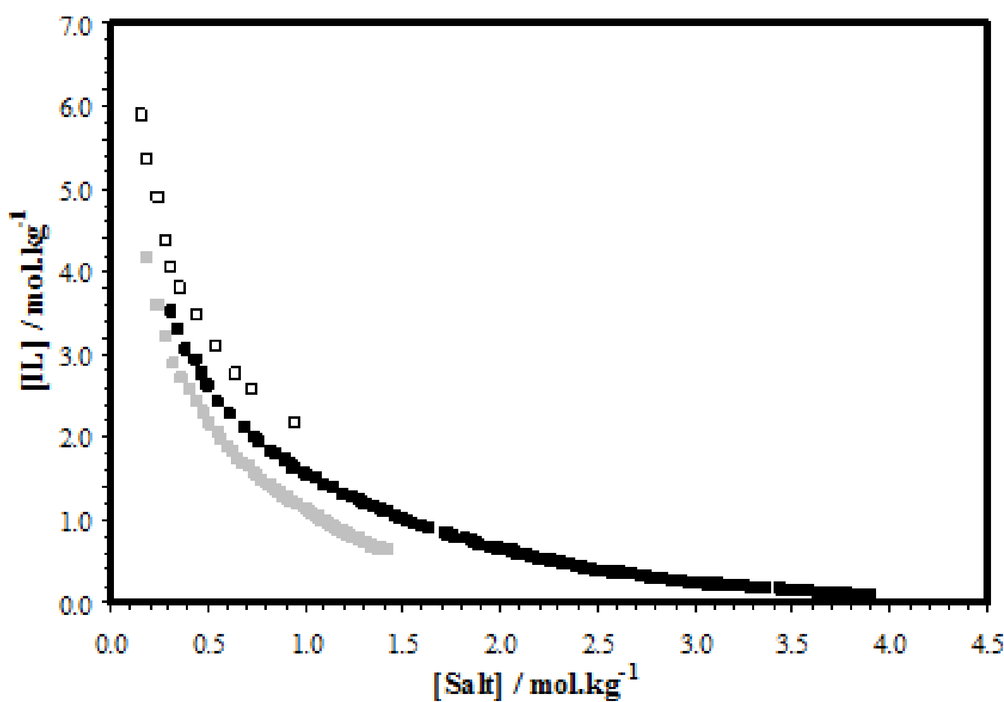


Figure 3.5.2. Phase diagrams for the [C_4mim]Cl and different potassium–phosphate-based salts ATPS at 298 K. (□) K_2HPO_4/KH_2PO_4 (pH = 7.0); (■) K_2HPO_4; and (■) K_3PO_4.

Despite their higher solubility in water and thus higher ability to promote the phase separation, the aqueous solutions formed by K_3PO_4 and K_2HPO_4 presented higher pH values, 13.7 and 9.1, respectively, which are undesirable when the objective is their application to enzymatic processes, due to the deleterious effect of the pH in some enzyme structures which could cause the decrease of their activity or even their inactivation [19]. Because KH_2PO_4 exhibits a weaker interaction with water molecules than the other phosphate-based salts, it is not capable by itself to promote the formation of IL-based ATPS [41]. Instead, a buffer composed of K_2HPO_4/KH_2PO_4, to achieve pH 7.0, was used and showed to be able to induce ATPS in several IL aqueous solutions. The influence of the three salts in the binodal curves with the same IL follows the Hofmeister series: K_3PO_4 > K_2HPO_4 > K_2HPO_4/KH_2PO_4. Strong salting-out inducing anions, PO_4^{3-} and HPO_4^{2-}, exhibit a better capability for creating ion-hydration complexes by excluding water from the IL-rich phase, favoring the formation of ATPS. The K_2HPO_4/KH_2PO_4 buffer has a lower ability for the formation of ATPS but was

used preferentially in this study due to the neutral pH value achieved. The remaining ATPS used for the separation tests, were already described in **Sections 3.3**. and **3.4**.

3.5.4.2. Lipase partition on IL based ATPS

The ATPS discussed in Papers 4 and 5 were used to study the partitioning of *C. antarctica* lipase B-CaLB (Lipozyme CALB). The 2D electrophoresis analysis, reported in Figure 3.5.3, shows the presence of residual contaminant proteins on the commercial enzyme used. The two lanes of this SDS-PAGE correspond to the molecular mass standards (Lane A) and CaLB (Lane B). The presence of multiple light bands confirms the presence of the CaLB enzyme (numbered with 1 in Figure 3.5.3) and further contaminant proteins, represented by the remaining bands (numbered with 2-5 in Figure 3.5.3.). The enzyme used in this work has a molecular weight of 35.3 kDa, that is consistent with what has been reported in literature by Ooi et al. [63] (39.5 kDa for lipase by *Burkholderia pseudomallei*) and Snellman et al. [6] (33 kDa for lipase by *Acinetobacter* sp.). Since the commercial enzyme was semi-purified, the expectable purification is thus limited.

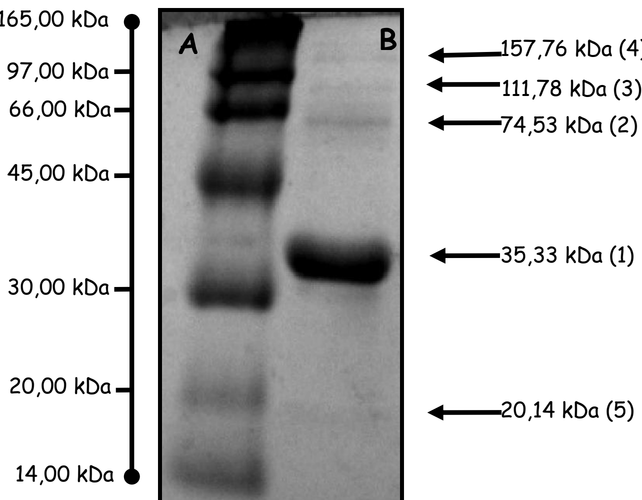


Figure 3.5.3. Sodium dodecyl sulphate-polyacrylamide gel (SDS-PAGE) patterns of CaLB. Lane A: molecular mass standard (14-97 kDa), Lane B: CaLB; 12 % acrylamide gel stained with coomassie blue R-250.

Nevertheless, the improvement in the purification factor using the ATPS here studied is reported in Tables 3.5.1 to 3.5.3 along with the partition coefficient of the enzyme, as well as the total protein, between the two phases.

The partition coefficients and purification efficiencies were determined at the biphasic region of each ATPS at the following composition: 25 % (w/w) of IL + 30 % (w/w) of KH_2PO_4/K_2HPO_4+ 45 % (w/w) of water. The results shown in the last column of Tables 3.5.1 to 3.5.3 suggest that the main IL feature that controls the enzyme purification is the IL cation alkyl chain length, followed by the anion and finally, by the cation core.

Table 3.5.1 shows that the increase in the alkyl chain length induces a stronger increase in K_P than on K_E that remains consistently very low. The increase in the alkyl chain length makes the IL more hydrophobic, decreasing the Coulombic interactions and increasing the dispersive forces that occur between the proteins and the IL at the IL-rich phase. These factors seem to favor the partitioning of the contaminant proteins into the IL-rich phase. Due to its low isoelectric point (p*I* = 6.0), the lipase is negatively charged at these pH conditions (pH 7.0) [64, 65] which results in the increase of its hydrophilic character, creating a higher affinity of the enzyme for the salt-rich phase. The differences observed in the partitioning behavior between the contaminant proteins and the enzyme, must result from differences in their hydrophobicity/hydrophilicity that will allow the partition of the enzyme and contaminant proteins into different phases. The highest value observed for the purification factor was 2.6 ± 0.1 (for $[C_8mim]Cl$). The enzyme recovery efficiencies obtained for all systems were consistently above 96%. Note that in this work the increase in purity and the PF is entirely defined by the increase in the enzyme activity. The presence of IL in the medium is reported to have a deleterious effect, though not very significant at this concentration level, on the enzyme activity. The increase in the PF must thus results mainly from the removal of the contaminant proteins, which are acting as enzymatic inhibitors.

Table 3.5.1. Effect of the IL cation side alkyl chain length in the partition and purification of CaLB, using 25 % (w/w) of IL and 30 % (w/w) of potassium phosphate buffer solution, at pH 7.0 and 298 K.

Ionic Liquid	R_v ± std	K_P ± std	R_B^P ± std (%)	K_E ± std	R_B^E ± std (%)	PF ± std (fold)
$[C_2mim]Cl$	1.6±0.3	0.07±0.02	89±3	0.006±0.002	99.096±0.06	1.05±0.04
$[C_4mim]Cl$	1.4±0.2	0.133±0.009	85±1	0.02±0.01	98±1	1.10±0.03
$[C_6mim]Cl$	1.1±0.2	0.184±0.003	90.954±0.06	0.015±0.002	98.1±0.2	1.068±0.004
$[C_7mim]Cl$	1.1±0.1	0.5±0.1	65±6	0.017±0.003	98.2±0.4	1.48±0.05
$[C_8mim]Cl$	0.9±0.1	1.6±0.2	40±3	0.046±0.001	95.9±0.2	2.6±0.1
$[C_7H_7mim]Cl$	0.97±0.03	0.16±0.01	85.1±0.7	0.031±0.003	97.1±0.2	1.14±0.03

The effects of both the IL anion and cation nature in the enzyme partitioning are described in Tables 3.5.2 and 3.5.3. Both the anion and cation nature were found to promote a small effect in the enzyme purification. However, the most effective anion studied was $[N(CN)_2]$ with K_p = 0.36 ± 0.07 and PF = 1.35 ± 0.08, while the most efficient cation evaluated was $[C_4mpyr]$ with K_p = 0.24 ± 0.02 and PF = 1.28 ± 0.08. The higher K_p and PF results are related with the enhanced hydrophobic character of IL-rich phases composed of such ions. The hydrogen-bond basicities (β) of ILs [66] are helpful in the interpretation of the anion influence results reported in Table 3.5.2. The increase in β values promotes the crescent organization of water molecules around the ionic liquid which in turn leads to an increase in the water content at the IL-rich phase and in the volume ratio (Table 3.5.2). In opposition to the volume ratio, the partition coefficients do not closely follow the ILs hydrogen-bond basicity. Those differences could be explained by the difference in the hydrophobic nature of the two phases, and on the IL anion ability to interact with the remaining components of the system. In what concerns the purification factor, the results suggest that the IL anion influence was not highly significant as stated before. Nevertheless, $[N(CN)_2]$ was found to be the most effective anion, among the studied IL anions. In this case, the partition system was represented by $K_E \ll 1$, a high K_p value and an enzyme bottom recovery of (99.5 ± 0.5)%.

Table 3.5.2. Effect of the IL anion nature in the partition and purification of CaLB, using 25 % (w/w) of IL and 30 % (w/w) of potassium phosphate buffer solution, at pH 7.0 and 298 K.

Ionic Liquid	R_v ± std	K_P ± std	R_B^P ± std (%)	K_E ± std	R_B^E ± std (%)	PF ± std (fold)
$[C_4mim][CF_3SO_3]$	0.50±0.04	0.10±0.03	95±1	0.017±0.003	98.6±0.4	1.05±0.05
$[C_4mim][N(CN)_2]$	0.76±0.02	0.36±0.07	75.1±0.8	0.011±0.002	99.5±0.5	1.35±0.08
$[C_4mim][CH_3SO_3]$	1.20±0.05	0.18±0.04	92.6±0.3	0.028±0.003	98.4±0.1	1.11±0.07
$[C_4mim]Cl$	1.4±0.2	0.133±0.009	85±1	0.02±0.01	98±1	1.10±0.03

Table 3.5.3 shows the results for the influence of distinct cation types. One more time, the results of the purification factors shows the minor effect of the cations (0.998 ± 0.002 < PF < 1.28 ± 0.08), indicating a mild removal of contaminants proteins from the enzyme solution. Despite the low purification values, the most effective cation was $[C_4mpyr]$. In what concerns the partitioning parameters, all the studied cations led to K_E << 1 indicating the higher affinity of the enzyme for the bottom phase (inorganic salt-rich phase). This was also reflected on the high enzyme recovery at this phase ($97.5 \pm 0.1 < R_B^E \approx 98.2 \pm 0.9$)%.

From the aforementioned ILs, the structural characteristics, which maximize the purification factor with high recuperations were selected and further investigated: the pyridinium cation core, the dicyanamide anion and the alkyl side chain with 8 carbons, which were tailored as $[C_8pyr][N(CN)_2]$ (Figures 3.5.4 and 3.5.5 and Table 3.5.3).

Table 3.5.3. Effect of the IL cation core in the partition and purification of CaLB, using 25 % (w/w) of IL and 30 % (w/w) of potassium phosphate buffer solution, at pH 7.0 and 298 K.

Ionic Liquid	R_v ± std	K_P ± std	R_B^P ± std (%)	K_E ± std	R_B^E ± std (%)	PF ± std (fold)
[C_4mpyrr]Cl	1.63±0.09	0.15±0.02	80.8±0.8	0.0152±0.0002	97.5±0.1	1.15±0.05
[C_4mim]Cl	1.4±0.2	0.133±0.009	85±1	0.02±0.01	98±1	1.10±0.03
[C_4mpyr]Cl	1.64±0.07	0.24±0.02	71.3±0.5	0.009±0.002	98.0±0.9	1.28±0.08
[C_4mpip]Cl	1.67±0.04	0.20±0.03	80±4	0.0076±0.0003	98.2±0.9	1.16±0.05
[C_8pyr][N(CN)$_2$]	0.6±0.0	0.00±0.00	100.00±0.00	0.003±0.002	99.8±0.2	0.998±0.002

The first step was to determine the binodal curve for the system [C_8pyr][N(CN)$_2$] + H_2O + K_2HPO_4/KH_2PO_4 (see binodal data in *Supporting Information Tables S8.3.43*). In this context, this binodal curve was compared in Figure 3.5.4 with some other curves previously determined [62, 67]. The ILs sequence regarding their ability for ATPS formation (Figure 3.5.4) with the K_2HPO_4/KH_2PO_4 buffer can be described as follows: [C_4mim]Cl < [C_8mim]Cl < [C_4mpyr]Cl < [C_4mim][N(CN)$_2$] < [C_8pyr][N(CN)$_2$].

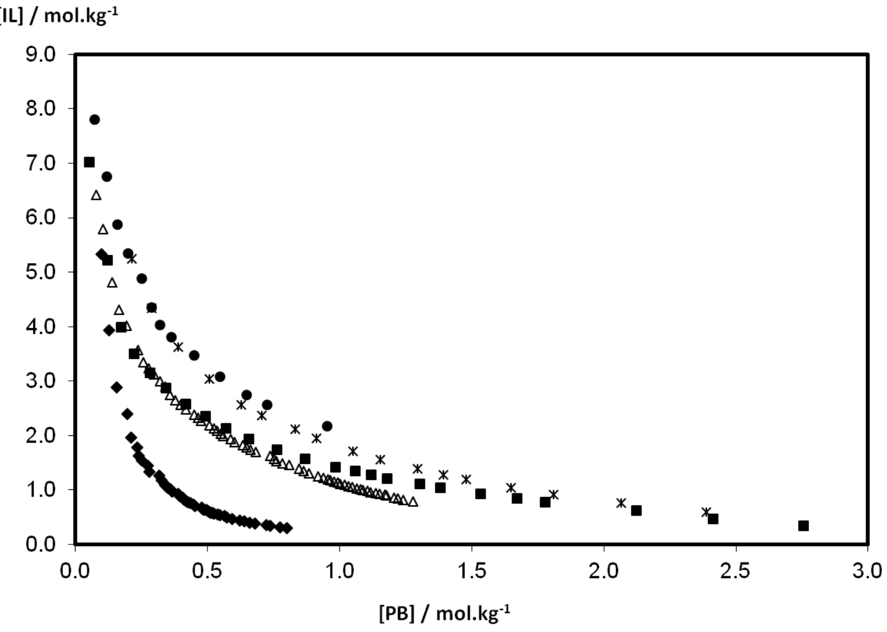


Figure 3.5.4. Phase diagrams for different ILs and K_2HPO_4/KH_2PO_4 ATPS (pH = 7.0) at 298 K. (∗) [C_8mim]Cl; (●) [C_4mim]Cl; (■) [C_4mpyr]Cl; (Δ) [C_4mim][N(CN)$_2$]; (◆) [C_8pyr][N(CN)$_2$].

To complete the phase diagram for the system containing $[C_8pyr][N(CN)_2]$, three different tie lines were additionally determined - Figure 3.5.5 and *Tables S8.3.44 - S6.8.45* from *Supporting Information*. The results indicate that, as previously observed for other systems [31, 39, 43], for shorter tie-line length (TLLs) the TLs were approximately parallel, whereas for longer TLLs the tie-line slopes started to deviate. These deviations in the TLs slopes were in agreement with literature [31, 39, 43] and were related with the fact that the salt-rich phase was increasingly free of IL at longer TLLs.

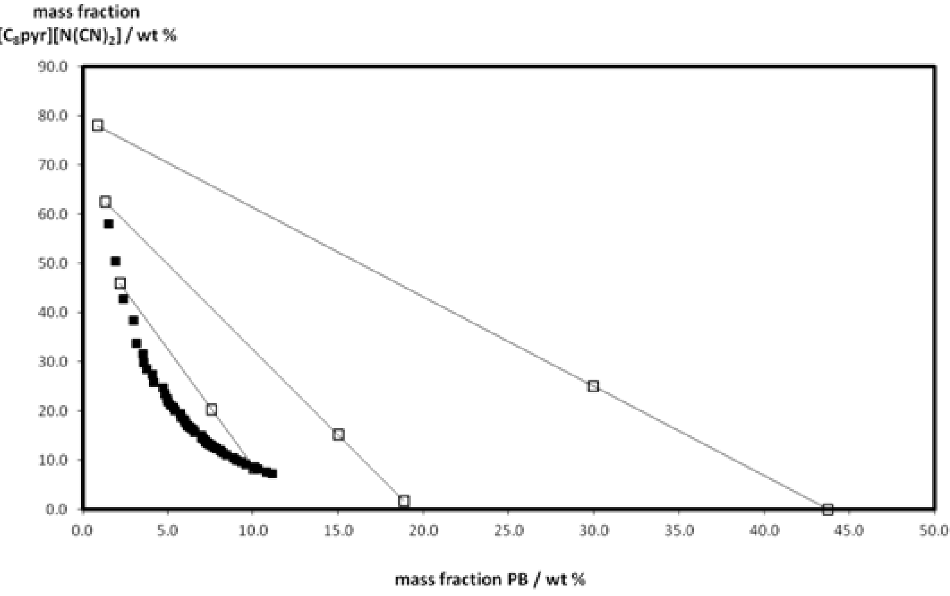


Figure 3.5.5. Phase diagram for the $[C_8pyr][N(CN)_2]$ and K_2HPO_4/KH_2PO_4 ATPS (pH = 7.0) at 298 K: (■) binodal curve data; (□) TL data.

In what concerns the ability for the lipase partitioning, surprisingly, this IL ($[C_8pyr][N(CN)_2]$) presenting all the optimized features previously studied, performed very poorly in the extraction/purification of lipase. Though the lipase partition into this IL is, as expected, very small, the unexpected behavior results from the extremely low partition observed for the contaminant proteins. The formation of self aggregates for ILs with sufficiently long chains changes the ATPS formation aptitude. It also seems to have a strong impact on the partition of the biomacromolecules and on their purification efficiencies. The optimization of the IL alkyl chain length based on the imidazolium cation (and probably other IL structural features) does not seem to be transferable to different cations. For reasons that are not entirely clear at present, but that may be

related with cohesion forces, contrarily to what is observed for [$C_n$mim]Cl, the formation of aggregates in [C_8pyr][$N(CN)_2$] seems to reduce the interaction with the contaminant proteins leading thus to a negligible extraction into the IL-rich phase and to a poor purification factor. This behavior was also previously observed by us for the extraction of small molecules (nicotine and caffeine [40], and vanillin [26]. A change in the partition coefficients was observed when the number of carbons of the IL alkyl chain increased from 6 to 8 probably due to micelle formation. In fact, recent results suggested that ILs composed of the [$N(CN)_2$] anion exhibit a higher capability for micelle formation when compared with the chloride anion for a common cation [68]. This difference is closely related with the size of the counterion [68, 69]. Moreover, it was suggested that large anions, as dicyanamide, favour the ILs aggregation since they decrease the charge repulsion at the micelle [68, 69]. On the other hand, strongly hydrated ions, such as chloride, are partially screened by the surrounding polar water molecules, and therefore, they are less effective at reducing charge repulsion [68, 69]. Regarding the cation core, it was also shown that critical micelle concentrations of imidazolium-based ILs are higher than the ones observed with pyridinium-based ILs (with similar anions and alkyl side chain lengths) [70-72]. As a result, [C_8pyr][$N(CN)_2$] will have a higher aptitude for micelle formation when compared to [C_8mim]Cl. The higher aptitude of [C_8pyr][$N(CN)_2$] for self aggregation compared to [C_8mim]Cl may be responsible for the unexpected poor results obtained with the pyridinium-based IL.

Using the partition coefficients and purification factors obtained in this work, a comparison was attempted with other literature data to evaluate the suitability of ATPS based in ILs for protein purification. In the majority of the works described in the introduction section, the PF values were all higher [7, 36, 47, 48] than those found in this work. However, a direct comparison with the results obtained in this work cannot be done due to the different nature of the proteins of the ATPS studied. More extensive and directly comparable data regarding the purification of lipase using polymer-based ATPS are available by Nandini and Rastogi [73] and Souza et al. [74]. Nandini and Rastogi [73] considered the same conditions of temperature and pH of the present work, but used instead the PEG 6000 + Na_2HPO_4 + H_2O ternary system. This system presented a PF = 1.56 but a recovery of only 88.45 %. Souza et al. [74] presented results for the partitioning and purification of the porcine pancreatic lipase considering the same conditions of temperature, type of salt solution and pH as in this work carried here. Souza et al. [74] reported the application of ATPS composed of 20 % (w/v) of

PEG 8000 + 18 % (w/v) of phosphate buffer solution (PB) + H_2O, with a purification factor of the enzyme of 1.13 ± 0.09 (fold) and a recovery yield of 83.40 %.

According to the results described above, more than 97% of the enzyme is recovered in the salt rich phase what makes possible to apply conventional techniques such as dialysis [74, 75] for the enzyme separation from the salt solution.

The results here obtained for the purification and recovery of lipase suggest that by using ATPS based in ILs, and with an appropriate choice of the IL structural features, it is possible to achieve improved purification coefficients and enhanced recoveries.

3.5.5. CONCLUSIONS

ILs have been shown to be able to induce aqueous phase separation in the presence of potassium phosphate buffer forming ATPS. Various IL structural features were studied in the partitioning and purification of *Candida antarctica* lipase B. The capacity of IL-based ATPS as prospective extraction and purification media in biotechnological processes was demonstrated since it was possible to increase the purity degree of the commercial enzyme, despite its high commercial purity level. The results obtained indicate that the higher purification factor values were found with an octyl side chain in the imidazolium cation, with the $[N(CN)_2]$ anion and with the $[C_4mpyr]$ cation as isolated effects. However, the additive properties were not verified for the extraction parameter, since the tailored $[C_8pyr][N(CN)_2]$ was further tested, and its purification capacity was found to be almost inexistent (PF = 0.998 ± 0.002). Nevertheless, a very large purification of the commercial enzyme was achieved with $[C_8mim]Cl$ with a purification factor of (2.6 ± 0.1) and an enzyme recovery at the bottom phase of (95.9 ± 0.2)%. The results obtained show that IL-based ATPS possess excellent ability for lipase purification and recovery.

3.5.6. REFERENCES

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3.6. PAPER 7

Production and Purification of an Extracellular Lipolytic Enzyme using Ionic Liquids-based Aqueous Two-Phase Systems

3.6.1. ABSTRACT

The ability of ionic liquid-based aqueous two-phase systems (ATPS) to purify a lipase produced by fermentation is here evaluated and compared against conventional PEG-based ATPS. Four ionic liquids, chosen after a screening of a larger number of ionic liquids, are evaluated with the maximum purification and higher recovery being obtained for the systems based on [C_8mim]Cl. It is shown that IL-based ATPS have a performance superior to PEG-based ATPS for the purification of this enzyme.

3.6.2. INTRODUCTION

Lipases are glycerol ester hydrolases (E.C. 3.1.1.3), which hydrolyze ester linkages of glycerides at water-oil interface. It is well known that lipases are the enzymes most widely used in organic synthesis and also that more than 20% of the biotransformations are performed with lipases [1]. In addition to their role in synthetic organic chemistry, they also find extensive applications in chemical, pharmaceutical, food and leather industries [2, 3]. These enzymes usually display exquisite chemo-selectivity, and stereo-selectivity. Moreover, the crystal structures of many lipases have been solved, facilitating considerably the design of rational engineering strategies. They do not require cofactors nor catalyze side reactions, and are readily available in large scale because most of them are produced by microbial fermentation processes. The traditional methods to purify macromolecules involve several steps, such as ammonium sulfate precipitation, dialysis, ionic and affinity chromatography [4] or electrophoresis [5], which increases the cost of the production [6]. Liquid-liquid extractive bioconversion processes seem to have, nowadays, a great potential, mainly with the use of aqueous two-phase systems here abbreviated as ATPS. Its advantages lie in the simplicity, low costs and easy scale-up. There is a very large number of works describing the use of ATPS based in polymer/polymer [7] and polymer/salt [8, 9] systems which were reported in the reviews of this field [10, 11]. These systems are usually considered as biocompatible, because of the high water content in both phases, and they possess partition properties favorable to their application in large scale to enzyme separation and purification [12, 13]. However, most of polymer-based ATPS display high viscosity [14-16] and normally form opaque aqueous solutions, which could interfere with the quantitative and qualitative analysis of the extracted compounds. During the last decade, ionic liquids (ILs) have emerged as alternative compounds for ATPS formation and

with potential to be used in extractive fermentation as solvents. They have unique properties such as their negligible vapor pressure, non-flammability, possess a wide electrochemical window, and high thermal and chemical stability [17]. Furthermore, they are known for the tunability of their chemical structures and physical properties, and by their strong solvation capability [17-19]. The first approaches to the use of ILs as extractants were carried out applying hydrophobic ILs [20, 21] in order to create IL/water biphasic systems. However, compared with the hydrophilic, the hydrophobic species are more expensive [22], toxic [23, 24] and their number is limited [22]. Moreover, the viscosity of the hydrophobic IL phases is characteristically high [22] and the partition coefficients observed are low [21]. Consequently, the attempts to the direct extraction of macromolecules from aqueous medium into an appropriate hydrophobic IL were considered as not practical. In 2003, Rogers and co-workers [25] reported, for the first time, that some hydrophilic ILs could form ATPS in presence of K_3PO_4, and these ILs-based ATPS could overcome the limitations of the ATPS based in hydrophobic ILs mentioned above. Since then, significant progresses have been done in the use of hydrophilic ILs-based ATPS [26-33]. The possibility of forming ATPS with different ILs and inorganic salts [22, 34, 35] has been explored and a number of works have been published on the effect of different ATPS conditions [26, 30, 32-34] and their use as separation techniques [22, 36, 37]. In what concerns their application in extraction, ILs were reported as attractive novel separation agents for various small compounds like metals [38, 39], alcohols [25], organic acids [40], and biomolecules such as amino-acids [30, 32, 41, 42]. Recently, the possibility of to extract some macromolecules, such as proteins and enzymes, was also assessed [43-50], having in mind that to use ILs as enzyme purification agents it is crucial to obtain both high enzyme activity and stability and also, significant extraction efficiencies [44].

The ATPS formed by ILs and inorganic salts are reported to present higher recoveries of the proteins studied than the conventional systems employing polyethylene glycol (PEG) and inorganic salt [49, 51]. In addition, IL-based ATPS also display better process properties, such as lower viscosity, little emulsion formation and fast phase separation [49, 51]. Considering the whole picture, it seems that the exploitation of hydrophilic ILs in the context of the separation of macromolecules, can be considered as an economical and efficient extraction approach for those separation systems [22, 44, 46, 47, 52].

This work reports the production and purification of a lipolytic enzyme produced by the bacterium *Bacillus* sp. ITP-001. In this work the separation and purification steps were performed on the fermentation broth after the end of the production phase, aiming to avoid toxicity [37, 40, 53], biocompatibility [43] and biodegradability [54] problems derived from the direct contact of the ILs with the bacterium [55]. This is, to the best of our knowledge, the first report of a comprehensive study showing the integration of IL-based ATPS in the process of production and purification of an enzyme. The pre-purification and purification stages are performed using an integrated process which comprises a salt precipitation with ammonium sulphate $(NH_4)_2SO_4$. The pre-purification is followed by a purification step using ATPS based in hydrophilic ILs and the phosphate buffer solution composed by K_2HPO_4 and KH_2PO_4, with the capacity to maintain a neutral pH [34, 35, 48].

3.6.3. EXPERIMENTAL SECTION

3.6.3.1. Materials

The present study was carried out using the inorganic salts K_2HPO_4 and KH_2PO_4 purchased at Sigma-Aldrich with purities higher than 98% (w/w). The ILs used are: 1-butyl-3-methylimidazolium chloride [C_4mim]Cl, 1-butyl-3-methylimidazolium dicyanamide [C_4mim][$N(CN)_2$], 1-methyl-3-octylimidazolium chloride [C_8mim]Cl, and 1-butyl-3-methylpyridinium chloride [C_4mpyr]Cl. They were all acquired at IoLiTec (Ionic Liquid Technologies, Germany) with mass fraction purities higher than 98%, confirmed by us using ^{1}H-NMR and ^{13}C-NMR. Their molecular structures, respective names and abbreviations are reported in Figure 3.6.1. Poly(ethylene glycols) of average weights 600 g.mol^{-1} and 4000 g.mol^{-1} (abbreviated as PEG 600 and PEG 4000) were supplied by Fluka and Sigma Aldrich, respectively.

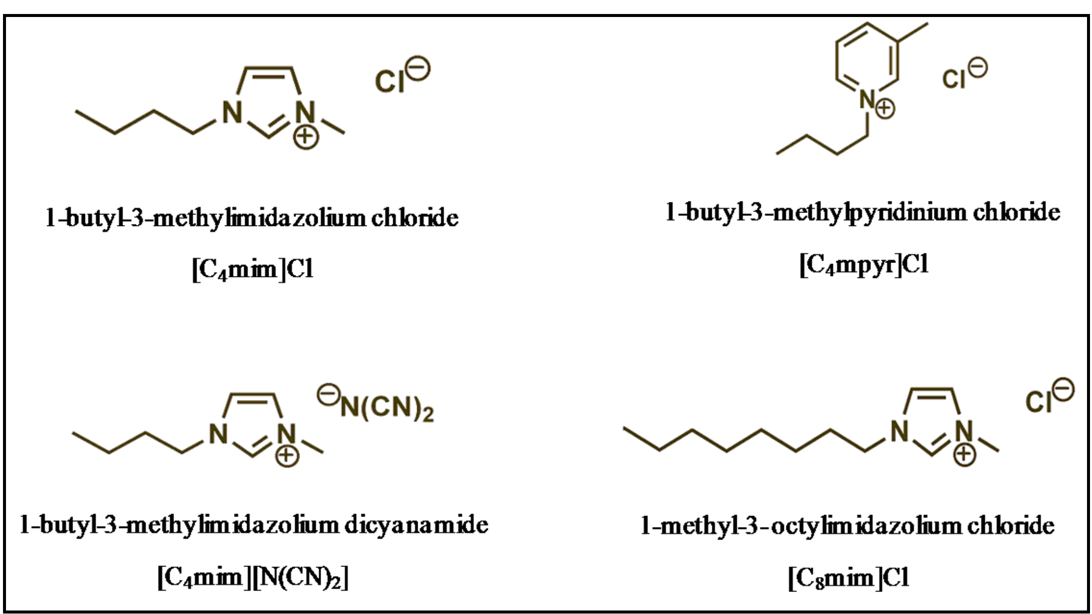

Figure 3.6.1. Chemical structure of the ILs used in the present work.

3.6.3.2. Methods

3.6.3.2.1. Fermentation conditions

Bacillus sp. ITP-001 is a bacterium strain isolated from soils contaminated with petroleum, stored in the Institute of Research and Technology (Aracaju-Sergipe, Brazil). The bacterium was maintained on nutrient agar slants at 37 ºC, stored at 4 ºC and cultured once a month.

The production of the extracellular lipase was carried out in a submerged fermentation conducted in 500 mL Erlenmeyer flasks and containing 200 mL of the fermentation medium, which composition (%, w/v) is the following: KH_2PO_4 (0.1), $MgSO_4.7H_2O$ (0.05), $NaNO_3$ (0.3), yeast extract (0.6), peptone (0.13), and starch (2.0) as the carbon source. The fermentation was carried at 37 °C, pH 7.0 and continuous agitation of 170 rpm. After 72 h of cultivation, coconut oil (4%, w/v) was added as inducer.

3.6.3.2.2. Pre-purification steps

3.6.3.2.2.1. Centrifugation of the fermentation broth

A sample of approximately 8 mL was centrifuged at 3000 rpm during 15 minutes. The bottom phase was discharged (biomass) and the supernatant was used to determine the enzymatic activity and the total protein.

3.6.3.2.2.2. Salt precipitation and Dialysis

Protein contaminants in the cell-free fermented broth were precipitated using a solution of ammonium sulphate $(NH_4)_2SO_4$ at 80% (w/v) and the broth was subsequently centrifuged at 10000 *g* for 30 minutes, aiming at separate the supernatant (with the target enzyme) from the precipitate (with the contaminant compounds). This procedure was performed several times until a much reduced amount of precipitate is achieved. All the supernatant phases obtained were dialyzed using dialysis membranes MD 25 (cut-off: 10000-12000 Da) against ultra-pure water. The dialysate solution containing the enzyme was then used to prepare the ILs-based ATPS [56].

3.6.3.2.3. Purification stage

3.6.3.2.3.1. Phase Diagrams and Tie-Lines

The binodal data were determined for all the ILs studied using the cloud point titration method [30, 32] at 25 (± 1) ºC. Repetitive drop-wise addition of the aqueous inorganic salt solution to the aqueous solution of IL (≈ 60 wt %) was carried out until the detection of a cloudy solution, followed by the drop-wise addition of ultrapure water until the detection of a monophasic region (limpid solution). The whole procedure was performed under constant stirring. The ternary system compositions were determined by the weight quantification of all components added within an uncertainty of $\pm 10^{-4}$ g. All these systems were already reported in literature [34, 35]. Those were all prepared at 25 ºC and pH 7.0.

Tie-lines (TLs) were determined by a gravimetric method previously described by us [30, 32] and adapted from Merchuck et al. [57]. A mixture at the biphasic region was prepared, vigorously stirred, and allowed to reach equilibrium by phase separation of both phases for 24 hours, at 25 ºC, using small ampules ($10\ cm^3$) especially designed for this purpose. After the separation step, both top and bottom phases were weighed. Each

individual tie-line (TL) was determined by application of the lever arm rule [57] as previously described [48].

3.6.3.2.3.2. Partition of the extracellular lipolytic enzyme

The extraction systems were prepared adding 25 wt% of IL + 30 wt% of potassium phosphate buffer + 45 wt% of the dialysate solution containing the lipolytic enzyme produced by *Bacillus* sp. ITP-001. The mixture point was the same for all the systems studied aiming to maintain the same compositions of IL and inorganic salt for all the experiments. The potassium phosphate buffer was obtained from the mixture of two inorganic salts, the K_2HPO_4 and KH_2PO_4 in the ratio of 1.087 (w/w) for the pH 7.0. In this case, the phosphate buffer solution was prepared with the dialysate solution and directly in the extraction system. Thus, the extraction systems were composed of 25 wt% of IL + 30 wt% of phosphate buffer aqueous solution pH 7.0 (the total mass of the extraction systems prepared is 5.0 g). All systems were prepared in graduated glass centrifuge vials of *circa* 10 cm^3. The mixture was gently stirred and centrifuged at 3000 rpm during 20 minutes. The graduated tubes were placed at 25.0 (± 0.1) ºC, for at least 12 hours to reach equilibrium, using a thermostatic bath (Marconi MA-127). After reaching equilibrium, both phases become clear and transparent and the interface was well defined. The two aqueous phases were carefully separated and cautiously collected for the determination of their volume and weight. For the determination of the viscosity data, two systems were prepared using the polymers PEG 600 and PEG 4000. The concentration used was the same as for the IL-based ATPS, but replacing the IL by the polymers. In this study, the partition and purification parameters were defined as shown before by Equations 3.3-3.8. This procedure was already used and validated before [51].

3.6.3.2.3.3. Enzymatic activity

Lipolytic activities were assayed using the oil emulsion method according to a modification proposed by Soares et al. [58]. The reaction was carried at 37 ºC and pH 7.0. One unit (U) of enzyme activity was defined as the amount of enzyme that produces 1 μmol of free fatty acid *per* minute ($\mu mol.min^{-1}$) under the assay established conditions (37 ºC, pH 7.0 and 100 rpm). The mentioned experimental techniques followed the protocols previously described by Carvalho et al. [59]. The possibility of

the hydrolysis of olive oil by the ILs was tested in control essays at the measurement conditions in absence of enzyme and verified to be negligible [48].

3.6.3.2.3.5. Protein Determination

Total protein concentration was determined by the Bradford's method [60], using a Varian 50 Bio UV-Vis Spectrophotometer at 595 nm, and a calibration curve previously established for the standard protein bovine serum albumin (BSA). To cancel the influence of the ILs' presence on the protein concentration analysis, a control system for each IL-based ATPS without enzyme was prepared under the same conditions. Equilibrium conditions (24 h, 25 ºC) and the phase separation procedure were those previously described [48].

3.6.3.2.3.6. Polyacrylamide Gel Electrophoresis

The molecular mass and purity level of the lipase were analyzed by a 12.5% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). The molecular mass standard was composed of bovine albumin (66 kDa), ovalbumin (45 kDa), glyceraldehyde-3-phosphate dehydrogenase (36 kDa), carbonic anhydrase (29 kDa), trypsinogen (24 kDa), trypsin inhibitor (20.1 kDa), α-lactalbumin (14.2 kDa) and were used as protein markers in this experimental method. The experimental procedure was focused in the protein silver staining method [61]. This method is made by some basic steps which are: i) fixation to get rid of interfering compounds; ii) sensitization and rinses to increase the sensitivity and contrast of the staining; iii) silver impregnation with a silver nitrate solution; iv) rinses and development to build up the silver metal image; and v) stop and rinse at the end to remove the excessive background formation and the excess of the silver ions and other chemicals prior to further processing [62].

3.6.3.2.3.7. Viscosity Determination

Measurements of viscosity for each one of the ATPS and both top and bottom phases, were performed in the temperature of 25 ºC at atmospheric pressure using an automated SVM 3000 Anton Paar rotational Stabinger viscometer. The temperature uncertainty is 0.02 ºC. The relative uncertainty of the dynamic viscosity obtained is less than 0.5% for the standard fluid SHL120 (SH Calibration Service GMbH), for the studied

temperature. This viscometer was previously tested for similar systems and presented a very good reproducibility [63, 64].

3.6.4. Results and Discussion

3.6.4.1. Production and pre-purification of the extracellular lipolytic enzyme

The present work addresses the process for the separation and purification of an extracellular lipolytic enzyme produced by *Bacillus* sp. ITP-001. The process, since the production until the final step of purification is depicted in Figure 3.6.2. It details, the whole route of production and pre-purification of lipase while Table 3.6.1 reports enzymatic activity (EA - U.mL^{-1}), total protein concentration (C - mg.mL^{-1}), specific activity (SA - U.mL^{-1}), and purification factor (PF - fold) on the fermentation broth and dialysate.

During the salt precipitation by $(NH_4)_2SO_4$ a great deal of contaminant proteins is removed and the lipase is concentrated in the supernatant. Following the salt precipitation, the supernatant was dialyzed to decrease the amount of low molecular weight compounds, including inorganic salts from the salt precipitation and fermentation processes. In this step, it was observed a small decrease in the enzymatic activity (EA = 4,245.40 U.mL^{-1}), probably due to losses of enzyme during the dialysis process.

Table 3.6.1. Purification factor, enzymatic activity, specific activity, and protein concentration at the end of each step of the production and pre-purification.

Process	EA (U.mL^{-1})	C (mg.mL^{-1})	SA (U.mL^{-1})	PF (fold)
Fermentation	4,662.06	1.19	3,922.53	1.00
Dialysate	4,245.40	0.06	76,501.46	19.50

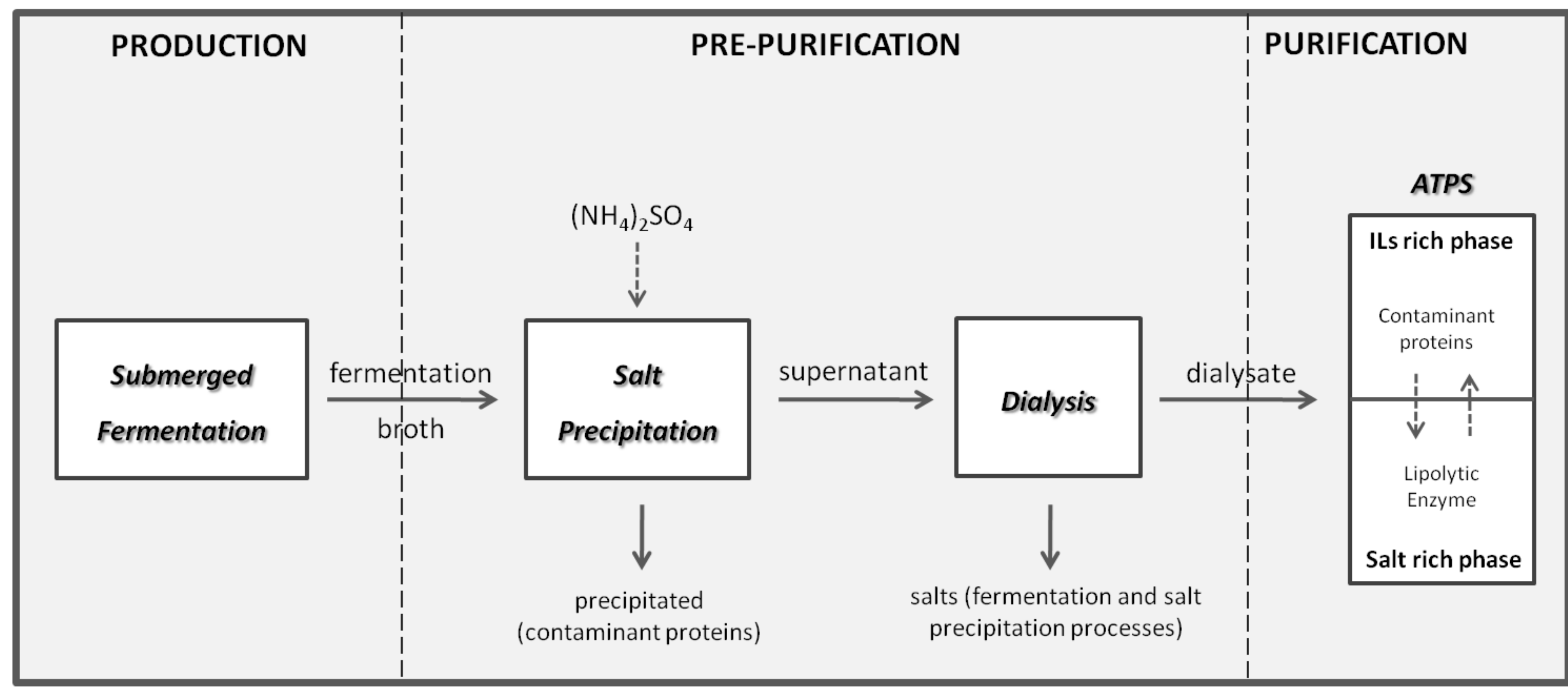

Figure 3.6.2. Representative scheme of the different steps followed for the production and purification of the lipolytic enzyme produced by *Bacillus* sp. ITP-001 *via* submerged fermentation.

3.6.4.2. Enzyme partition and purification on ILs-based ATPS

As described in Figure 3.6.2, after the pre-purification stage, the IL-based ATPS were used for further purification of the enzyme. The ATPS studied were based in four different ILs. Those were chosen by taking into account a previous study based in the partition and purification of *Candida antarctica* lipase B (CaLB) in several IL-based ATPS [48]. Figure 3.6.3 shows the binodal curves of the ATPS used for the preparation of the extraction systems [34, 35]. The compositions of the extraction system (25 wt % of IL + 30 wt % of phosphate buffer solution at pH 7.0) are the same for all the ILs studied to exclude the influence of the IL and salt compositions, since it is reported in the literature [44, 45, 65] that these conditions can significantly affect the purification/separation performances.

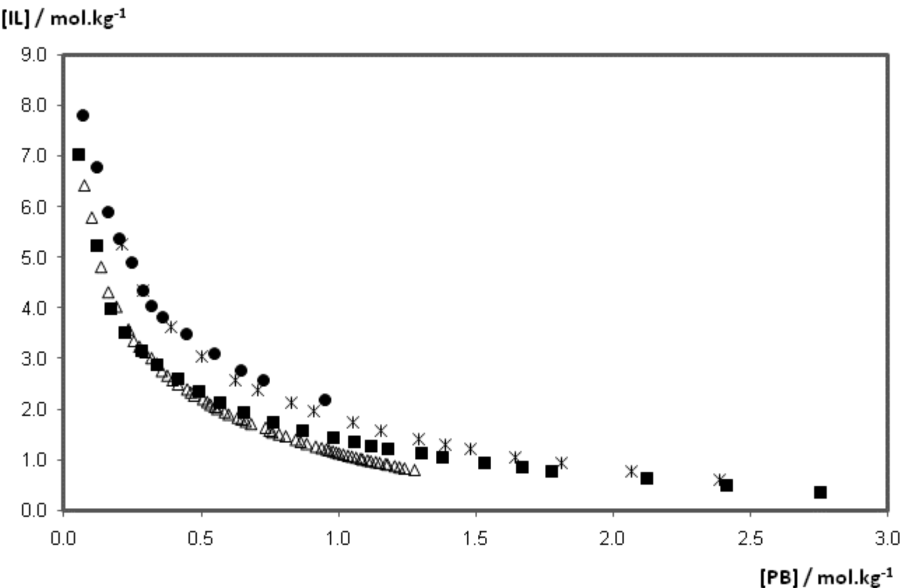

Figure 3.6.3. Phase diagrams for the ILs used in the partitioning and purification studies: (Δ) [C_4mim][NCN_2]; (■) [C_4mpyr]Cl; (✳) [C_8mim]Cl; and (●) [C_4mim]Cl.

Table 3.6.2 shows the purification and partition parameters obtained after the extraction of the enzyme and total protein present in the dialysate. The data suggests that the purification of the enzyme was controlled mainly by the alkyl chain length, followed by the cation core and finally, by the anion moiety. The increase in the alkyl chain length leads to a stronger increase in PF and K_P than on K_E, which remains consistently very low. This happens due to an increase in the hydrophobic nature of the ILs from [C_4mim] to [C_8mim], that decreases the Coulombic interactions and increases the dispersive

forces that occur between the enzyme and the ILs at the IL-rich phase. These factors seem to favor the partitioning of the contaminant proteins into the IL-rich phase. Due to its very low isoelectric point (p*I* = 3.0) [56], the lipase is negatively charged at this pH 7.0 [66-68], which results in the increase of its hydrophilic character, creating a higher affinity of the enzyme for the salt-rich phase. The differences observed in the partitioning behavior between the contaminant proteins and the lipolytic enzyme, must result from differences in their hydrophobic/hydrophilic nature, that will allow their partition into different phases. Note that in this work, the increase in the purity and, consequently in the PF, is entirely defined by the enzymatic activity. The increase in the PF from 40.8 ± 0.4 to 51 ± 2 fold, must thus result mainly from the removal of the contaminants which act as inhibitors [48].

Table 3.6.2. Effect of the different IL structural features in the partition and purification of the enzyme before the pre-purification steps, using 25% (w/w) of IL and 30% (w/w) of potassium phosphate buffer solution, at pH 7.0 and 298 K.

Ionic Liquid	R_v ± std	K_p ± std	K_E ± std	R_B^E ± std (%)	PF ± std (fold)
$[C_4mim][N(CN)_2]$	0.90 ± 0.09	0.063 ± 0.003	0.049 ± 0.003	96.14 ± 0.08	26 ± 1
$[C_4mpyr]Cl$	1.04 ± 0.04	1.137 ± 0.008	0.059 ± 0.003	94.2 ± 0.3	36.7 ± 0.9
$[C_4mim]Cl$	1.35 ± 0.04	0.27 ± 0.02	0.082 ± 0.007	90.6 ± 0.1	40.8 ± 0.4
$[C_8mim]Cl$	1.11 ± 0.04	14 ± 2	0.076 ± 0.001	92.2 ± 0.2	51 ± 2

The influence of the anion in the enzyme partitioning was investigated using the ILs $[C_4mim][N(CN)_2]$ and $[C_4mim]Cl$ as shown in Table 3.6.2. The $[C_4mim][N(CN)_2]$ was responsible for the poorest partition coefficient of the proteins (K_P = 0.063 ± 0.003) and the lowest purification performance (PF = 26 ± 1 fold). It presents low values of K_P and K_E that were significantly lower than the unit (<< 1), which indicates that with this IL both enzyme and contaminant proteins, partition preferentially into the salt-rich phase (bottom phase) leading to low PF. One more time, those results were combined with higher enzyme recovery efficiencies at the bottom phase (90.6 ± 0.1 < R_B^E < 96.14 ± 0.08)%. The results in Table 3.6.2 also provide information on the effect of the cation core on the purification by comparing the systems based on $[C_4mim]Cl$ and $[C_4mpyr]Cl$.

In what concerns the ability for the lipase purification, the cation core seems to play a small role when compared with the effect of the other IL features. Indeed, the substitution of the imidazolium ring by a pyridinium is responsible for a small decrease on the purification factor from 40.8 ± 0.4 to 36.7 ± 0.9. The enzyme and protein partition coefficients for the pyridinium-based IL suggest that the migration of the contaminant proteins for the top phase decreased when comparing with [C_4mim]Cl, while the enzyme remained in the bottom phase (salt-rich phase). The high purification factors were also followed by high enzyme recovery efficiencies at the bottom phase $(90.6 \pm 0.1 < R_B^E < 96.14 \pm 0.08)\%$.

To support our interpretation of the results concerning the purification capacity of the IL-based ATPS, a 2D electrophoresis analysis was performed using samples of the bottom and top phases from the ATPS based in the [C_8mim]Cl (considered in this work the best purification/separation system). The three lanes shown in Figure 3.6.4 correspond to the molecular mass standard (Lane *P*), the bottom phase (Lane *B*) and top phase (Lane *T*). The presence of multiple light bands in Lane *T* confirms the presence of some contaminant proteins, designed as *A*, *B* and *C*. In Lane *B*, it is possible to see the presence of the target enzyme with a molecular weight of around 54 kDa (here abbreviated as *Enz*) and the absence of contaminant compounds. The results from the electrophoresis show the excellent purification ability of the [C_8mim]Cl ATPS that made possible the complete separation of the enzyme from the contaminant compounds.

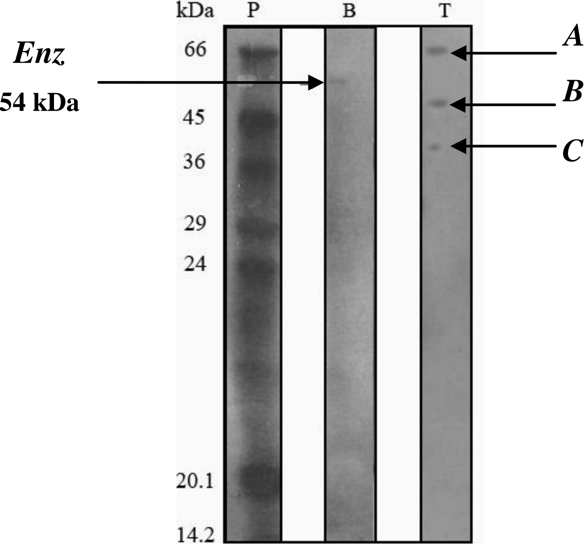

Figure 3.6.4. Sodium dodecyl sulphate-polyacrylamide gel (SDS-PAGE) patterns of the enzyme produced by *Bacillus* sp. Lane *P*: molecular mass standard (14.2-66 kDa), Lane *B*: bottom phase obtained from the [C_8mim]Cl-based ATPS; Lane *T*: top phase obtained from the [C_8mim]Cl-based ATPS. 12.5% acrylamide gel stained with silver nitrate solution.

3.6.4.3. Comparison of the performance of polymer and IL-based ATPS

Since the aim of IL-based ATPS is to replace the polymer-based ATPS, the purifications achieved using these two systems were here compared. The study-case investigated in this work (production and purification of the enzyme produced by *Bacillus* sp. ITP-001) was also investigated elsewhere using ATPS based in polyethylene glycol (PEG 8000) and potassium phosphate [56]. Despite some differences in the operation conditions of both systems (inorganic salt, temperature, and pH), the results obtained on this work are in general superior, with PF ranging from 37 to 51, than those using PEG-based ATPS that have purification factors below 30 [56]. This comparison was carried considering similar conditions in both works (20% (w/v) of PEG 8000 + 30% (w/v) potassium, pH 6.0, and 4 ºC of temperature). As described in literature [9, 48, 56], the pH of the extraction system is a very important parameter. The work with PEG-based ATPS was carried with a pH of 6.0 while the pH used in this work is 7.0. As described by Barbosa et al. [56] the increase in the pH leads to a strong decrease in the PF, which means that the purification factor of 30 (fold) here described, would be lower at the pH value used in this work.

A further advantage of the IL-based ATPS in comparison with PEG-based systems is the low viscosity of the aqueous phases on these systems. To make a comparison between the two systems the dynamic viscosity at the top and bottom phases were measured at 298 K for the four IL-based and two polymer-based ATPS (PEG 600 and PEG 4000). As shown in Figure 3.6.5. the viscosity of the salt-rich phase (bottom phase) is low and similar for the ILs and polymer-based ATPS. While the viscosity of the IL-rich phases is comparable with those of the salt rich phases, the viscosity of the PEG-rich phase is larger by an order of magnitude. This appears as an excellent advantage of the ILs-based ATPS, since the lower viscosities make the fluid transport and the mass transfer between the phases easier.

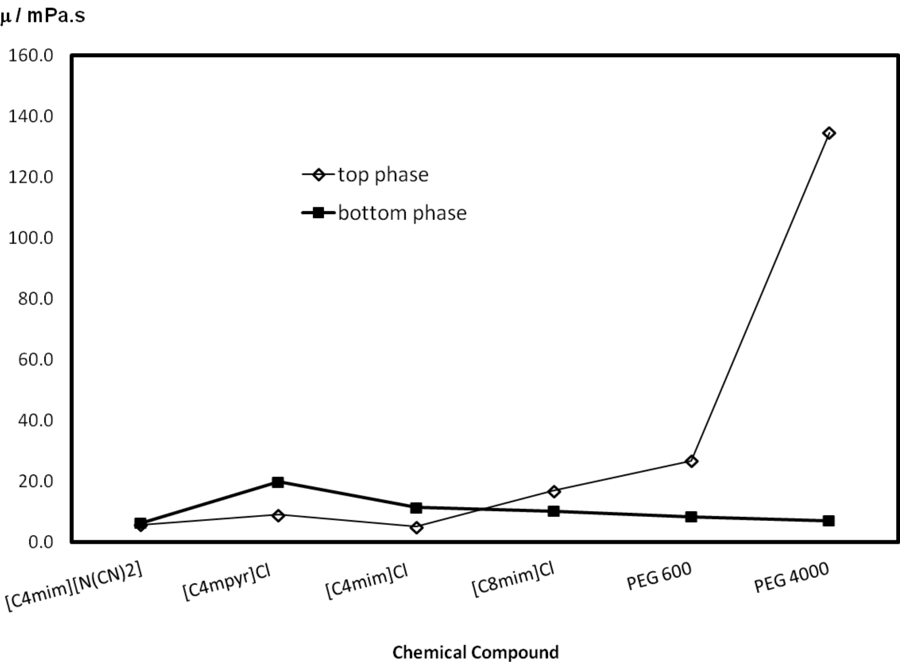

Figure 3.6.5. Dynamic viscosity (μ) results for different ATPS based in the phosphate buffer inorganic salt conjugated with the ILs reported in this work and two distinct polymers, PEG 600 and PEG 4000, for both phases at 298 K.

This work shows that IL-based ATPS are well succeeded in the purification of proteins from real systems, constitute an improvement over the polymer-based systems, and may become an important operation in the design and future industrial implementation of new biocompatible purification techniques.

3.6.5. CONCLUSIONS

Ionic liquid-based aqueous two-phase systems were here successfully applied to the purification of lipase, produced by the bacterium *Bacillus* sp. ITP-001, from a fermentation broth. Both high purification factors and enzyme recovery efficiencies at the salt-rich phase were obtained for all systems ($90.6 \pm 0.1 < R_B^E < 96.14 \pm 0.08$)%. It is shown that ILs-based ATPS are more efficient and significantly improve the extraction capacity of the commonly used polymers-based ATPS.

3.6.6. References

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3.7. PAPER 8

Candida antarctica lipase B - The effect of ILs' nature on the enzymatic activity

3.7.1. ABSTRACT

During the last two decades, the use of biocatalysts in organic solvents has received growing attention due to the advantages they offer, such as the possibility of carrying out processes involving hydrolytic enzymes which are thermodynamically unfavourable in water and their ability to increase the solubility of organic substractes. Ionic liquids have recently emerged as alternatives to classical organic solvents for a wide variety of non-aqueous biocatalytic processes. A systematic study of the effects of the ionic liquids structure and concentration on the activity of *Candida antarctica* lipase B (CaLB) is here reported. In what concerns the effects of different alkyl side chains, it was proved that the increase in the number of carbons leads to the decrease in the enzymatic activity which is promoted by the increase in the hydrophobic nature of the alkyl chain. Moreover, it is here shown that the anion effect may be explained by the water activity, here described by its hydrophobic nature and also by the interactions (mainly dispersion forces and hydrogen bonds) established by each one of those anions. Finally, these results suggest that the increase in the ionic liquid' concentration leads to the decrease in the relative enzymatic activity and that while the water activity plays an important role in respect to the enzymatic activity maintenance, it is not the single factor controlling the enzyme activity.

3.7.2. INTRODUCTION

It is well established that many enzymes are active in hydrophobic organic solvents [1]. Lipases are so stable that have been shown to remain active even in anhydrous organic solvents. One of the most important reasons for applying lipases under such conditions is to avoid hydrolysis when non-hydrolytic transformations are performed, which constitute a large number of industrial applications [2]. The minimum water requirement is enzyme-dependent and ranges from a few tightly bound water molecules per molecule of enzyme [3] to a nearly intact hydration shell. The enzyme *Candida antarctica* lipase B (CaLB) is very stable, even when compared with other lipases. CaLB is routinely used in anhydrous organic media and does not require a hydration shell to be active. This characteristic, as well as its quite relaxed reactant specificity and its operational stability, make of CaLB a valuable tool for various synthesis [4].

Ionic liquids (ILs) have recently emerged as alternatives to classical organic solvents for a wide variety of non-aqueous biocatalytic processes [5-10]. The appropriate

combination of the cationic and anionic parts of the solvent might increase the substrate' solubility, improve the enzyme selectivity or enhance the enzyme activity and/or stability. The results reported in literature suggest that the enzymes basically follow the same catalytic mechanism in ionic liquids as in water or organic solvents. As in any other organic solvent, the enzyme works with a micro-aqueous phase surrounding its structure. One of the major problems using ionic liquids, is that they might decrease, or even destroy, the active site and/or the water layer around its surface, consequently decreasing or even extinguishing its activity and/or stability. In fact, some studies about the influence of different ionic liquids on the activity of enzymes such as lipases [11-25], peroxidases [10, 26, 27], laccases [28], oxiredutases [29], cellulases [30] or even tyrosinases [31, 32] have been reported. Recently, some interesting works have been published reviewing these issues [32-38]. One of the principal conclusions described in those works is that an IL may play the same role as an organic solvent [31, 39-41] in affecting the enzyme performance, by: (1) stripping off the essential water associated with the enzyme; (2) penetrating into the micro-aqueous phase to interact with the enzyme by changing the protein dynamics, the protein conformation, and/or the enzyme's active site; and (3) interacting with the substrates and products by either direct reactions with them or by altering their partitioning between the aqueous and non-aqueous phases [42]. It was shown that, for some ionic liquids, an enhanced enzyme activity can be obtained when compared to organic solvents [22,43]. Until now, biocatalysis has been performed in several ionic liquids with several classes of enzymes, where a high sensitivity of the enzyme performance regarding the ion constituents of the ionic liquid has been observed [37, 44]. These results show that the anion has an important role in what concerns the maintenance of CaLB activity.

Most of the work done in this area suggests that, although hydrophilic ionic liquids and their aqueous solutions dissolve enzymes, most of them establish strong interactions (*via* hydrogen-bonding) resulting in enzyme deactivation. Due to the ionic character of the ILs, it is important to understand the specific influence of the individual ions (cations and anions) on the enzyme performance, mainly in what concerns its activity and stability [37]. In the case of lipases, for example, it has been found that the enzyme activity is particularly sensitive to the choice of the anions. Hydrophilic ionic liquids that contain nitrate or lactate [14, 45], led to the deactivation of lipases, while a high activity was yielded in hydrophobic ionic liquids containing hexafluorophosphate [PF_6]

or tetrafluoroborate $[BF_4]$ and bis(trifluoromethylsulfonyl)imide $[NTf_2]$ anions [14, 22]. It has also been observed that large anion concentrations deactivated lipases [46].
Regarding the IL cation, the enzyme activity has been reported to gradually increase with the length of the alkyl group [14, 47, 48]. However, this trend is not generally accepted since other authors report the opposite effect [49] remaining this an issue that needs to be clarified. To understand the dynamics of the phenomenon occurring, it is necessary to know what physical properties are important to the catalytic behavior of enzymes in ILs. It is well known that the enzyme' performance in ionic liquids is affected by some factors, such as the water activity of the system, pH, excipients and impurities [35]. The solvent properties of ionic liquids are also important, as reflected by the number of works on this subject. The most studied were the ILs polarity [22, 38, 50-52], hydrogen-bond basicity and nucleophilicity of anions [11, 22, 53, 54], viscosity [7, 17, 37, 50], enzyme dissolution [11, 14, 17], ion kosmotropicity [32, 34, 55-58] and finally, hydrophobicity [14, 59, 60].
In most of these works, it appears that the ability to dissolve enzymes and the potential stabilization of enzymes are mutually exclusive qualities of ionic liquids [11]. The results indicate that strong interactions of anions with lipases impair the enzyme activity. Recently, the hydration of the enzyme when exposed to the ionic liquids' structures has been taken into account, since the hydration level of the enzyme is one crucial condition to maintain its best performance, both in terms of stability and enzymatic activity [9, 22, 39, 61]. However, due to the limited number of ILs investigated, principally in what concerns hydrophilic anions, more comprehensive studies aiming at achieving a deeper understanding of these phenomena are required.
The aim of this study is to enlarge the range of ionic liquids tested on their effects on the activity of CaLB. All the ILs here studied belong to the imidazolium family. Thus, the imidazolium head was conjugated with ethyl-, butyl- and octyl- alkyl chains and several hydrophilic anions (chloride Cl, bromide Br, trifluoracetate $[CF_3COO]$, methanesulphonate $[CH_3SO_3]$, hydrogenosulphate $[HSO_4]$, dicyanamide $[N(CN)_2]$, trifluoromethanesulfonate $[CF_3SO_3]$, acetate $[CH_3COO]$). Moreover, a range of concentrations were also investigated for all the ILs tested. Finally, the level of hydration represented by the thermodynamic water activity (a_w) was determined and investigated for some of the hydrophilic anion-based ILs, aiming at understand the influence of the free water molecules around the enzyme on the enzyme activity.

3.7.3. EXPERIMENTAL SECTION

3.7.3.1. Material

The ionic liquids used in this study were: 1-ethyl-3-methylimidazolium chloride - $[C_2mim]Cl$, 1-methyl-3-octylimidazolium chloride - $[C_8mim]Cl$ 1-butyl-3-methylimidazolium methanesulfonate - $[C_4mim][CH_3SO_3]$, 1-butyl-3-methylimidazolium trifluoromethanesulfonate - $[C_4mim][CF_3SO_3]$, 1-butyl-3-methylimidazolium acetate - $[C_4mim][CH_3COO]$, 1-butyl-3-methylimidazolium chloride - $[C_4mim]Cl$, 1-butyl-3-methylimidazolium bromide - $[C_4mim]Br$, 1-butyl-3-methylimidazolium trifluoracetate - $[C_4mim][CF_3COO]$, 1-butyl-3-methylimidazolium hydrogenosulphate - $[C_4mim][HSO_4]$, and 1-butyl-3-methylimidazolium dicyanamide - $[C_4mim][N(CN)_2]$. Their molecular structures are shown in Figure 1, with their respective names and abbreviations. All ILs were acquired at IoLiTec (Ionic Liquid Technologies, Germany) with mass fraction purities higher than 99 %, confirmed by us using ^{1}H-NMR, and ^{13}C-NMR. The enzyme *Candida antarctica* lipase B, here abbreviated by CaLB, was produced in submerged fermentation of a genetically modified *Asperghillus oryzae* microorganism and was supplied by the Novozymes® company. For the determination of the enzymatic activity by the spectrophotometric method, *para*-nitrophenyl laurate (*p*-NFL) from Fluka (purity ≥ 98.0 % by GC analyse), dimethyl sulfoxide (DMSO) from Lab-Scan and *para*-nitrophenol (*p*NF) from Sigma-Aldrich were used.

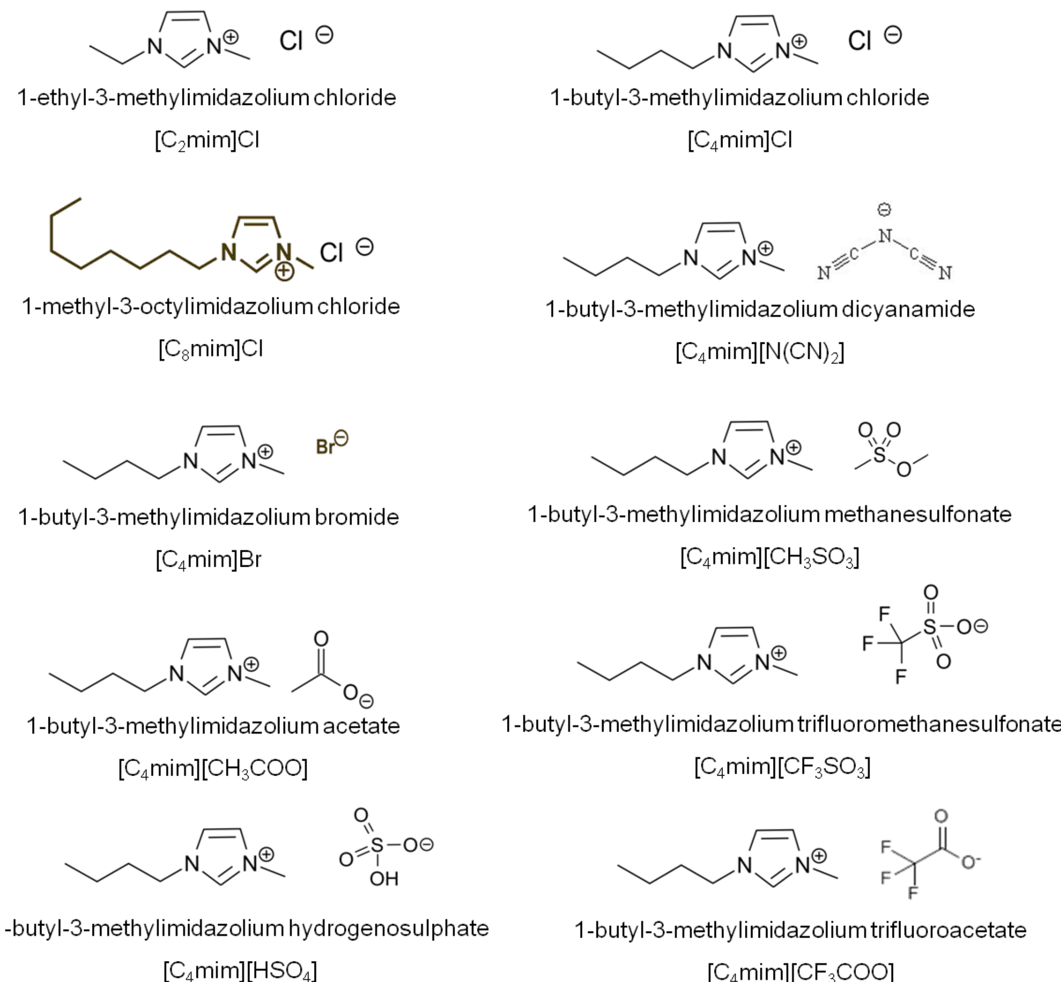

Figure 3.7.1. Chemical structures of the ILs used in the experiments.

3.7.3.2. Experimental Methods

3.7.3.2.1. Enzymatic Activity

The lipase activity was assayed spectrophotometrically using a SHIMADZU UV-1700, Pharma-Spec Spectrometer. The substrate solutions composed of *p*-NFL were prepared mixing 0.018 g of *p*-NFL in 1 mL of DMSO. This solution was then diluted 100 times in a phosphate buffer solution (50 mM and pH 7.0). The enzymatic solution was prepared adding 25 μL of CaLB to 300 μL of the phosphate buffer solution (50 mM and pH 7.0). Each solution of substrate and enzyme was acclimatized at 310 K during 15 minutes and at 413 K during 20 minutes, respectively. To measure the lipase activity, 200 μL of the incubated enzymatic solution (containing different amounts of ILs, depending of the concentration tested or the phosphate buffer solution) was added to 1.8 mL of *p*-NFL solution. After the addition of both solutions (substrate and enzymatic

solutions), the absorbance variation at 410 nm was determined during 150 seconds at 298 K, and the enzymatic activity was calculated according to Eq. 3.9. One unit was defined as the amount of enzyme that oxidized 1 μmol of *p*-NFL per min and the enzymatic activities were expressed in $U.L^{-1}$,

$$A = \frac{\Delta Abs \times D \times f \times V_r}{\Delta t \times V_s} \qquad \text{Eq. 3.9.}$$

where A represents the enzymatic activity in $U.L^{-1}$, ΔAbs means the variation of absorbance during a certain time Δt (between 30 to 120 seconds) previously determined through the linear phase. D corresponds to the dilution of the enzymatic solution, V_r is the total volume of the reaction system, V_s correspond to the total volume of the enzyme in each reaction, both in litters. Finally, f represents the conversion factor, obtained from the inverse of the slope determined from the calibration curve previously determined ($\mu mol.L^{-1}$). In this work the results are reported as the ratio between the activity of the enzyme with and without IL (A/A_0).

3.7.4. RESULTS

In this work, the capacity to maintain the enzymatic activity after the exposure of the enzyme to several ILs was tested. The conditions of preparation of both the enzyme solution (20 minutes at 313 K) and substrate solution (15 minutes at 310 K) were maintained as well as the reaction conditions (pH 7.0 at 298 K). All the results are reported as molar concentrations to allow comparisons with the same number of moles of ionic liquid around the enzyme.

3.7.4.1. Effect of the cation alkyl chain length

Figure 3.7.2 shows that the increase in the alkyl chain length of the cation induces a significant decrease in the enzymatic activity at all IL' concentrations tested. This behavior was previously observed by Attri et al. [49] and it may be explained by the increase of the cation' hydrophobicity with the elongation of the alkyl chains. The increase in the hydrophobicity leads to the increase in the van der Waals interactions between the alkyl chains and the non-polar domains of the enzyme [62]. The increase in these interactions is responsible for the spontaneous formation of aliphatic domains,

comprised of those non-polar alkyl chains [62]. The formation of those domains was proved by molecular dynamics simulation and later by some experimental studies [63-65]. These aliphatic domains tend to accumulate around the non-polar active site, leading to its partial or total obstruction (depending of the IL' concentration on the system). As described by Klähn et al. [62], with the obstruction of the enzyme' active site, the diffusion of the substrate is compromised causing mass transfer problems, which are responsible for the decrease in the enzymatic activity.
Other works reporting the opposite trend of the enzymatic activity with the increase in the length of the alkyl chains [14, 47, 48] use hydrophobic ILs based in the water unstable [PF_6] anion and/or immobilized enzymes and they generally fail to provide an adequate explanation for the enhanced activities observed.

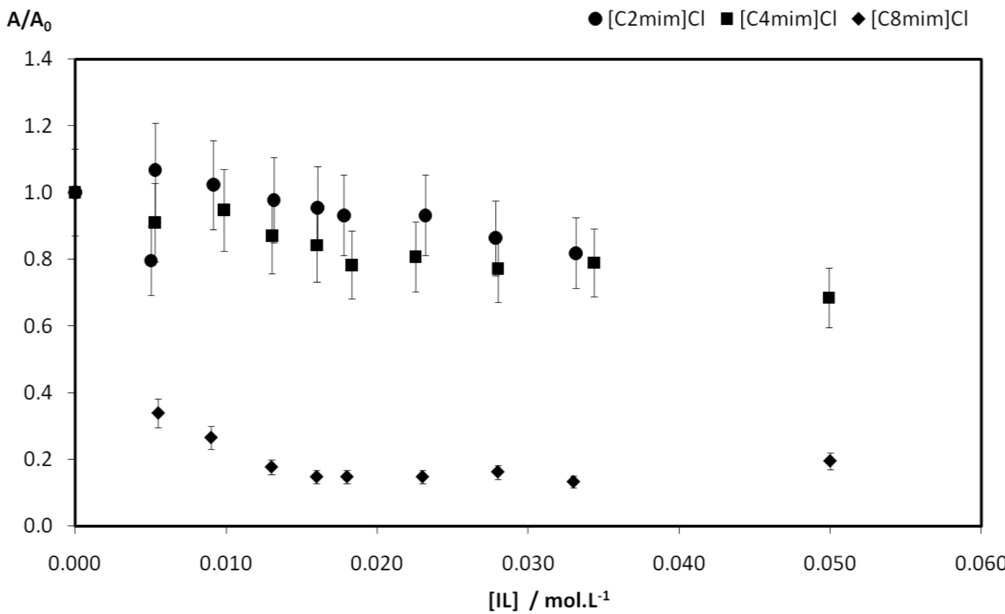

Figure 3.7.2. Enzymatic activity of CaLB in the presence of different [$C_n$mim]Cl - based ILs at 298 K.

3.7.4.2. Effect of the ionic liquid concentration

One of the major problems using ionic liquids as solvents for the enzymes is that their use decreases the water hydration layer around its surface. The effect of the concentration of the various ionic liquids studied on the enzymatic activity is shown in Figures 3.7.2 (cation effect) and 3.7.3 (anion effect). It can be observed that in all cases an increase in the IL concentration leads to a decrease in the relative enzymatic activity.

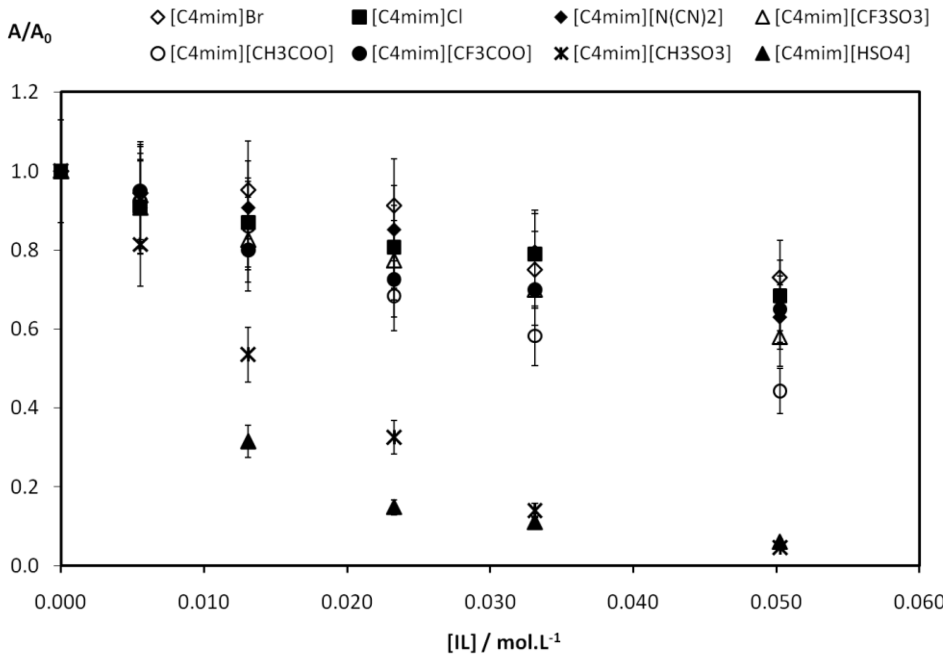

Figure 3.7.3. Enzymatic activity of CaLB in the presence of different IL' concentrations (mol.L^{-1}) for several hydrophilic ILs [C$_4$mim]X, at 298 K.

To take into account the availability of water for the hydration of the enzyme, since the level of hydration of an enzyme is crucial for its performance [9, 61, 66] the enzyme loss of activity was plotted as a function of the water activity, here abbreviated for a_w in Figure 3.7.4. The highly diluted solutions used in this work made the direct measurement of the water activity impossible by conventional means and thus, an alternative approach was used. Given the ability of COSMO-RS to predict the non ideality of diluted aqueous solutions of ionic liquids [67], the water activity was here estimated using the predictive method COSMO-RS, for the various ILs studied on this work. From the results presented in Figure 3.7.4., it can be observed that, as expected and previously reported, [42] the enzymatic activity decreases with the water activity. Although the results clearly point for an important and direct relationship between the enzymatic activity and the water activity, the differences observed among the curves for the various ionic liquids show that this parameter is by no means the sole responsible for the loss of activity and that other factors must be taken into account to explain its loss of activity.

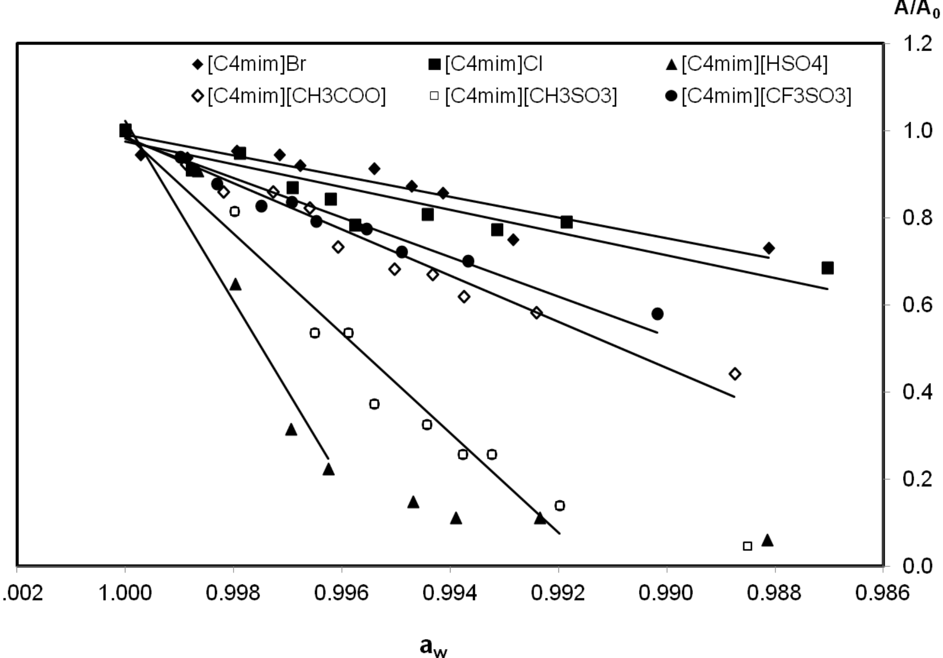

Figure 3.7.4. Dependency of the residual enzymatic activity (A/A_0) on the water activity (a_w) at 298 K.

3.7.4.3. Effect of the anions' nature

Having shown that besides the water activity other factors are also relevant to understand the activity loss of CaLB, in presence of ionic liquids, the nature of the anion was investigated. In this context, the type of interactions between the ionic liquid anion and the enzyme were considered, aiming at understand the different impacts in the enzymatic activity observed for the anions, and in particular the large effect of [HSO_4] and [CH_3SO_3]. The various interactions that can be established by the anions with the enzyme were studied using Kamlet-Taft solvatochromic parameters [68-70], α (hydrogen bond donor) and β (hydrogen bond acceptor), and π representing, respectively, the polar and dispersive interactions described in Table 3.7.1 for the ionic liquids studied.

Table 3.7.1. Solvatochromic parameters, α β, and π for several hydrophilic anion-based ILs [68-70].

Ionic Liquid	α	β	π
$[C_4mim]Cl$	0.32	0.95	1.13
$[C_4mim]Br$	0.36	0.87	1.14
$[C_4mim][CF_3SO_3]$	0.63	0.52	0.98
$[C_4mim][CH_3COO]$	0.36	0.85	1.06
$[C_4mim][CH_3SO_3]$	0.36	0.85	1.04
$[C_4mim][HSO_4]$	-	0.66	1.11

To remove from the analysis the influence of the water activity previously established, the dependency of the enzymatic activity with the water activity estimated from the slopes of the lines in Figure 3.7.4, are used in this study. Their relations with the solvatochromic parameters reported in Table 3.7.1. are shown in Figure 3.7.5. The results show that α seems to have no influence in the enzymatic activity. However there seems to be a clear relation between the activity decrease with the water activity and the β and π solvatochromic parameters. This suggests that the anions act as hydrogen bonding acceptors with the protein and that dispersive forces also play an important role in the loss of enzymatic activity, in agreement with what was previously discussed concerning the cation alkyl chain effect. According to Figure 3.7.5, $[CF_3SO_3]$ appears as an exception, but it was not possible to understand its anomalous behavior.

The results here reported make possible to relate the different declines of the enzymatic activity with the interactions enzyme – ionic liquid. It is shown that by increasing both β and π parameters, the interactions (hydrogen bonds and van der Waals interactions), between the ionic liquid and the enzyme become more important decreasing the enzymatic activity.

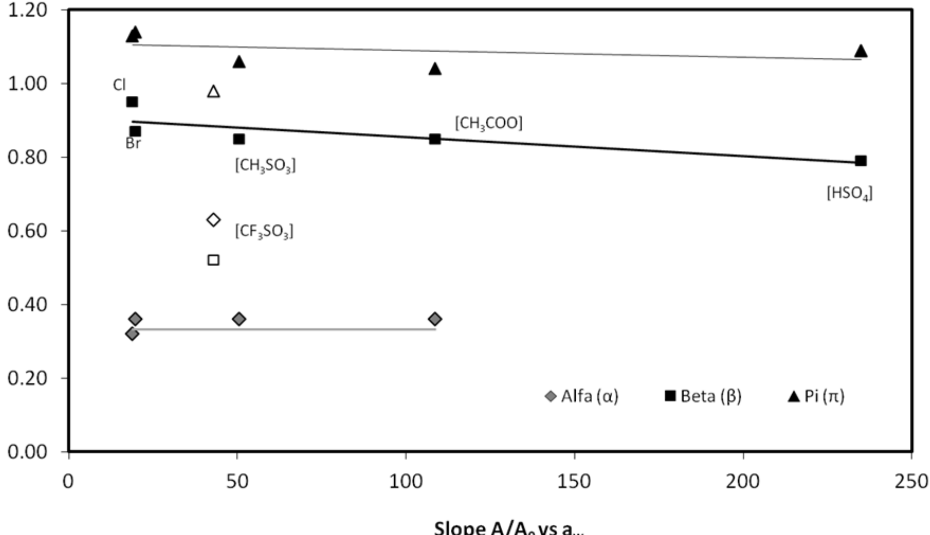

Figure 3.7.5. Dependency of the decrease in enzymatic activity with the water activity and the solvatochromic parameters α, β, and π.

3.7.5. Conclusions

This work studied the effect of various ionic liquid structures and concentrations on the enzymatic activity of *Candida antarctica* lipase B (CaLB). The ionic liquid features investigated were the alkyl chain length and the nature of the anion. It was shown that the ionic liquid structures have a deleterious influence upon the enzymatic activity, which means that the residual activity determined for the lipase decreased for all the ionic liquids studied. The ionic liquid concentration is one of the main factors with negative impact on the enzymatic activity. It was however shown that although the water activity could explain a part of the deactivation observed, it cannot explain all the observed effects. For the ionic liquids studied it was shown that the strength of the interactions established between the enzyme and the different anions, dominated by dispersion forces and hydrogen bonding, correlated well with the loss of activity observed. Concerning the increase in the alkyl chain, the impact on the activity seems to be related with the hydrophobic nature of the alkyl side chain, which promotes the ability of the ionic liquid to obstruct the non-polar active site of the enzyme. There seems to be no simple explanation as to whether an enzyme is active or not in a certain ionic liquid, because the enzyme activity depends largely on the enzyme-medium-substrate relationships and characteristics.

3.7.6. References

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4. (ECO)TOXICOLOGICAL EVALUATION

In the past few years, the image of ILs has changed. In the beginning, the whole substance class was discussed in an undifferentiated way as being "green" and "environmentally benign". Those statements were justified with the negligible vapor pressure, which results in reduced air emissions, non-flammability and non-explosiveness. Indeed, this pronounced advantage for the operational safety includes a "green" potential, but without a sound knowledge of the (eco)toxicological behavior, there is no justification for this classification. Recently, it has been demonstrated that many commonly used ILs have a certain level of toxicity. This fact has been proved in many (eco)toxicological investigations on the effect of ILs in several organisms (different species and trophic levels), the effect of different IL' properties on these toxicities, the effect of the surrounding medium, and finally, the use of the experimental toxicological profiles for the development of promising predictive tools on toxicities. Moreover, some new and important initiatives have appeared, such as the Biodegradability and Toxicity of Ionic Liquids BATIL meetings (Berlin 2007 and Frankfurt 2010) and the UFT/Merck Ionic Liquids Biological Database (http://www.il-eco.uft.uni-bremen.de) available online.

The following section - **Chapter 4** focuses on the (eco)toxicological evaluation of a large set of ILs and also in the study of the bioaccumulation capacity of some ILs entities, through the investigation of the ecotoxicity (EC_{50}) - Papers 9 to 11 and 1-octanol-water distribution coefficients (D_{ow}) - Paper 12. Were tested ILs from different families, such as the imidazolium, piperidinium, pyrrolidinium, pyridinium, phosphonium and guanidinium, with various anions (hydrophilic and hydrophobic) and side chains (oxygenated and non-oxygenated). Moreover, the influence of those ILs was investigated in respect to distinct aquatic species (the marine luminescent bacteria *Vibrio fischeri*, two freshwater green algae *Pseudokirchneriella subcapitata* and *Chlorella vulgaris* and two cladoceran species *Daphnia magna* and *Daphnia longispina*). Finally, the bioaccumulation level of some hydrophobic ILs was tested according the slow stirring method. The end-point of this methodology, D_{ow}, the 1-octanol-water distribution coefficient was addressed.

4.1. PAPER 9

Assessing the Toxicity on $[C_3mim][NTf_2]$ to Aquatic Organisms of Different Trophic Levels

Aquatic Toxicology, 2010, 96, 290-297

4.1.1. ABSTRACT

Ionic liquids (ILs) are an exciting class of neoteric solvents that are being object of great attention as a potential replacement to conventional environmental damaging solvents in industrial applications. Despite some progresses concerning ILs' toxicity and their environmental impact, the information about these compounds is still scarce.

In this work, biological tests were performed to establish the toxicity of 1-methyl-3-propylimidazolium bis(trifluoromethylsulfonyl)imide, $[C_3mim][NTf_2]$, in five aquatic species at different trophic levels. Freshwater algal growth inhibition (*Pseudokirchneriella subcapitata* and *Chlorella vulgaris*), freshwater cladocerans' immobilisation and chronic traits (*Daphnia magna* and *Daphnia longispina*) and viability of luminescent marine bacteria (*Vibrio fischeri*) were investigated. The sensibility of the different species to the IL was compared in order to determine further repercussions in trophic food web.

It is shown that the studied IL is moderately toxic being *Pseudokirchneriella subcapitata* and *Daphnia magna* the most tolerant species and *Chlorella vulgaris* and *Daphnia longispina* the most sensitive to its presence.

4.1.2. INTRODUCTION

Ionic liquids (ILs) are a novel class of technologically advanced solvents, composed by large organic cations, such as ammonium, imidazolium, pyridinium, piperidinium or pyrrolidinium with alkyl side chains that can vary in length, number and position, and organic or inorganic anions of variable nature. The huge number of different cation / anion combinations and, consequently, the potential for structure adjustability, allows property control and the design of the molecule for task specific applications [1]. The most relevant and unique physicochemical properties of ILs include their negligible volatility, non-flammability, high thermal, chemical and electrochemical stabilities and the ability to be easily recycled, with favourable solvation behaviour, because many organic, organometallic and inorganic compounds can be dissolved in ILs [2]. Nowadays, ILs are being studied for multiple technological applications being seen as potential "green" substitutes of conventional organic solvents [3]. These "green" credentials are mainly derived from their negligible vapour pressures that reduce the risk of air pollution.

The information about the (eco)toxicological risk profile of ILs is still scarce and, in some cases, even inexistent, as Ranke et al. [4] have recently pointed. The main conclusion was that the experimental data in several contexts, such as bioaccumulation, biodegradation of ILs in the environment, photodegradation, release to soil and water and aquatic toxicity are extremely incomplete. Thus, the main objective of this study was to generate new information about the toxicity of ILs to aquatic organisms.

Despite their low vapour pressure, as we previously mentioned, even the water immiscible ILs shown some solubility in water [5, 6] and some even a limited stability in presence of water [7] that allows its dispersion into the aquatic systems resulting in water pollution. Furthermore most ILs seem to be also poorly decomposed by microorganisms. The adsorption of these solvents onto a range of bacterial surfaces has also been found to be minimal, also implying that their transport through subsurface groundwater would be unimpeded [4]. Based on this information it can be hypothesized that ILs may pose environmental risks to aquatic ecosystems and accurate data on their toxicities are likely to be of foremost importance but are still scarce, especially for hydrophobic ILs. Microtox® Acute Toxicity Test is quick, simple, cost-effective and sensitive being a widely accepted method [8] for toxicity determination, providing a fast yet accurate estimate of the toxicity of a compound. This test is a prokaryotic microscale toxicity bioassay using a luminescent and gram negative marine bacteria. The endpoint of this methodology, EC_{50}, is the effective concentration value that corresponds to the concentration of toxic that produces 50% inhibition of light emission from a specific strain of bioluminescent bacteria. Recent works [9-11] illustrated the effects of ILs in *Vibrio fischeri* (formerly *Photobacterium phosphoreum*), where the bioluminescence inhibition was studied. Some authors [12] studied the toxicity of imidazolium - based ILs with Cl, $[BF_4]$, $[(CF_3SO_2)_2N]$, octylsulfate and bis(1,2-benzenediolato)borate anions [13] fitted the EC_{50} values of different imidazolium - based ILs with the $[BF_4]$, $[PF_6]$, Br and Cl anions.

This work is part of a project aiming at using water immiscible ionic liquids in biotechnological applications. Here, the toxicity of a water stable and immiscible ionic liquid from the most used cation family, the imidazolium, viz. the 1-methyl-3-propylimidazolium bis(trifluoromethylsulfonyl)imide, $[C_3mim][NTf_2]$, shown in Figure 4.1.1 was evaluated, thinking in the idea of the possible impact of the presence of these compounds in aquatic environments. Thus, the organisms were chosen to cover various trophic levels and some autochthonous species (*Chlorella vulgaris* and *Dapnhia*

longispina), to perform a comparative study between species, considering the same trophic level. Furthermore, these autochthonous species belongs to the Portuguese freshwater ecosystems.

There are reports about the ILs' toxicity in aquatic organisms of higher trophic levels, such as freshwater green algae - primary producers [14-16] and cladocerans - primary consumers [17-20]. The widespread distribution of algae makes these organisms ideal for toxicological studies, because they have a short life cycle and quickly respond to environmental changes [21, 22]. The cladoceran studies report the toxicity for various ILs' families with different anions on *Daphnia magna*, since this daphnid is a widely accepted model organism for toxicity testing because of its rapid rate of reproduction and sensitivity to different conditions. However, there is limited or no information about the effects of the ILs tested on other species of algae and cladocerans, namely those studied on this work - *Chlorella vulgaris* and *Daphnia longispina*. Moreover, dose-response bioassays on test aquatic organisms such as freshwater algae and crustaceans, are always required for defining toxicological endpoints of chemicals. The main goal of the present study is to provide further information about the toxicological impacts of an immiscible imidazolium - based IL in different species and trophic levels. We assessed also, the chronic effects of $[C_3mim][NTf_2]$ on the populational parameters of *Daphnia magna* (clone A, *sensu* [23]) and *Daphnia longispina* (clone EM7, *sensu* [24]).

4.1.3. MATERIALS AND METHODS

4.1.3.1. Test chemicals

The molecular structure of 1-methyl-3-propylimidazolium bis(trifluoromethylsulfonyl)imide, $[C_3mim][NTf_2]$ is provided in Figure 4.1.1. The IL was purchased at Iolitec (Ionic Liquid Technologies, Germany). To reduce the water and other volatile compounds content to negligible values, the IL was dried under constant stirring at moderate vacuum and temperature (≈ 353 K) for a minimum of 48 h prior to be used. After this procedure, its purity was further checked by ^{1}H and ^{13}C NMR spectra and found to be > 99 w/w %. The water used was ultra-pure water, double distilled, passed by a reverse osmosis system and further treated with a Milli-Q plus 185 water purification apparatus.

Figure 4.1.1. Chemical structure of the IL studied: [C_3mim][NTf_2].

4.1.3.2. Microtox® tests

Microtox® test [3] was used to evaluate the inhibition of the luminescence in the marine bacteria *Vibrio fischeri* (Beijerinck) Lehmann and Neumann. This test was performed using a range of diluted aqueous solutions (from 0 to 100%) of IL, where 100% of IL corresponds to a saturated solution - 12.0 g.L^{-1}[4]. After 5 and 15 minutes of exposure to the IL, the light output of the luminescent bacteria was measured and compared with the light output of a blank control sample. The toxicity was evaluated and a 50% reduction in luminescence was computed using Microtox® Omni™ Software version 4.3.0.1. [5]

4.1.3.3. Freshwater Green Algae

4.1.3.3.1. Algae Cultures

Cultures of *Pseudokirchenriella subcapitata* and *Chlorella vulgaris* may be initially obtained from commercial sources and subsequently cultured using a sterile technique. Upon receipt of an algal culture not previously maintained in a facility, a period of six weeks culturing is recommended to establish the ability to successfully maintain a healthy, reproducibly-growing culture. Information on culturing algae can be found in the references listed in literature [28]. Aseptic stock transfer should be maintained in or near the logarithmic growth phase and standard procedures was followed [29, 30]. The algal inoculum used to initiate toxicity testing was from a liquid culture shown to be actively growing in at least two subcultures lasting 7 days each prior to the start of the definitive test.

Algae cultures were reared under a $16h^L:8h^D$ photoperiod (provided by cool fluorescent white lights) and a temperature of 293 ± 2 K.

4.1.3.3.2. Algae Tests

The green algae *Pseudokirchneriella subcapitata* (Korshikov) Hindak and *Chlorella vulgaris* Beijerinck were used to test the effects of the Imidazolium on the algal growth. These two species of microalgae were obtained by Bacteria - Free protease Agar, Carolina Biological Supply Company – Burlington, North Carolina 27215 and Algal - Gro® Freshwater and Carolina Biological Supply Company - Burlington, North Carolina 27215, respectively.

Marine Biological Laboratory medium (MBL – Sterilized Hoods Hole Culture [31] was used as nutritive culture medium, being this prepared in accordance with Gonçalves et al. [28]. The medium was sterilized by autoclaving. The vitamins (sterilized by filtration) were only added after medium sterilisation under cold conditions. The freshwater green algae mostly recommended for algal growth inhibition testing procedures is *Pseudokirchneriella subcapitata* [29, 30, 32]. Nevertheless, the performance of *Chlorella vulgaris* as test organism was assessed by comparison with *Pseudokirchneriella subcapitata* growth behaviour under the same conditions. For each alga, the samples were placed in 100 mL borosilicate Erlenmeyer flasks with 40 mL of final test volume [28]. Three days before starting the experiments, an inoculum was incubated under the same conditions as the test cultures to adapt each alga to the test conditions and achieve exponential growth as usually recommended [30].

The initial cell density used for all green algae corresponded to 10^4 cells.mL^{-1}. The preparation of MBL medium and flasks handling was carried out aseptically. The IL concentrations were obtained by successive dilutions of a saturated solution of [C_3mim][NTf_2] with MBL. Clean MBL medium was used as the negative control. To each treatment corresponded a set of three replicates.

The tests were performed in an incubation chamber with continuous agitation at 100 rpm, under the same photoperiod and temperature conditions as described for algae cultures. Standard growth inhibition procedures recommend that algal growth tests should be terminated after 72 h of incubation under continuous light. In this experiment, the test was finished after 4 days of incubation (after 96 h) due to the photoperiod and the biomass parameter determined was cell counting [28]. Algal density was directly counted using a Neubauer chamber for both species of algae [33].

4.1.3.4. Cladocerans

4.1.3.4.1. Daphnid Cultures

Monoclonal cultures of *Daphnia magna* Straus (clone A, *sensu* [34]) and *Daphnia longispina* O. F. Müller (clone EM7, *sensu* [24]) have been reared in our laboratory for several generations using widespread procedures for the standard organism [34-36]. Daphnids were cultured in American Society for Testing and Materials hard water medium [37], to which an organic additive, *Ascophyllum nodosum* extract [34], was added. ASTM media is prepared using sterile distilled water, adding 2.40 g of $CaCO_3$ previously dissolved in 2 L of distilled water, vitamins and 200 ml of $NaHCO_3$ (19.2 $g.L^{-1}$), $MgSO_4.7H_2O$ (24.57 $g.L^{-1}$) and KCl (0.8 $g.L^{-1}$). Cultures were reared under a $16h^L:8h^D$ photoperiod (provided by cool fluorescent white lights) and a temperature of 293 ± 2 K. The medium was renewed every other day, to a total of three times per week. Cladocerans were fed with *Pseudokirchneriella subcapitata* (Korshikov) Hindak every other day, at a concentration of $3.0x10^5$ $cells.mL^{-1}$ and $1.5x10^5$ $cells.mL^{-1}$ for *Daphnia magna* and *Daphnia longispina*, respectively. Algal ratio was determined spectrophotometrically at 440 nm, as described for *Chlorella vulgaris* Beijerinck by Carvalho et al. [38].

4.1.3.4.2. Acute test method

Tests were performed according to standard protocols [39-41] under the same temperature and photoperiod regimes as described for rearing procedures. For both *Daphnia* species, experiments were initiated with neonates (< 24-h-old) obtained from the same bulk culture, born between the 3rd and 5th broods. IL concentrations were obtained by successive dilutions of the saturated solution of $[C_3mim][NTf_2]$ in the synthetic hard water medium (ASTM). The culture medium was used as the control treatment.

Acute tests were carried out in four glass beakers per treatment containing 100 mL of test solutions. A static design was employed, using 20 neonates (randomly divided into four groups of five organisms) per control and IL concentration. *Daphnids* were exposed to different IL concentrations during 48 h without food or organic extract. Vessels were checked for immobilised individuals, at 24 and 48 h, for posterior determination of EC_{50} values.

4.1.3.4.3. *Chronic test method*

Chronic tests were conducted for 21 days according to standard protocols [35, 42, 43]. The body length (from the base of the spine to the top of the head) of a subsample of neonates (n = 20) and from the same batch were measured under stereoscope magnification in order to determine their size at the beginning of the test (*li* – initial body length). Experiments were carried out using glass beakers (10 per treatment) containing 50 mL of test solution, including organic extract, unlike in the acute tests. A semi-static design was employed, using 10 individualised organisms (specifically one per replicate) randomly assigned to the control and to each IL concentration. Daphnids were fed daily, with *Pseudokirchneriella subcapitata*, and transferred to freshly - prepared test solutions every other day. Cladocerans were checked every day at the same approximate hour for mortality and reproductive state (presence of eggs or offspring). When neonates were released, they were counted and discarded. A life history table was built with the mortality and fecundity data. At the end of the test, all the surviving mothers were measured (from the base of the spine to the top of the head) under stereoscope magnification (*lf* – final body length). This allowed the calculation of the somatic growth rate, which was estimated from the initial (*li*, in mm) and final (*lf*, in mm) body size of the daphnids, according to the following expression:

$$growth\ rate = \ln(l_f) - \ln(l_i) / \Delta t \qquad \text{Eq. 4.1}$$

where growth rate is expressed in day^{-1} and Δt is the time interval (21 days). Survival and fecundity estimates were also used to compute the intrinsic rate of population increase (*r*). This demographic parameter was iterated from the Euler – Lotka equation:

$$1 = \sum_{x=0}^{n} e^{-rx} l_x m_x \qquad \text{Eq. 4.2}$$

where *r* is expressed in day^{-1}, *x* is the age class (1…n days), l_x is the probability of surviving to age *x*, and m_x is the fecundity at age *x*. Pseudovalues and standard errors for *r* were estimated using the jack-knifing technique described by the literature work [44].

4.1.4. Statistical Analysis

The results of the different algae treatments, expressed as optical density for both algae species, were compared using analysis of variance (ANOVA). If applicable, a Tukey multiple comparison test was applied with statistically significant differences in growth

reported for $p < 0.05$ [45]. The EC_{50} values based on inhibition concentrations were also determined by Probit analysis for each species.

The EC_{50} values for immobilisation (acute tests) and fecundity (chronic tests) were calculated by Probit analysis [46], for both daphnids. To test the significance of the IL effect on the life history parameters, an one-way ANOVA was employed on the chronic test data. When the ANOVAs were statistically significant, a Dunnett test was then applied to the data in order to determine which concentrations were significantly different from the control group. A significance level (α) of 0.05 was used in the analysis.

4.1.5. RESULTS AND DISCUSSION

The experimental EC_{50} values determined by Microtox® bioassays for 5 and 15 minutes of IL exposure were presented (Table 4.1.1). These results indicate that $[C_3mim][NTf_2]$ can be considered moderately toxic. Various imidazolium-based ILs were previously tested and reported in literature [9, 47]. However, only Matzke et al. [12] have presented the EC_{50} for an imidazolium-based IL with a $[NTf_2]$ anion. Despite of the differences on the alkyl chain length and time of exposure between the two works, the toxicity values are consistent. These results show that, as the time of exposure of *Vibrio fischeri* increases, a reduction of EC_{50} value is observed.

Table 4.1.1. Microtox® EC_{50} values ($mg.L^{-1}$) of $[C_3mim][NTf_2]$ after 5, 15 and 30 minutes of exposure to the luminescent marine bacteria *Vibrio fischeri*, with respective 95% confidence limits (in brackets).

Ionic Liquid	**EC_{50} ($mg.L^{-1}$)** (lower limit; upper limit)	**Reference** (time of exposure)
$[C_3mim][NTf_2]$	480.00 (240.00; 840.00)	This study (5min)
	240.00 (120.00; 840.00)	This study (15 min)
$[C_4mim][NTf_2]$	125.81	Matzke et al. [12] (30 min)

Having established the toxicity of $[C_3mim][NTf_2]$ towards marine luminescent bacteria it is important to evaluate its relative toxicity when compared with organic solvents that the ILs are supposed to replace. The EC_{50} results relatively to some common organic solvents for 30 minutes of exposure were presented in Table 4.1.2. Although the exposure time is not the same and knowing that the higher the exposure time the lower the EC_{50} value, it was observed that $[C_3mim][NTf_2]$ presents a somewhat lower toxicity than other common solvents.

Table 4.1.2. EC_{50} values ($mg.L^{-1}$) of organic solvents after 30 minutes of exposure to the luminescent marine bacteria *Vibrio fischeri*, by other studies.

Compound	EC_{50} ($mg.L^{-1}$)	Reference
Phenol	30.76	Kaiser and Palabrica, Steinberg et al. [48, 49]
Toluene	31.74	
Benzene	108.05	
Ethylene glycol	621.00	
Chloroform	1199.33	
Dichloromethane	2532.33	
Ethyl acetate	5822.00	
Acetone	19311.14	
Methanol	101068.50	

The growth of both algae species is affected by the presence of the IL as shown in Figures 4.1.2 and 4.1.3. *Pseudokirchneriella subcapitata* was more tolerant ($EC_{50} \equiv 14.40$ $mg.L^{-1}$) than *Chlorella vulgaris* ($EC_{50} \equiv 10.29$ $mg.L^{-1}$) to the IL (Table 4.1.3). No previous EC_{50} values were reported for $[C_3mim][NTf_2]$.

Table 4.1.3. EC_{50} values ($mg.L^{-1}$) of [C_3mim][NTf_2] for freshwater microalgae (*Pseudokirchneriella subcapitata* and *Chlorella vulgaris*).

Species	**EC_{50} ($mg.L^{-1}$)**
Pseudokirchneriella subcapitata	14.40 (6.00; 25.00)
Chlorella vulgaris	10.29 (7.53; 13.02)

However, the toxicity for [C_4mim][NTf_2] on *Pseudokirchneriella subcapitata* (EC_{50} = 26.49 $mg.L^{-1}$) was reported in literature [17], being this in good agreement with our study. Similarly, Fritz and Braun [50] obtained comparable results using chemical compounds that alter processes universally important, such as oxidative phosphorylation. Toxicants like herbicides, that are designed to inhibit the photosynthesis electron transport process, have a mode of action that makes them potentially lethal to a wide variety of non-target species of primary producers in which freshwater microalgae are included [51]. Moreover, algal size is also an important factor in the interaction between algae and toxicants [14].

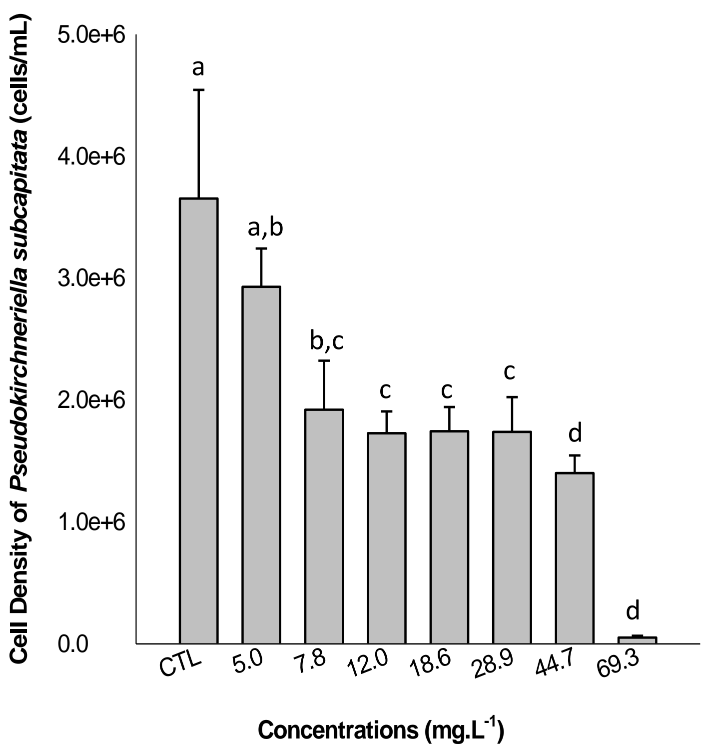

Figure 4.1.2. Growth inhibition of *Pseudokirchneriella subcapitata* when exposed to successive dilutions of [C_3mim][NTf_2], after 96h of incubation. Error bars represent standard error and the letters correspond to significant differences between the treatments ($p < 0.05$).

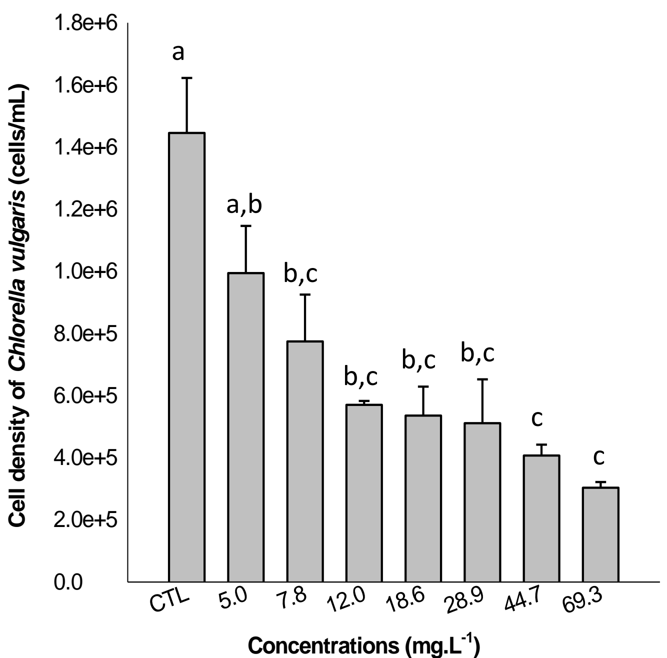

Figure 4.1.3. Growth inhibition of *Chlorella vulgaris* when exposed to successive dilutions of [C_3mim][NTf_2], after 96h of incubation. Error bars represent standard error and the letters correspond to significant differences between the treatments ($p < 0.05$).

In order to compare ILs and organic solvents, the toxicity of some common solvents towards *Pseudokirchneriella subcapitata* and *Chlorella vulgaris* is presented in Table 4.1.4. Again, the toxicity of this IL is comparable to other aromatic solvents such as aniline, methanol and 2- propanol, and lower than the toxicity of xylenes.

Table 4.1.4. EC_{50} values of organic solvents calculated in other studies to freshwater green algae.

Compound	**Algal Species**	**Chronic EC_{50} ($mg.L^{-1}$)**	**Reference**
Dichloromethane	*P. subcapitata*	662.0	[52]
Aniline	*P. subcapitata*	19.0	[53, 54]
Toluene	*P. subcapitata*	> 100	[18]
Xylenes	*P. subcapitata*	~ 5	[18]
Methanol	*P. subcapitata*	11.1	[53]
2-propanol	*P. subcapitata*	11.92	[53]
Phenol	*C. vulgaris*	370.0	[17]

The acute and chronic EC_{50} values reported in Table 4.1.5 for *Daphnia magna* and *Daphnia longispina* show that the former species is more tolerant to IL concentrations than the last one. Indeed, *Daphnia magna* revealed to tolerate IL concentrations twice as higher than those tolerated by *Daphnia longispina*. This behaviour can be explained by the smaller size of the latter species, and the consequent greater surface-to-volume ratio [55], would lead to a great exposition of the organism to the chemical. This pattern has been observed by several authors when comparing acute toxicities of different chemicals to both daphnids [56-59]. The acute assays' outcome, denoting a lower sensitivity of the larger species, was confirmed in the chronic exposures.

Table 4.1.5. Acute and chronic EC_{50} values ($mg.L^{-1}$) of $[C_3mim][NTf_2]$ for cladocerans species (*Daphnia magna* and *Daphnia longispina*), with respective 95% confidence intervals (in brackets).

Species	Acute EC_{50} ($mg.L^{-1}$) (lower limit; upper limit)	Chronic EC_{50} ($mg.L^{-1}$) (lower limit; upper limit)
D. magna	146.80 (141.20-.153.20)	111.56 (106.40-115.85)
D. longispina	74.41 (69.56-80.01)	i.d

i. d. – impossible to determine, since the mortality was inferior to 20%.

No previous EC_{50} values were reported for $[C_3mim][NTf_2]$ but Pretti et al. [17] report acute EC_{50} values for $[C_4mim][NTf_2]$ on *Daphnia magna* (EC_{50} = 18.91 $mg.L^{-1}$). However, the acute EC_{50} value of *Daphnia magna* when exposed to $[C_4mim][NTf_2]$ demonstrates that this IL is more toxic than $[C_3mim]NTf_2]$ for this cladoceran species. The comparison between the acute toxicity of $[C_3mim][NTf_2]$ and organic solvents for *Daphnia magna* reported in Table 4.1.6 shows that this IL toxicity compares favourably with the toxicity of some common solvents.

Table 4.1.6. Acute EC_{50} values of organic solvents to *Daphnia magna*, determined in other studies.

Compound description	Acute EC_{50} ($mg.L^{-1}$)	Reference
Methanol	24.50	[17]
Dichloromethane	1.68	[17]
Acetonitrile	3.60	[17]
Aniline	80-380	[17]
Triethylamine	200	[17]
Chlorine	0.12-0.15	[60]
Ammonia	2.90-6.93	[60]
Phenol	10-17	[61]

In general, all life history parameters of both daphnids were affected by $[C_3mim][NTf_2]$ with the exception of the number of broods and somatic growth rate (SGR) for *Daphnia longispina* (Figures 4.1.4 and 4.1.5).

The mortality of *Daphnia magna* increased in the two higher concentrations of IL (50 and 90%, correspondingly). For *Daphnia longispina*, the mortality was inferior to 20%, which explains the absence of the correspondent EC_{50} value. A concentration-dependent decrease in the number of offspring was observed for both species, being it more noticeable for *Daphnia magna.* This decrease was accompanied by a developmental delay, indicating an increase in the age at first reproduction. A decrease in the somatic growth rate of both species was also observed, being more in evidence again in *Daphnia magna*. On the other hand, the number of broods produced per female was also significantly reduced on the *Daphnia magna* case. As a consequence of the lower fecundity and of the developmental delay, the intrinsic rate of increase was significantly reduced in both species, being more obvious for *Dapnhia magna*, where this reduction was preceded by a stimulatory effect at low concentration of $[C_3mim][NTf_2]$ - 96.37 $mg.L^{-1}$. IL is a contact product and its main intake route is likely to be through body surface rather than via filtration-related mechanisms. Actually, *Dapnhia longispina* is smaller than *Dapnhia magna*, with consequent greater surface-to-volume ratio.

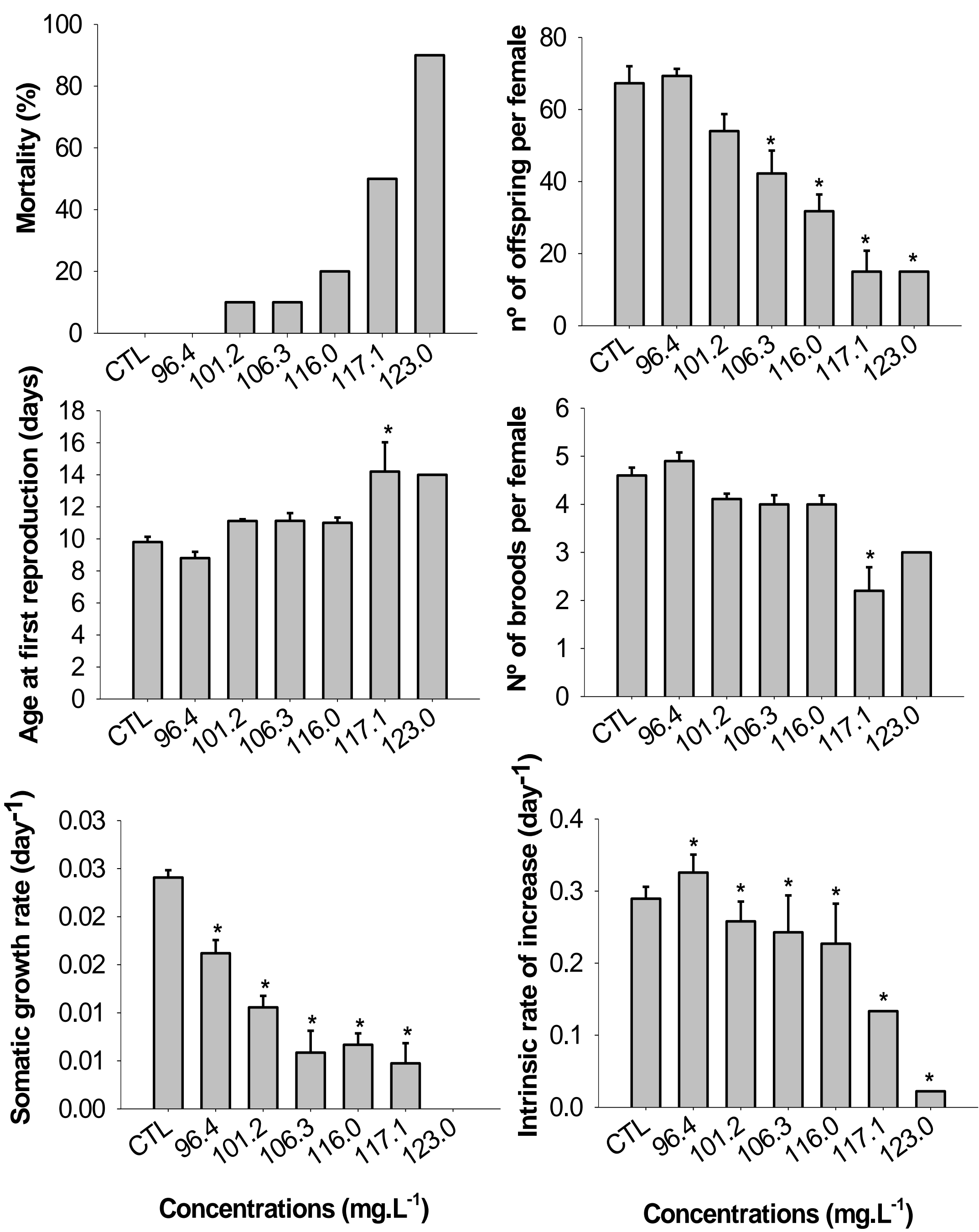

Figure 4.1.4. Life history parameters of *Daphnia magna* exposed to different IL concentrations for 21 days. Error bars represent standard error and * indicates statistically significant differences (Dunnett test, p≤0.05) between the test concentrations and the control (CTL).

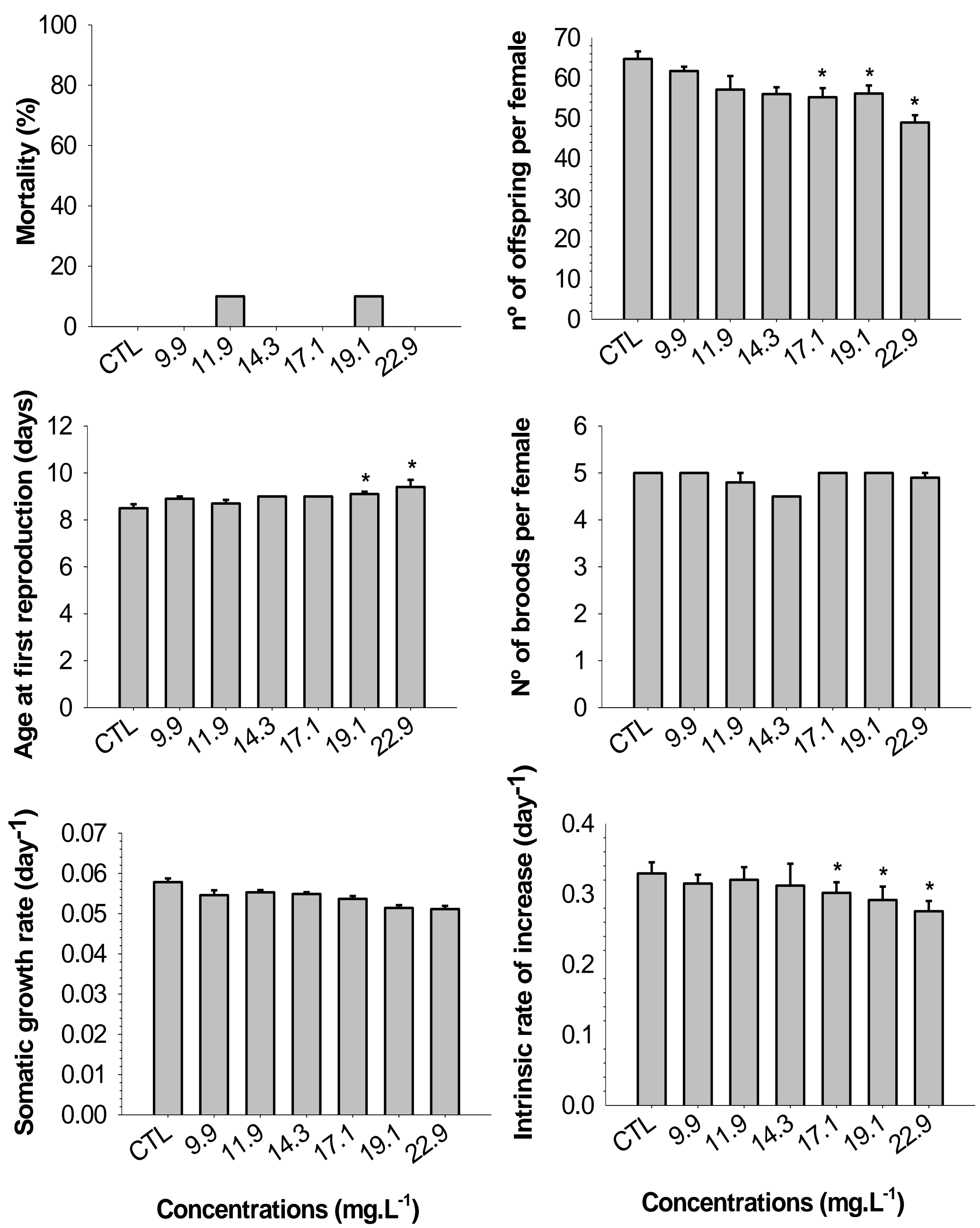

Figure 4.1.5. Life history parameters of *Daphnia longispina* exposed to different IL concentrations for 21 days. Error bars represent standard error and * indicates statistically significant differences (Dunnett test, p≤0.05) between the test concentrations and the control (CTL).

As mentioned in the introduction section, once in industrial use, the main pathway of ILs into the environment is through aqueous effluents. In previous works [54, 62] has been shown that the concentration of ILs in aqueous solutions can be reduced significantly by treating them with salts. They show that a number of salts have a strong salting-out ability allowing them to be used in water treatment. Essays carried by us show that using aluminium sulphate concentrations of 0.8 $mol.L^{-1}$, it is possible to reduce the solubility of $[C_3mim][NTf_2]$ from 75000 to 500 ppm. We are currently carrying studies to identify the possibility of using inorganic salts in the treatment of effluents contaminated with ILs. Whether this treatment can bring the concentration of the IL below the desired value or can be used as a primary treatment that must be followed by adsorption or another option it remains to be seen. Both the moderate toxicity of the IL studied and the possibility of treat the aqueous effluents contaminated with it open good perspectives to its industrial application.

4.1.6. CONCLUSIONS

The toxicity of the ionic liquid $[C_3mim][NTf_2]$ was studied for different species and trophic levels and it was shown to present a moderate toxicity equivalent to other aromatic industrial solvents.

This study demonstrates that the sensitivity to the $[C_3mim][NTf_2]$ differs among species. Within the species of the same trophic levels *Pseudokirchneriella subcapitata* is shown to be somewhat more tolerant than *Chlorella vulgaris* when exposed to the IL. When compared with *Dapnhia longispina*, *Dapnhia magna* is shown to be far more sensitive to the presence of the IL.

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4.2. PAPER 10

Designing ionic liquids: How to enhance their hydrophobic nature while lowering toxicity

4.2.1. ABSTRACT

Ionic liquids (ILs) are a novel promising class of solvents with interesting physicochemical properties. Many different applications have been described as alternatives to organic solvents in chemical processes. Despite their low vapor pressure, even the water immiscible ILs shown some solubility in water that allows their dispersion into the aquatic systems resulting in water contamination.

This work aims at enlarging the restricted knowledge about the ILs toxicity and inhibitory effects in aquatic ecosystems and to investigate the possibility of designing hydrophobic ionic liquids of low toxicity. It reports a set of toxicity results, which correspond to aromatic and non-aromatic immiscible ILs, through the use of different cations (pyridinium, piperidinium, pyrrolidinium and imidazolium) and hydrophobic anions (bis(trifluoromethylsulfonyl)imide [NTf_2] and hexafluorophosphate [PF_6]). In this context, biological assays were performed using organisms of different trophic levels, such as the decomposer *Vibrio fischeri*, the producer *Pseudokirchneriella subcapitata* and the first consumer *Daphnia magna*.

Contrary to the common belief that the ILs toxicity increases with their hydrophobicity it is here shown that it is possible to design ILs with an enhanced hydrophobic character, expressed as water solubility, and lower toxicity, as determined by the tests here conducted, by elimination of their aromatic nature.

4.2.2. INTRODUCTION

One of the principles of green chemistry aims at the reduction of the toxicity of the chemical compounds used in industrial processes or product formulations. Legislation concerning this subject is nowadays more stringent as REACH [1] (Registration, Evaluation, Authorization and Restriction of Chemicals), requires the registration of new commercial chemicals and holds the suppliers responsible for their products. Therefore, in the development of new products, the optimization of technical performance must run in parallel with the minimization of hazard potentials. This strategy makes sense not only from an ecological point of view but also economically because of the reduced risk of failure in the process of registration of a new chemical compound [2].

A new class of chemicals composed exclusively by ions that are liquid at room temperature, known as Ionic Liquids (ILs), is under development and have found favor

of both academia and industry as replacement for volatile organic compounds in many applications [3], solvents [4], reaction media for chemical [5, 6] and biochemical reactions [7-9], engineering fluids [6], among others. ILs are composed of organic cations, such as imidazolium, pyridinium, piperidinium and pyrrolidinium and halogen, fluorinated or organic anions. They are considered “designer solvents” due to the possibility of freely manipulating their characteristics by combining these cations and anions to meet the requirements for a specific application [10, 11].

One of the driving forces behind the interest on ILs is not only their extended performance when compared with conventional solvents, but also them being touted as “green solvents”. The rationale for labeling them “green” is based on four arguments: their synthesis [12], their negligible vapor pressure [13, 14], meaning that the inhalative exposure of workers is reduced as compared to conventional molecular solvents, their non-flammability, being the risk of fast and exothermic oxidations in the case of an accident strongly reduced, and finally, the low toxicity of many ionic liquids [15, 16]. While these issues are certainly important in the discussion concerning their industrial application, the latest point in particular, has been repeatedly challenged. Most ILs are poorly decomposed by microorganisms [17-20] and their adsorption onto a range of bacterial surfaces is also minimal [21]. Moreover, their properties such as resistance to photodegradation [22], bioaccumulation [23], water solubilities and water stabilities [24-29] concur to the idea that they may pose a threat to aquatic organisms. However, subjects such as biodegradability and bioaccumulation, are poorly studied, which is very disturbing [30]. Recently, some authors have been addressing the issue of the biodegradability of various ILs, studying the effect of their anions [17, 31], cations [2, 17] and the type and number of carbons in their alkyl chains [17-19], demonstrating the importance of this issue in the total risk assessment approach [16]. The enhanced biodegradability of ionic liquids achieved by introducing oxygenated groups on their alkyl chains was successfully established in literature [18]. Nevertheless, a range of biodegradability studies for ILs and their metabolites is required to correctly establish their effect on the risk assessment [16].

In what concerns the (eco)toxicity of these compounds, there is still a great uncertainty regarding their distribution in the different environmental compartments [16]. According to our recent work [32] and other reports from literature [15, 33] is well known that the ILs’ (eco)toxicity varies widely across organisms and trophic levels. The most tested trophic levels are decomposers [32, 34, 35] (bioluminescent marine

bacterium), producers [30, 32, 36-40] (freshwater green algae) and primary consumers [15, 32, 41] (daphnids).

Freshwater green algae are extensively used as biological assay tools for environmental impact studies due to their vulnerability to chemical stressors that results from their small size, large population numbers and short life cycles [42]. Moreover, as producers of organic matter required by small consumers in freshwater food chains, their ecology is crucial in providing the energy for sustaining higher trophic levels.

Latala and his co-workers [38, 43, 44] have recently reported a series of works, where two green algae (*Chlorella vulgaris* and *Oocystis submarina*), three diatoms algae (*Cyclotella meneghiniana*, *Skeletonema marinoi* and *Bacillaria paxillifer*) and one blue green alga (*Geitlerinema amphibium*) were studied. The authors observed a pronounced effect of the cation alkyl chain length for all these algae species. The results also showed that diatoms were far more sensitive than green algae. This was explained by differences in the cell wall structure. The silica-based cell wall of the diatoms appears to be more sensitive than the cellulose wall of green algae. Moreover, the cell size also plays an important role in the toxic effects since a tenfold difference in cell size results in 100 % more sensitive reaction to ILs on the part of both the green and diatoms algae. Since no information on EC_{50} values were previously reported for algae, the data reported in this work provides useful information for the assessment of the fate of ILs in marine environments. Most publications about the (eco)toxicity of ILs on algae deal with *Pseudokirchneriella subcapitata*. Several authors have reported studies on the toxicological effects of distinct ILs towards algae [15, 30, 36-40, 43-46]. Some [39] have observed that long alkyl chains in the IL cation increase their toxicity. Others [36, 37] have used this standard alga to study the effect of different head groups, side chains and anions of ILs on the algal growth rate and photosynthetic activity. These results revealed that the toxic influence of ILs on growth rates were more significant than on photosynthetic performance [47]. Other authors [15, 47] have confirmed the results about the effects of the cation alkyl chain length and the anions towards *Pseudokirchneriella subcapitata*.

Daphnia magna is an important link between producers and higher trophic levels [48], being of fast reproduction rate and sensitivity to environmental conditions. Usually, this aquatic organism is subjected to both acute and chronic tests [49]. Acute bioassays expose an organism to a range of chemical concentrations for a short period of time, evaluating the concentration that produces a 50 percent mortality in a test population

over a specific time period [30]. If an effect other than mortality is investigated (such as growth or reproduction), the term EC_{50} is used to denote the Effect Concentration that is estimated to cause a 50 percent decrease or increase in the effect (chronic test). The acute and chronic toxicity of imidazolium cation-based ILs on *D. magna* was first studied by Bernot and his co-workers [50]. This work was followed by other studies [15, 31, 35, 39, 41, 51] about the influence of different cation structures on the IL toxicity. These studies have shown that ILs have different degrees of toxicity to cladocerans. An increase on the cation alkyl chain length [50] produces a strong effect on the growth and reproduction of cladocerans. Recently, there has also been evidence that anions can contribute to toxicity, but in most cases the anion effects are less important than the cation alkyl chain length effect [33, 45].

The Microtox® bioassay is a bioluminescence inhibition method based on the bacterium *Vibrio fischeri*. It is today one of the most widespread toxicological bioassays due to its speed, simplicity and cost-effective implementation [52]. This test was widely used in different approaches, such as the study of the influence of the anion [31, 34, 45], cation [51] and alkyl chain length [2, 31, 35, 45] of several ILs with results that are qualitatively in agreement with those carried for species of higher trophic levels. For this organism, the ILs toxicity also correlates directly with the alkyl chain length of the cation [53]. In what concerns the anionic influence, although it has been claimed that modifications of the anion lead to changes in chemical and physical properties of ILs, [54] no clear increase in toxicity caused by the anion could be observed. Although the anion is not considered responsible for the high toxicity values, there are some exceptions, such as the $[NTf_2]$ anion, which shows high values of toxicity, independently of the cation [33]. Couling and his collaborators [51] have extended the bioluminescence inhibition assays to pyridinium derivatives and it was noted that the quaternary ammonium compounds seem to be less toxic than the pyridinium and imidazolium analogues. The toxicity results available in the literature concur in showing that the cation is the main responsible for the toxicity of the IL [34]. This is explained by the possible intercalation of the lipophilic part of the molecules into the organism membrane, whereas their ionic head group is, at least, partially solvated in the aqueous solution [55]. Additionally, it has been proposed that the mode of toxic action for ILs takes place through membrane disruption because of the structural similarity of imidazolium-based ILs to detergent, pesticides and antibiotics [2]. Recently, there have been attempts at understanding the relationship between the toxicity of the ILs and other

important properties, such as their hydrophobic nature (normally determined according to the solubilities of ILs in water) [56], membrane water partitioning [33] and lipophilicity [33, 45, 57, 58].

Although distinct, the concepts of lipophilicity and hydrophobicity are often confused. While the lipophilicity describes the ability of a compound to interact with the non polar compounds such as lipids and cell membranes, the hydrophobicity addresses the poor affinity of the compounds towards water molecules. The water solubility is a measure of the IL hydrophobicity/hydrophilicity while the lipophilicity is measured by partition coefficients between an aqueous and an organic phase such as K_{ow} or log P. Although correlated for many compounds for which a high hydrophobicity corresponds to a high lipophilicity, this is not always true for complex molecules or fluorinated compounds.

Based on these ideas a number of approaches were proposed trying to develop correlations to predict the toxicity of the ILs. Some of the most important works on this area [33, 45, 58] reported the relation between the lipophilicity and/or hydrophobicity of the ILs and their correspondent toxicity/cytotoxicity. In particular, Ranke and his contributors [58] proposed a correlation between the cytotoxicity, expressed as EC_{50}, and the hydrophobicity, expressed as log k_0. These results suggest a potential conflict on the design of ionic liquids between the contradictory goals of minimization of the toxicity and maximization of the hydrophobicity of these solvents as required for fermentative extraction processes, among others.

The main objective of the present study is to establish the impact of some little studied parameters on the toxicity of ILs, evaluating the possibility of designing ionic liquids of lower toxicity and higher hydrophobicity. For that purpose, the aromatic nature of the ILs will be studied for various cations and anions by measuring the EC_{50} values of four different families of IL cations (1-methyl-3-propylimidazolium, 1-methyl-1-propylpyrrolidinium, 3-methyl-1-propylpiridinium and 1-methyl-1-propylpiperidinium) for *V. fischeri* (Microtox® bioassays [59]), *P. subcapitata* (OECD guidelines [60]) and *D. magna* (ASTM protocols [61]). These cations were combined with two different hydrophobic anions bis(trifluoromethylsulfonyl)imide [NTf_2] and hexafluorophosphate [PF_6], to produce the hydrophobic ionic liquids here studied. The results reported are analyzed to establish a relation between the impacts of the ILs' aromatic nature on their toxicity. The results suggest the possibility of designing IL cations that are simultaneously more hydrophobic, *i.e.* less water soluble, and less toxic, as determined by the tests here conducted, for the various environmental compartments.

The choice of the anions was done attending at their hydrophobic character but does not consist on an endorsement of these anions as being environmentally friendly. Actually the $[PF_6]$ anion is poorly water stable releasing in certain conditions HF into the aquatic environment [28], while the $[NTf_2]$, though being water stable, has the potential to persist in the environment. The search for hydrophobic environmentally friendly anions is ongoing and it is a field where there is great scope for improvement.

4.2.3. MATERIALS AND METHODS

4.2.3.1. Test chemicals

The ILs used in this study are 1-methyl-3-propylimidazolium bis(trifluoromethylsulfonyl)imide, $[C_3mim][NTf_2]$ 1-methyl-1-propylpyrrolidinium bis(trifluoromethylsulfonyl)imide, $[C_3mpyrr][NTf_2]$, 3-methyl-1-propylpyridinium bis(trifluoromethylsulfonyl)imide, $[C_3mpyr][NTf_2]$, 1-methyl-1-propylpiperidinium bis(trifluoromethylsulfonyl)imide, $[C_3mpip][NTf_2]$ 1-methyl-3-propylimidazolium hexafluorophosphate, $[C_3mim][PF_6]$, 1-methyl-1-propylpyrrolidinium hexafluorophosphate, $[C_3mpyrr][PF_6]$, 3-methyl-1-propylpyridinium hexafluorophosphate, $[C_3mpyr][PF_6]$ and 1-methyl-1-propylpiperidinium hexafluorophosphate, $[C_3mpip][PF_6]$. The molecular structures of all ILs are shown in Figure 5.2.1. The ILs used in this work were purchased from Iolitec (Ionic Liquid Technologies, Germany) with purities of 99%. The ILs were submitted to a purification step and their purity was further checked by ^1H and ^{13}C NMR spectra.

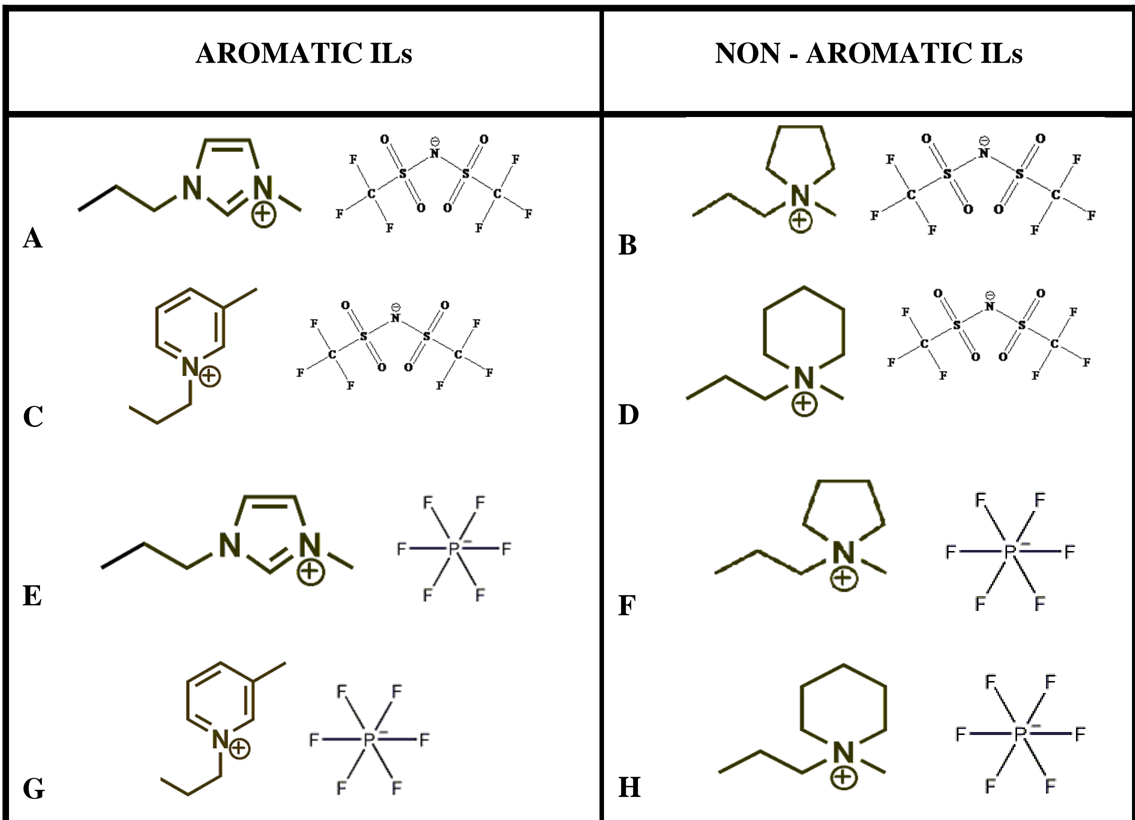


Figure 4.2.1. Chemical structure of the ILs studied: [C_3mim][NTf_2] (A), [C_3mpyrr][NTf_2] (B), [C_3mpyr][NTf_2] (C), [C_3mpip][NTf_2] (D), [C_3mim][PF_6] (E), [C_3mpyrr][PF_6] (F), [C_3mpyr][PF_6] (G) and [C_3mpip][PF_6] (H).

4.2.3.2. Ecotoxicological tests

The present work reports a range of toxicological tests using the marine bacteria *Vibrio fischeri* (Beijerinck) Lehmann and Neumann (Microtox® tests), the alga *Pseudokirchneriella subcapitata* (Korshikov) Hindak and the cladoceran *Daphnia magna*. The first tests aforementioned [59] were used to evaluate the inhibition of the luminescence in the bacteria. This test was performed using a range of diluted aqueous solutions (from 0 to 100%) of each IL, where 100% of IL corresponds to the concentration of a stock solution previously prepared (with concentration in IL well known and dependent of the IL) [24]. The Microtox® tests were performed following the same procedure described in the **Section 4.1.3.2**. The toxicity was evaluated and a 50% reduction in luminescence was computed using Microtox® Omni™ Software

version 4.3.0.1. [62]. The toxicity results reported in this work for the Microtox® assays were carried accordingly to the Basic Test [59].

The *Pseudokirchneriella subcapitata* was used in this work due to their widely utilization in algal growth inhibition testing procedures [61, 63, 64]. The preparation of these tests was done according to **Section 4.1.3.3.** These tests were performed as detailed elsewhere [32].

The acute tests with cladocerans were performed according to standard protocols [60, 65, 66] and **Section 4.1.3.4.**

4.2.4. Results and Discussion

One of the most appealing characteristics of ILs is the capacity to design them with a specific set of properties to meet the multiple requirements for a specific application [10, 11, 69]. In previous works, the mutual solubilities of ILs with water were investigated [24-27, 70, 71]. It was found that aromatic cations, such as imidazolium and pyridinium, have a larger solubility in water than non aromatic cations such as pyrrolidinium and piperidinium. The water solubilities of the ILs here studied are reported in Table 4.2.1. Water solubilities for [C_3mpyrr][PF_6] and [C_3mpip][PF_6] were measured in this work using a methodology well described in literature [72]. The toxicological data for different freshwater planktonic species obtained from tests with *Vibrio fischeri*, *Pseudokirchneriella subcapitata* and *Daphnia magna* are reported in Tables 4.2.1, 4.2.2 and 4.2.3.

Table 4.2.1. Solubilities in water ($mg.L^{-1}$) and Microtox® EC_{50} values ($mg.L^{-1}$) of each IL after 5 and 15 minutes of exposure to the luminescent marine bacteria *Vibrio fischeri*, with respective 95% confidence limits (in brackets).

Ionic Liquid	Solubility in water ($mg.L^{-1}$) at 298.15 K	EC_{50} ($mg.L^{-1}$) 5 min (lower limit; upper limit)	EC_{50} ($mg.L^{-1}$) 15 min (lower limit; upper limit)
$[C_3mim][NTf_2]$	1.22×10^4 [24]	4.80×10^2 (2.40×10^2; 8.40×10^2)	2.40×10^2 (1.20×10^2; 8.40×10^2)
$[C_3mpyrr][NTf_2]$	9.79×10^3 [72]	1.10×10^3 (6.00×10^2; 2.10×10^3)	8.00×10^2 (2.00×10^3; 3.30×10^4)
$[C_3mpyr][NTf_2]$	8.67×10^3 [27]	1.70×10^1 (0.00; 8.50×10^1)	1.70×10^1 (0.00;5.10×10^1)
$[C_3mpip][NTf_2]$	8.64×10^3 [72]	5.53×10^2 (4.42×10^2; 6.85×10^2)	3.76×10^2 (3.54×10^2; 3.76×10^2)
$[C_3mim][PF_6]$	2.94×10^4 [73]	1.70×10^3 (1.31×10^3; 2.20×10^3)	9.69×10^2 (6.03×10^2; 1.55×10^2)
$[C_3mpyrr][PF_6]$	1.01×10^4	3.90×10^3 (3.60×10^3; 4.20×10^3)	2.80×10^3 (2.40×10^3; 3.30×10^3)
$[C_3mpyr][PF_6]$	2.40×10^4 [73]	7.01×10^2 (5.04×10^2; 9.86×10^2)	4.38×10^2 (3.50×10^2; 5.48×10^2)
$[C_3mpip][PF_6]$	1.01×10^4	1.44×10^3 (6.65×10^2; 3.14×10^3)	1.12×10^3 (4.26×10^2; 2.79×10^3)

The Microtox® bioassays reported in Table 4.2.1 were carried using imidazolium, pyrrolidinium, pyridinium and piperidinium-based ILs with the same alkyl chain length (propyl) and conjugated with the hydrophobic anions $[NTf_2]$ and $[PF_6]$ presented in Figure 4.2.1. Under the conditions used in this work, the hydrolysis of $[PF_6]$ can be considered negligible [27]. The EC_{50} values obtained show that the ILs based on the aromatic imidazolium and pyridinium cations are always more toxic than those based on non-aromatic cations such as pyrrolidinium and piperidinium. Also, cations with 6 member rings are always more toxic than 5 member ring cations and $[NTf_2]$-based ILs

are more toxic than the [PF_6]-based Ils, as already suggested by other authors. [31, 33, 45, 53] For both anions the increase in toxicity follows the tendency:

$$[C_3mpyrr] \langle\ [C_3mpip] \langle\ [C_3mim] \langle\ [C_3mpyr]$$

The relation between the ILs toxicities measured by Microtox® and the respective water solubilities is reported in Figure 4.2.2.

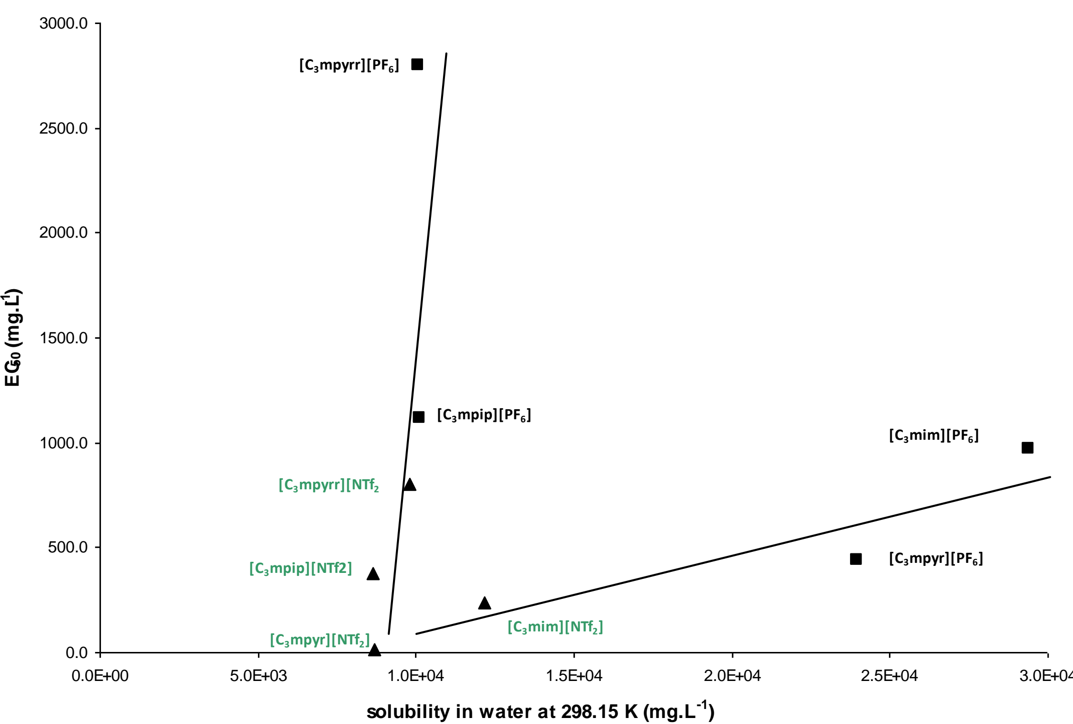


Figure 4.2.2. Relation between the water solubility of the different ILs studied and their toxicities (EC_{50} values) obtained by Microtox® assays.

The results clearly show that the studied ILs can be divided in two groups presenting very different dependencies of the toxicity with the water solubility. One of the groups comprises the non aromatic ILs, based on the piperidinium and pyrrolidinium, while the other comprises the aromatic ILs-based on the imidazolium and pyridinium cations. The non aromatic ILs present a much lower water solubility and toxicity than the aromatic ILs while the anions have a little impact on the groups with both [PF_6] and [NTf_2] anions, being the same toxicity dependency on the water solubility. While for both groups the toxicity does increase with the hydrophobicity (EC_{50} values decrease with the increase in water solubility), following the trend generally accepted, the differences

in the behavior between these two groups show that it is possible to design ILs of enhanced hydrophobicity while decreasing their toxicity, in a paradigmatic example of the "designer solvent" character of the ILs.

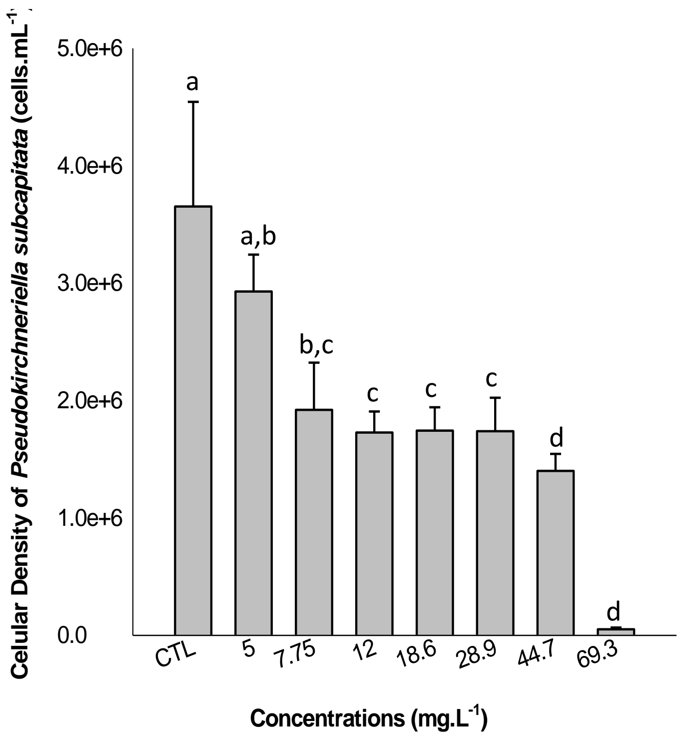
The suggestion that ionic liquids based on non aromatic cations are less toxic than those based on aromatic cations was recently proposed by Pretti et al. [15]. A compilation of toxicity data from the literature reported in *Tables S.8.4.1* and *S8.4.2* from *Supporting Information* shows that this hypothesis is sustained for all the pairs of imidazolium/pyrrolidinium and pyridinium/piperidinium ionic liquids available in good agreement with the observation reported in this work.

Having established the toxicity of the ILs towards marine luminescent bacteria it is important to compare their toxicity with common organic solvents. The EC_{50} values for the luminescent marine bacteria *Vibrio fischeri* reported in literature [31] for some common organic solvents (alcohols, acetone, chlorinated compounds...) have values close to 0.5 $g.mL^{-1}$, meaning that ILs have a much lower toxicity than these compounds. EC_{50} values for the standard algae *Pseudokirchneriella subcapitata*, were also determined and reported in Table 4.2.2. These results were obtained after 96 h of incubation with various IL concentrations and were compared by analysis of variance and subjected to a Tukey multiple comparison test, when statistically significant differences in inhibition were reported. The EC_{50} values obtained indicate that the ILs were considered moderately toxic, except the pyridinium-based IL, which may be considered only slightly toxic according to the hazard ranking proposed by Pretti and his collaborators [15] and adapted from literature [74].

Table 4.2.2. EC_{50} values for the freshwater green algae *Pseudokirchneriella subcapitata*.

Ionic Liquid	**EC_{50} ($mg.L^{-1}$)**
$[C_3mim][NTf_2]$	1.44×10^1
$[C_3mpyrr][NTf_2]$	1.82×10^1
$[C_3mpyr][NTf_2]$	3.01
$[C_3mpip][NTf_2]$	1.41×10^1

Figures 4.2.3 (*a* and *b*) and 4.2.4 (*c* and *d*) describe the algae growth for successive concentrations of each IL, showing that this parameter was significantly affected by the presence of the ILs. Analyzing the EC_{50} values (Table 4.2.2) for the freshwater green algae *Pseudokirchneriella subcapitata*, was possible to recognize that the relation between the cation toxicity and the different cations was the same described above for the Microtox® assays.



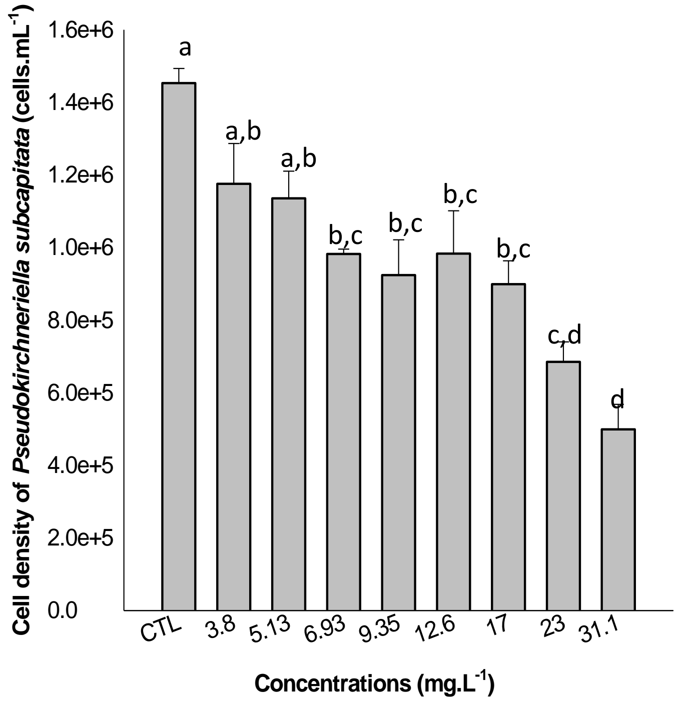
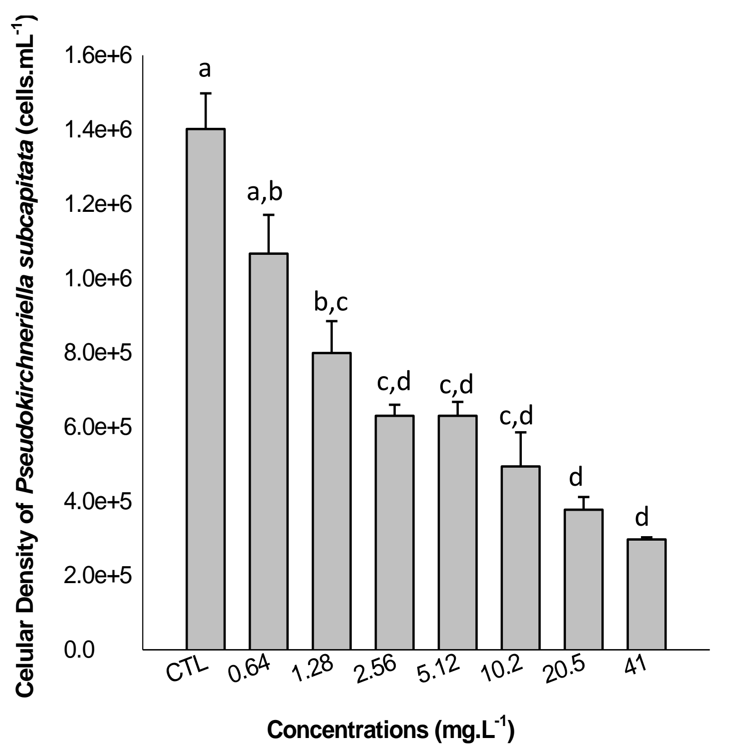
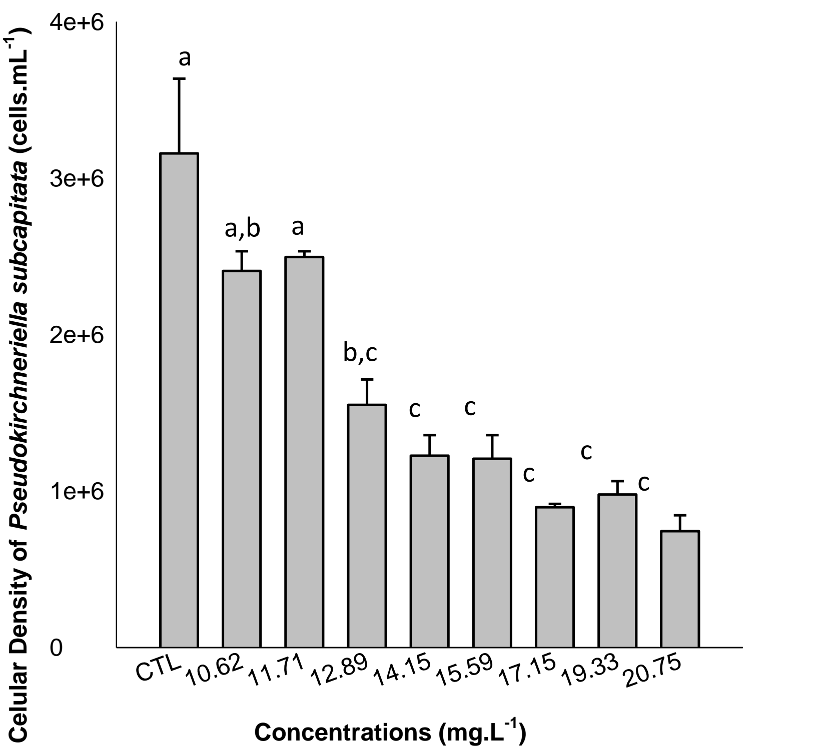


Figure 4.2.3. Growth inhibition of *Pseudokirchneriella subcapitata* when exposed to successive dilutions of [C_3mim][NTf_2] (*a*) and [C_3mpyrr][NTf_2] (*b*), after 96 h of incubation. Those values represent the mean of at least 3 replicates. Error bars represent standard error and the letters correspond to significant differences between the treatments ($p < 0.05$).



c



d

Figure 4.2.4. Growth inhibition of *Pseudokirchneriella subcapitata* when exposed to successive dilutions of [C_3mpyr][NTf_2] (*c*) and [C_3mpip][NTf_2] (*d*), after 96 h of incubation. Those values represent the mean of at least 3 replicates. Error bars represent standard error and the letters correspond to significant differences between the treatments ($p < 0.05$).

The toxicity of the ILs here studied on *Pseudokirchneriella subcapitata* when compared with common solvents such as dichloromethane [65], dimethylformamide [36], methanol [36] and aniline [75] is somewhat higher with the exception of aniline that presents a toxicity comparable to these ILs.

Acute EC_{50} values are reported in Table 4.2.3 for the crustacean *Daphnia magna*. The acute EC_{50} values were determined for [C_3mim][NTf_2], [C_3mpyrr][NTf_2] and [C_3mpyr][NTf_2] while the chronic EC_{50} values were determined for [C_3mim][NTf_2] - 111.56 $mg.L^{-1}$ (106.40-115.85) and [C_3mpyrr][NTf_2] - 123.21 $mg.L^{-1}$ (118.79-127.87) . These data show that the chronic toxicity values were only slightly lower than the acute toxicity results.

Table 4.2.3. EC_{50} values ($mg.L^{-1}$) of [NTf_2] - based ILs for *Daphnia magna*, with the respective 95% confidence intervals (in brackets).

Ionic Liquid	**Acute EC_{50} ($mg.L^{-1}$)** (lower limit; upper limit)
[C_3mim][NTf_2]	1.47×10^2 (1.41×10^2-1.53×10^2)
[C_3mpyrr][NTf_2]	1.59×10^2 (1.48×10^2-1.70×10^2)
[C_3mpyr][NTf_2]	9.89×10^1 (9.67×10^1-1.00×10^2)
[C_3mpip][NTf_2]	1.17×10^2 (8.94×10^1-1.36×10^2)

These can thus provide a good guide for the chronic values. Considering the acute toxicity tests, it was seen that *Daphnia magna* shows to be affected by the presence of all the ILs. However, the biggest effect corresponds to [C_3mpyr][NTf_2], which is considered, according to the hazard ranking [74] aforementioned, as only slightly toxic (EC_{50} < 100 $mg.L^{-1}$) while the other ILs are considered practically harmless. The acute EC_{50} values show the same tendency with the different cations as was shown for the *Pseudokirchneriella subcapitata* results.

A similar tendency was observed on *Daphnia magna* for the butyl analogues of the ILs here studied [15]. When compared with common solvents, the toxicity of these ILs is equivalent to aromatic compounds such as aniline (EC_{50} = 80 - 380 $mg.L^{-1}$) [15] or nitrogen containing compounds such as amines (EC_{50} = 200 $mg.L^{-1}$) [76] and much less toxic than phenol (EC_{50} = 10 -17 $mg.L^{-1}$) [77] or ammonia (EC_{50} = 2.90 - 6.93 $mg.L^{-1}$) [76]. In crustaceans and algae, the mechanism of toxicity induced by ILs is not yet known. Nevertheless, some authors have suggested that this property could be related to enzymatic inhibition and membrane disruption of daphnids.

The toxicity data here reported show that the ionic liquids here studied present toxicities that are identical or inferior to commonly used organic solvents, and that their toxicity can be further lowered by an appropriate choice of the cation and anion, in particular the non aromaticity of the cation can reduce their toxicity. It is thus possible to design the IL not only to achieve improved physico-chemical properties but also to obtain inherently safer ILs.

4.2.5. CONCLUSIONS

This work shows that it is possible to overcome the conflicting goals of minimization of the toxicity and maximization of the hydrophobic nature of ionic liquids as required for their use in various processes. In the present study, toxicological data for different freshwater planktonic species was obtained from tests with *Vibrio fischeri*, *Pseudokirchneriella subcapitata* and *Daphnia magna*. Results clearly show that the toxic character of the ILs studied can be subdivided in two groups - the aromatic and the non aromatic ILs - presenting very different dependencies of toxicity with the water solubility.

While for both groups the toxicity increases with the hydrophobicity, following the trend generally accepted, the differences in behavior between these two groups show that it is possible to design ILs by enhancing their hydrophobic character while simultaneously decreasing their toxicity, in a paradigmatic example of the “designer solvent” character of the ionic liquids. If the potential of this “designer solvent” character is fully understood and applied, ILs may certainly become promising compounds for use in chemical processes, not only with enhanced performances, but also with a lower environmental impact and a “greener” character.

4.2.6. References

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4.3. PAPER 11

Toxicity Assessment of Various Ionic Liquid Families towards *Vibrio fischeri* Marine Bacteria

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4.3.1. ABSTRACT

The increasing interest on the application of ionic liquids (ILs) to a wide range of processes and products has been hampered by a lack of toxicological data, mainly in what concerns novel cations, such as guanidinium, phosphonium, and functionalized and non-functionalized imidazolium-based ILs. The present study reports the toxicity of five guanidinium- six phosphonium- and six imidazolium-based ILs towards the luminescent marine bacteria *Vibrio fischeri*. These new results clearly show that guanidinium-, unlike the imidazolium- and phosphonium-based ILs, do not follow the trend of increasing toxicity with the increase in the alkyl chain length. Moreover, the introduction of oxygenated groups on the alkyl chains, such as ether and ester, leads to a decrease of the toxicity of guanidinium and also imidazolium compounds. In what respects the effect of the different cations, it is possible to conclude that the phosphonium-based ILs seems to be more toxic when compared to the analogue imidazolium-based ILs (with the same anion and alkyl chains).

4.3.2. INTRODUCTION

Ionic liquids (ILs) are attracting increasing attention due to their unique properties, such as negligible vapor pressure, chemical and thermal stability, non-flammability, high ionic conductivity, wide electrochemical potential window and solvation ability. A huge number of different ILs can be synthesized by the combination of different anions and cations, or by the manipulation of their characteristics such as varying their alkyl chain length or by the introduction of oxygenated groups. This makes possible to control physical properties such as, hydrophobicity, viscosity, density, their solubility behavior, and also, to influence their biodegradation ability or toxicological features [1-4].

In the light of their recent widespread commercial availability, the synthesis of ILs has been object of a huge number of developments. If in the past, the synthesis of ILs was focused on obtaining unique physico-chemical properties (1st IL generation); to achieve a specific behavior considering the potential final industrial application (2nd ILs generation); nowadays the main goal is to produce ILs with the desired biological features (3rd ILs generation) [5] for the final application and also to facilitate the registration processes. Although ILs can lessen the risk of air pollution due to their virtually zero vapor pressure, they do present some water solubility [6-10] which was already correlated with the different ILs' parameters, such as the anion [11] and cation

hydrophobicity [12]. This is, consequently, the most likely medium for ILs to be released into the environment. In view of the crescent number of industrial applications, their accidental discharge and environmental contamination are realities to take into consideration [2, 13, 14]. The properties that make them of industrial interest (high chemical and thermal stabilities and non-volatility) suggest potential problems with degradation or persistence in the environment. Moreover, due to the use of non-renewable resources as starting materials, the economic/environmental impact and the cumulative energy demand, the environmental fate and the (eco)toxicological behavior are extremely important parameters to take into account for a complete analysis of ILs sustainability, above all in order to improve the chances of large-scale applications [15, 16]. As a consequence, the number of studies involving the environmental fate analysis (chemical degradation, bioaccumulation, biodegradability and the distribution of ILs in different environmental compartments, such as soil, water and sediments) and the toxicological effects (cytotoxicity, (eco)toxicity, phytotoxicity, and antimicrobial and antibacterial activities) of ILs are increasing [2, 13, 14]. The toxicological impact of ILs has been analyzed in a series of interdisciplinary studies [2, 17-19] which have underlined the importance of a preventive evaluation in the ILs design, because of the influence of different structures on pertinent technological and (eco)toxicological properties. Moreover, as previously demonstrated by our group [20, 21] the need of a full understanding of the biological effects at different biological levels has a fundamental importance, and such knowledge should became a key factor in the modulation and selection of ILs features.

The most used methods to evaluate the toxicological risk of a substance in an aqueous media are those measuring their toxicity by an inhibition assay. Different aquatic species have been used in these inhibition measurements (daphnids, algae and fish) and also the test which uses the *Vibrio fischeri* (formely known as *Photobacterium phosphoreum*) bioluminescence inhibition assay. This is a rapid, cost-effective, and well-established method for toxicity determination [22] focusing on environmental issues, and also a standard (eco)toxicological bioassay in Europe (DIN EN ISO 11348). Several different luminescence inhibition tests of *Vibrio fischeri* have been developed so far, most being designed for analysis of aqueous samples, such as Microtox® Toxicity Test. This test can be used into a wide range of applications, as the analysis of industrial effluents and discharges, waters, soils and sediments, and new products. This test is normally used as a possible approach for determination of the toxicity for both

organic solvents [23-25] and ILs [2, 13, 14, 18-21, 26-33]. There are different approaches in what concerns the use of Microtox® and ILs. The published data on ILs toxicity towards *Vibrio fischeri* were comprehensively interpreted in literature [34]. Several authors have discussed the effect of different alkyl chain lengths, anions and different cations. Usually, the increase in the alkyl chain length leads to a pronounced augment in the toxicity [19, 26-28, 31, 32, 35]. This was explained by the increase in the lipophilicity of the cation that, according to the baseline toxicity, is the responsible for a non-specific disturbance of the structure functioning of biological membranes [11]. Thus, the long alkyl chains are able to be incorporated into the phospholipidic bilayer of the membranes, the same toxicity mechanism described in literature for some surfactants [36]. Considering the cation core, some reports show that the aromatic cations (imidazolium and pyridinium) are, in general, more toxic than the non-aromatic ILs, such as pyrrolidinium, piperidinium, phosphonium and ammonium [19, 20, 26, 29, 30, 33, 35, 37]. The anion seems to contribute to the toxicity in spite of the large disagreement among the literature data [20, 28, 38, 39]; tetrafluoroborate and hexafluorophosphate were considered the less toxic and the bis(trifluoromethylsulfonyl)imide and octylsulfate the most toxic anions [18-20, 26-28, 31, 32, 40]. Various authors have attempted to find a correlation between EC_{50} and different ILs' properties, such as lipophilicity [11, 19, 33, 39], or hydrophobicity [12], and recently, solubility of ILs in water [20].

More recently, some authors have applied Quantitative Structure-Activity Relationships (QSAR) models to data sets of ionic liquids to detect general assumptions on the toxicity and behavior of these compounds [26, 29, 35, 41-43]. The aim is the development of predictive tools to help in the sustainable design of ILs safer for humans and for the environment [44]. The picture that emerges is that a number of characteristics play an active role on toxicity, being most of them still poorly understood [20].

In this context, it is easy to understand the necessity of to contribute to the enlargement of the toxicological databases considering the different families and structural features of ILs. Imidazolium-based ILs appears as the family most studied in the toxicological field, being the phosphonium-based ILs one of the less studied, despite their high industrial interest (for example in biotransformation processes such as xenobiotics-degradation [45]). Moreover, this family shows some interesting properties such as the possibility to decrease its antimicrobial activity for the longest alkyl chains

(phosphonium based in alkyl chains of 8 and 14 carbons and conjugated with the chloride anion) [45]. Moreover, it was also focused that the exchange of the halide by other anions has resulted in a loss of antimicrobial activity [45] thus rendering a higher interest in this family. The apparently high toxicity of other phosphonium halides against *Vibrio fischeri* [26], *Daphnia magna* [46], and *Pseudokirchneriella subcapitata* [46, 47] was also demonstrated. Still, the information for this family about its hazard to the environment is still scarce and not conclusive.

Recent toxicological studies have been focused on a new class of ILs with increased biodegradability through the incorporation of oxygenated alkyl chains [48-50]. The oxygenation is usually carried at two different parts of the IL structure, the cation core, represented for example by the morpholinium family, [14] or at the cation alkyl chains, which can be achieved by the introduction of hydroxyl (-OH) [27, 28, 31], ester (O-C=O) [28] or ether (-O-) [2, 19, 51-56] groups. The oxygenated imidazolium-based ILs are a promising class of alternative solvents with some interesting properties, such as high solubility for polar substrates [57, 58], suitable features as reaction media for some biocatalytic processes [59] and for catalytic asymmetric reaction [60], high carbon dioxide selectivity useful for gas separation processes [61] and nanoparticles stabilizing properties [62], which justifies the increased interest in the toxicity study of those structures. The reports previously published about oxygenated imidazolium-based ILs were focused on their biodegradability [28], antimicrobial activity [63] and toxicity towards *promyelocytic leukemia* cells (IPC-81) [19], human colon carcinoma cells (*CaCo-2* and *HT-29*) [51, 52], *Daphnia magna* [54, 55], marine diatoms algae [56] and the luminescent bacteria *Vibrio fischeri* [54, 55]. More recently, a review on the behavior of oxygenated imidazolium-based ILs was reported [14]. The results published show that the introduction of one oxygen atom into the lateral chain of imidazolium-based ILs seems to decrease the toxicity of the IL with respect to alkyl counter parts towards the crustacean *Daphnia magna* and the bacterium *Vibrio fischeri* [55]. Moreover, it also increases their biodegradability using organisms of the soils, as described in literature [28, 53]. Samori's group [55] have described a comparison between the response of *Vibrio fischeri* and *Daphnia magna* to them. Apparently, the main contribution for the reduction on the toxicity is achieved by the introduction of a single oxygen atom in the lateral chain [54]. In this work, the authors have described that the addition of more than one ethoxy moiety in the alkyl chain as a matter of fact, increases their toxicity towards the bacterium *Vibrio fischeri*, in opposition to *Daphnia*

magna. They justify that the bacterium was more sensitive to an elongation of the chain than the crustacean, in spite of the increasing number of oxygen units, which should increase the overall polarity of the cation [54].

The guanidinum cations are the basis of a bioactive family of ILs, yet poorly studied [14, 19, 52]. The toxicological reports for this family are scarce [14] and, at the same time the knowledge of their physico-chemical properties is also very limited [64-66]. The toxicity observed for guanidinium-based ILs seems to be very anion dependent, being the hexafluorophosphate [PF_6] anion the responsible for the most negative effect, when compared with dicyanamide [$N(CN)_2$] and bis(trifluoromethylsulfonyl)imide [NTf_2] [51, 52]. Accordingly, the introduction of an ether group in the guanidinium cation core is responsible for a remarkable decrease of the effect upon *CaCo-2* cells viability [51, 52].

This work addresses the measurement of new toxicological data for the luminescent marine bacterium *Vibrio fischeri* for three distinct IL families: guanidinium-, phosphonium- and imidazolium-based ILs, being the latest family used to determine the influence of cation functionalization on the ionic liquid toxicity.

4.3.3. MATERIALS AND METHODS

4.3.3.1. Test chemicals

The molecular structure of the ILs used is provided in Figure 1. The ILs used were: di-butyl-tetramethyl-guanidinium iodide [$TMGC_4$]I, di-heptyl-tetramethyl-guanidinium iodide [$TMGC_7$]I, di-dodecyl-tetramethyl-guanidinium iodide [$TMGC_{12}$]I, tetrahexyl-dimethyl-guanidinium chloride [$(di\text{-}h)_2DMG$]Cl, N''N''-dimethyl-N,N,N',N'-tetra-(2-methoxyethyl)-guanidinium chloride [$(C_3O)_4DMG$]Cl, (3-methyl-1-(ethoxycarbonyloctyl)imidazolium bromide [$C_{10}C(O)OEtmim$]Br, 1-(2-(2-methoxyethoxy)ethyl)-3-methylimidazolium chloride [C_5O_2mim]Cl, trihexyltetradecylphosphonium bromide [$P_{6,6,6,14}$]Br (purity > 97%), trihexyltetradecylphosphonium chloride [$P_{6,6,6,14}$]Cl (purity > 98%), trihexyltetradecylphosphonium methanosulfonate [$P_{6,6,6,14}$][CH_3SO_3] (purity > 98%), tetrabutylphosphonium bromide [$P_{4,4,4,4}$]Br (purity > 98%), and tributyl(methyl)phosphonium methylsulfate [$P_{4,4,4,1}$][CH_3SO_4] (purity > 99%), triisobutyl(methyl)phosphonium tosylate [$P_{i(4,4,4)1}$][TOS] (purity > 99%),1-butyl-3-

methylimidazolium bromide [C_4mim]Br (purity > 99%), 1-butyl-3-methylimidazolium methanosulfonate [C_4mim][CH_3SO_3] (purity > 99%), and 1-butyl-3-methylimidazolium tosylate [C_4mim][TOS] (purity > 98%).

The guanidinium- and oxygenated imidazolium-based ILs were synthesized according to reported procedures: [(C_3O)$_4$DMG]Cl [66], [(di-h)$_2$DMG]Cl [67], [C_5O_2mim]Cl [68], [TMGC_7]I [66], [TMGC_{12}]I [66], [TMGC_4]I [66] and [$C_{10}O_2$mim]Br [69]. The phosphonium-based ILs were supplied by Cytec Industries and the hydrophilic imidazolium based ILs were purchased at IoLiTec (Ionic Liquid Technologies, Germany) and their mass fraction purities were further confirmed by ^{1}H NMR and ^{13}C NMR.

Figure 4.3.1. **Chemical structures of the ILs studied.**

4.3.3.2. Microtox® tests

The Microtox® Acute Toxicity Test [70] was used to evaluate the inhibition of the luminescence in the marine bacteria *Vibrio fischeri*. This test was performed using a range of diluted aqueous solutions (from 0 to 100 %) of each IL, where 100 % of IL corresponds to a known concentration of a stock solution. After 5, 15, and 30 minutes of exposure to the IL solution (depending of the IL), the light output of the luminescent bacteria was measured and compared with the light output of a blank control sample. The toxicity was evaluated and a 50% reduction in luminescence was computed using Microtox® Omni™ Software version 4.3.0.1. [71]

4.3.4. RESULTS AND DISCUSSION

The present work shows the impact of various ILs features, such as the elongation of the alkyl chain length (guanidinium and phosphonium families), the influence of different cation cores and anions (non-functionalized imidazolium and phosphonium families), the increase in the number of long alkyl chains (guanidinium), and finally the effect of the side chains' functionalization with oxygenated groups (ester and ether structures) in distinct cation cores (guanidinium and imidazolium) in their toxicity towards trhe marine luminescent bacteria *Vibrio fischeri*.

Table 4.3.1 shows the EC_{50} values for the guanidinium- and oxygenated imidazolium-based ILs. It can be observed that the increase in the toxicity of guanidinium family follows the tendency $[TMGC_4]I < [TMGC_{12}]I < [TMGC_7]I$, which is not in agreement with the established idea about the influence of the alkyl chain length on the toxicity. From $[TMGC_4]I$ to $[TMGC_7]I$ there is an increase in toxicity as expected. This effect is currently known as the "side chain effect" [44], and describes the increase in the toxicity derived from the elongation of the IL' alkyl side chain. There are a few works [27, 28, 31, 32] reporting this effect also for the bioluminescent bacteria *Vibrio fischeri*. The EC_{50} values reported in those articles shows that there is a correlation between the toxicity and the number of carbons on the alkyl chains, independently of the exposure period. However, there is a change in the toxicity tendency when $[TMGC_7]I$ and $[TMGC_{12}]I$ were compared, since in this case the elongation of the alkyl chain leads to the decrease in the IL' toxicity. In fact, it is also described in literature that the dependence between the increase in both the alkyl side chain length and toxicity no longer holds true for very long alkyl chains. At a certain chain length, the toxicity

cannot be increased any further, being this phenomenon described as the “cut-off” effect [33, 34]. As discussed in literature [44], different explanations for this phenomenon are proposed based either on insufficient solubility (nominal concentration deviating from real test concentration) or kinetic aspects (uptake is slowed because of steric effects for compounds with a large molecular size). Despite the inexistence of literature toxicity data for *Vibrio fischeri*, these guanidinium-based ILs were previously tested in the human colon cancerous cells *CaCo-2* [52] and the same trend was observed for these cells. The [$TMGC_4$]I is considered as non toxic, but the EC_{50} values reported for [$TMGC_7$]I and [$TMGC_{12}$]I were, respectively < 750 and 955 μM [52]. Also for this biological system it was observed the same “cut-off” effect, which supports our results.
The EC_{50} values for [$TMGC_7$]I and [(di-h)$_2$DMG]Cl, presented in the same Table, can be used to study the effect on the toxicity of the number of long alkyl chains in the guanidinium core. It was not possible to compare directly the influence of TMG- and DMG-based ILs because those have distinct anions, which may be responsible for significant differences in the ILs’ toxicity [18, 20]. However, and despite the difference of one carbon in the alkyl chains of [TMGC7]I and [(di-h)$_2$DMG]Cl, the toxicity results described for both the ILs in Table 4.3.1., seem to indicate that the increase in the number of long alkyl chains is responsible for a slightly increase in the toxicity (the EC_{50} = 3.72 (0.00; 24.80) for the [$TMGC_7$]I and EC_{50} = 3.81 for [(di-h)$_2$DMG]Cl, at 15 minutes of exposure). This could be explained by the increase in the hydrophobic/lipophilic nature of the cation core, when the number of substituted alkyl chains was enhanced. According to the hazard ranking described by Passino’s group [72], the level of toxicity for both the ILs is not different, since both are considered as “slightly toxic”. This again seems to be in good agreement with the results reported by Frade et al. for the cytotoxicity data towards the human colon carcinoma cells (*CaCo-2* cells and *HT-29*) [45, 46].
Considering the oxygenation of the side chain of the cation, and aiming at understanding the effect of these oxygen groups in different cation cores, two different imidazolium-based ILs and one guanidinium were also studied. Table 4.3.1 shows the toxicity results for [$(C_3O)_{4DMG}$]Cl, [C_5O_2mim]Cl and [C_{10}C(O)OEtmim]Br for 5 and 15 min of exposure time. The guanidinium can be classified as “practically harmless”, [C_5O_2mim]Cl can be classified as “moderately toxic” while [C_{10}C(O)OEtmim]Br is “slightly toxic” to *Vibrio fischeri*, according to literature [72]. For these compounds no statistical differences are observed between the two exposure times and no other works

reporting their toxicological profiles towards different organisms or (biological) test systems were described. However, it was expected that the inclusion of those oxygenated groups in the side chains leads to the decrease in their toxicity as was explained by Frade et al. [13]. These authors reported that the introduction of the group (O-C=O) can be considered advantageous in the reduction of the ILs' toxicity, making them biocompatible. This behavior is not exclusive for the guanidinium- or imidazolium-based ILs, as described in different works [33, 41, 44, 54, 73-76]. In this context, a compilation of toxicity data reported in *Supporting Information Table S8.4.3* was carried aiming at showing that the effect of the oxygenation of the side chain is normally independently of the cation core and the biologic test system used.

Table 4.3.1. Microtox® EC_{50} values ($mg.L^{-1}$) for all the ILs tested (guanidinium, imidazolium, and phosphonium) after 5, 15 and 30 minutes of exposure to the luminescent marine bacteria *Vibrio fischeri*, with respective 95% confidence limits (in brackets) obtained in the fit of the data.

Ionic Liquid	**EC_{50} ($mg.L^{-1}$) 5 min** (lower limit; upper limit)	**EC_{50} ($mg.L^{-1}$) 15 min** (lower limit; upper limit)	**EC_{50} ($mg.L^{-1}$) 30 min** (lower limit; upper limit)
$[TMGC_4]I$	61.20 (51.00; 71.40)	30.60 (20.40;51.00)	----
$[TMGC_7]I$	4.96 (1.24; 17.36)	3.72 (0.00; 24.80)	----
$[TMGC_{12}]I$	26.00 (15.60; 52.00)	15.60 (5.20; 57.20)	----
$[(C_3O)_4DMG]Cl$	139.17 (106.43; 180.11)	98.24 (65.49; 147.36)	----
$[(di\text{-}h)_2DMG]Cl$	8.47	3.81	----
$[C_5O_2mim]Cl$	34.76 (11.59; 75.31)	40.55 (5.79; 144.83)	----
$[C_{10}C(O)OEtmim]Br$	5.51 (4.03; 7.54)	4.49 (1.67; 12.08)	----
$[P_{6,6,6,14}]Br$	i.d.	6.38	----
$[P_{6,6,6,14}][CH_3SO_3]$	i.d.	7.43 (3.68; 15.23)	2.67 (0.87; 8.26)
$[P_{6,6,6,14}]Cl$	i.d.	7.10 (1.13; 44.40)	2.95 (0.92; 11.49)
$[P_{i(4,4,4)1}][TOS]$	169.60 (84.80; 275.60)	169.60 (21.20; 1462.80)	----
$[P_{4,4,4,1}][CH_3SO_4]$	----	237.60 (99.18; 569.60)	232.20 (75.04; 720.10)
$[P_{4,4,4,4}]Br$	216.00 (21.60; 1382.40)	172.80 (0.00; 3218.40)	----
$[C_4mim]Br$	1651.68 (1228.03; 2221.48)	735.93 (494.69; 1094.83)	----
$[C_4mim][CH_3SO_3]$	1123.54 (645.87; 1955.26)	901.99 (435.22; 1717.93)	----
$[C_4mim][TOS]$	957.60 (604.20; 1517.50)	653.22 (511.00; 835.10)	----

i.d. – impossible to determine.

On the other hand, the same Table 4.3.1 shows the results for various phosphonium-based ILs conjugated with different anions and alkyl chains, for 5, and 15 minutes of exposure. The toxicity results obtained for the phosphonium family, suggest that the long alkyl chains promote higher toxic effects towards the bacterium, which is shown

for example in the toxicity differences observed for $[P_{6,6,6,14}]Br$ EC_{50} = 6.38 $mg.L^{-1}$ and $[P_{4,4,4,4}]Br$ EC_{50} = 172.80 $mg.L^{-1}$, respectively. This behavior falls into the conventional "side chain effect" previously discussed [44]. These compounds had previously been studied by Couling et al. [26] and although a good agreement was observed for $[P_{6,6,6,14}]Br$ its value for $[P_{4,4,4,4}]Br$ of 0.51 mg L^{-1} seems to be far underestimated and does not make sense in what concerns the effect of the alkyl chain elongation.

At the same table are reported EC_{50} values for the $[P_{6,6,6,14}]$ cation moiety, conjugated with different anions, such as bromide, Br, chloride, Cl, and methanosulfonate, $[CH_3SO_3]$. In this case, the anions seem to have an insignificant influence in the EC_{50} results, independently of the exposure time. The toxicity in this case, was also determined for 30 minutes of exposure time Table 4.3.1. It is clear from the results of this table that the increase in the time of exposure of the *Vibrio fischeri* to ILs is responsible for the increase on toxicity. This can be shown by the comparison between the toxicity values described for $[P_{6,6,6,14}]Cl$ and $[P_{6,6,6,14}][CH_3SO_3]$. The marginal influence of the anions was also shown for other systems in literature [31, 33]. These results can be explained in one hand, by the poor affinity of the anions to the *Vibrio fischeri* structure and, in the other hand, by the overlapping effect of the long alkyl chains substituted in the cation core.

The remaining results reported in this table show the effect of three different imidazolium-based ILs and phosphonium with short alkyl chains, composed by three different anions. In this context, and comparing the effect of the phosphonium and imidazolium families, it was demonstrated that the toxic effect of the acyclic family is significantly higher when compared to the toxic effect of the imidazolium cation core. This is shown when comparing the results here reported for the pairs $[P_{4,4,4,4}]Br/[C_4mim]Br$, $[P_{6,6,6,14}][CH_3SO_3]/[C_4mim][CH_3SO_3]$, and $[P_{i(4,4,4)1}][TOS]/[C_4mim][TOS]$. These results support those previously reported by other authors [26, 33]. The $[P_{4,4,4,1}][CH_3SO_4]$ was also studied, however, due to the differences on the alkyl chain and anion moiety, a comparison was not possible since this work is the first reference describing its toxic effect towards the luminescent bacterium *Vibrio fischeri*. Also for the imidazolium cation, the effect of three different anions was assessed. It was observed that the anion effect is more pronounced for this cation than for the phosphonium, in particular for the $[CH_3SO_3]$ anion, probably due to the short alkyl chains of the cation core.

4.3.5. CONCLUSIONS

The present study was focused on the toxicity determination of various guanidinium-, phosphonium- and imidazolium-based ILs towards the bioluminescent marine bacteria *Vibrio fischeri*. The guanidium-based ILs seem to follow the established trend that the increase in the alkyl chain length ($[TMGC_4]I$ and $[TMGC_7]I$) promotes the increase in the toxicity of the IL, normally designated by "side chain effect", but seems to suffer also the "cut-off" effect ($[TMGC_{12}]I$).

The introduction of ether or ester groups in the IL side chain leads to the decrease of the toxicity, independent of the cation core (phosphonium or guanidinium).

The generality of the ILs studied (imidazolium, guanidinium and phosphonium) are shown to be "moderately" or "slightly toxic" towards the marine bacteria. The toxicity for the phosphonium-based ILs was shown to increase with the alkyl chain from $[P_{4,4,4,4}]$ to $[P_{6,6,6,14}]$. For the $[P_{6,6,6,14}]$ cation the anion effect on the toxicity was observed to be residual. Finally, according to the results here reported was possible to conclude that the phosphonium-based ILs are more toxic than the analog imidazolium-based ILs (same anion and alkyl chain length).

4.3.6. REFERENCES

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4.4. PAPER 12

Ecotoxicological Risk Profile of Ionic Liquids: Octanol-water distribution coefficients and toxicological data

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4.4.1. ABSTRACT

The knowledge of the ionic liquids (ILs) toxicity and their influence on the aquatic ecosystems must be assessed before an accurate judgment of their environmental benefits and prior to their industrial applications. In the present work, the *overall* octanol-water distribution coefficients (D_{ow}) of imidazolium-based ILs were measured using the slow-stirring method. Biological tests were performed to establish the toxicity of some of these imidazolium-based ILs and relate it with the D_{ow}.

The D_{ow} values of the investigated ILs are low and concentration dependent, indicating that these ILs, at low concentrations, will not accumulate or bioconcentrate in the environment. Moreover, it is shown that the most hydrophobic anions present higher D_{ow} and the higher toxicity, which means lower EC_{50} values (the concentration of IL needed to decrease the luminescence of the marine bacteria *Vibrio fischeri* in 50% compared to the control organism luminescence).

A new and more accurate interpretation of the partition of ionic liquids between water and 1-octanol is presented. These results allowed the measurement of new D_{ow} data and the study of their relation with the water solubility and the EC_{50} values.

4.4.2. INTRODUCTION

Volatile organic compounds released into the atmosphere due to industrial chemical processes are contributing to a crescent pollution, climatic changes and human health-related problems. The growing environmental and health concerns lead to the creation of more stringent regulations on their emissions. This is spurring a growing interest in more environmentally friendly technologies among which there is a major interest in identifying alternative solvents [1]. In the last few years, ionic liquids (ILs) have been looked upon as one of the most promising alternatives to traditional solvents [1-10]. They are expected to help reduce the use of hazardous and polluting organic solvents in separation, synthesis and in the design of new products, due to their unique characteristics [11-13]. In spite of their undeniable interest, it should be noted that not all ILs are “green” or environment friendly. The general questions of “greenness” and sustainability of the use of ILs as alternative reaction media were already discussed by Jastorff et al. [14] As for the organic solvents, a risk analysis of these substances [15] is imperative. The concept of a risk analysis requires that a certain minimal set of data

about both exposure and effects of a chemical under scrutiny is available. Since the large majority of ILs is still in a very early phase of development, it is not yet clear which of them will ever be produced at an industrial scale. Thus, a more flexible method of risk analysis that can be applied to compounds with sparse and heterogeneous data, such as the ionic liquids, is needed. Such a method has been devised for a comparative risk evaluation of chemical substances based on five risk indicators (release, spatiotemporal range, bioaccumulation, biological activity, and uncertainty) forming ecotoxicological risk profiles for each substance [16]. The crescent number of ionic liquids synthesized and the lack of information about their ecotoxicological risk profiles, requires that more studies are carried out. Finally, it must be noted that due to the structural variability of ILs, it is extremely important that researchers and industries consider not only their technological advantages but also, both must take into account their (eco)toxicological risks [17].

Nowadays, there are publications showing some results concerning the assessment of toxicity for different organisms and species - decomposers [18, 19], producers [19-22] and consumers [19, 23, 24]. One of the most used toxicological tests is the Microtox® Acute Toxicity Test, which uses the gram negative marine bacterium *Vibrio fischeri*. The Microtox® Acute Toxicity Test is quick, simple, cost-effective and sensitive being a widely accepted method by the industrial and academic communities for toxicity determination, providing a fast, yet accurate, estimate of the toxicity of a compound [25]. The endpoint of this methodology, EC_{50}, is the effective concentration value that corresponds to the concentration of chemical that produces 50% inhibition of light emission from a specific strain of bioluminescent bacteria. Some authors studied the toxicity of imidazolium-based ILs with chloride, hexafluorophosphate, tetrafluoroborate, bis(trifluoromethylsulfunyl)imide, octylsulfate and bis(1,2-benzenediolato)borate anions [18, 19, 26].

Before the unavoidable release of ILs into the environment, due to their use in processes and products, adequate knowledge is required about the mechanisms of “attack” of the ILs to various organisms, their biodegradability, the distribution between different environmental compartments and the bioaccumulation [27, 28].

This work is part of a project aiming at studying these properties of the ionic liquids and attempting to relate them with the ILs chemical structure. Being ionic species fully dissociated in the aqueous phase when highly diluted, it is not possible to define an octanol-water partition coefficient (K_{ow}) for the ionic liquids, instead, their octanol-

water distribution coefficient, D_{ow}, is here defined and measured for a number of hydrophobic ionic liquids. The effect of the anion, cation alkyl chain length and the concentration of different ILs in the D_{ow} values is discussed. Correlations between D_{ow} and the toxicological parameters (EC_{50}) were investigated, aiming at acquiring a better knowledge about the environmental risk profile of those imidazolium-based ILs.

Octanol-water partition (K_{ow}) and distribution (D_{ow}) coefficients

One of the most used parameters for assessing the environmental impact of a chemical specie is the octanol-water partition coefficient (K_{ow}). The 1-octanol is an amphiphilic solvent whose dielectric properties are similar to those of a generalized lipid phase [29]. This parameter is useful in the ecosystem risk analysis because partition coefficients in octanol-water systems display similarities to the partition of biological compounds between water and living organisms. Correlations between environmental parameters for natural systems and K_{ow} have been successful because the 1-octanol ability to mimic a lipid phase behaviour.

Many direct and indirect methods are available to experimentally measure K_{ow}, being each used by different authors for different purposes. K_{ow} values for several 1-alkyl-3-methylimidazolium ILs have been reported by several groups [30-34]. The data is quite scattered and for given compounds sometimes differ by up to 2 orders of magnitude between authors. Differences in values have been related to different concentration ranges investigated, [30] and also the experimental method used. Domanska et al. [32] have estimated the K_{ow} values from the ratio of solubilities of ILs in pure 1-octanol and water using the synthetic and visual method, but the very small range of these data for ILs with cation chain lengths varying from 4 to 12, suggests that their method is not adequate. The values reported by Ropel et al. [30] seem to be carefully measured using the slow stirring method and are consistent with expectations concerning the influence of alkyl chain length and the anion hydrophobic nature. They are however strongly concentration dependent unlike a true K_{ow} value. Using an alternative approach Stepnowski and Storoniak [35] have suggested using the group contribution method of Hansch and Leo [36] for estimating log K_{ow} values for ionic liquids. However, since the imidazolium cation is not covered by the original method, they resorted to using the fragment constant for the nitrogen in quaternary ammonium compounds for both nitrogens in the imidazolium ring. While the resulting log P parameters should not be

used as absolute estimates of experimental log K_{ow} values, they do provide a measure of lipophilicity [37].

The major problem concerning the measurement of K_{ow} values is that most authors dealing with this, subject treats the ionic liquids as non-ionizable compounds, failing to take into account that, at low concentrations, they may undergo an important dissociation in aqueous solution and even some dissociation in the organic phase. This precludes the use of K_{ow} values defined according to Eq. 4.3 since the analytical techniques used will not account just for the $[IL]_w$ but to the total amount of the cation present in the water phase, both as ion pair and as dissociated ion. In this context, the available results were useless with regard to the effect of ILs in the bioaccumulation area. Contradicting these wrong ideas, was the work of Lee and Lee [34] and, more recently, that of Gardas et al. [38]. In these studies the importance of taking into account the ionic nature of the IL to establish the 1-octanol water distribution coefficients was recognized. However, Lee and Lee [34] failed to take the electroneutrality condition into account on their analysis.

The octanol-water partition coefficient refers specifically to the ratio of concentrations in both phases of the neutral, non-associated and non-complexed solute,

$$K_{ow} = \frac{C_{oct}}{C_w} \quad \text{Eq. 4.3}$$

where C_{oct} and C_w are, respectively the equilibrium concentrations of the compound in the 1-octanol and water rich phases.

In situations where competing equilibria (ionization, association, and complexation) participate, the partition coefficient must be replaced by the distribution coefficient also known as apparent partition coefficient (K_{app}). The distribution coefficient (D_{ow}) is defined as the ratio of the sum of the concentrations of all solute's forms (ionized plus unionized) in 1-octanol to the sum of the concentration of all solute's forms in the aqueous phase, at a given pH [29, 39].

$$D_{ow} = \frac{\sum (C_{oct})_i}{\sum (C_w)_i} \quad \text{Eq. 4.4}$$

This coefficient is pH and concentration dependent, reason why the conditions at which it is measured must be specified. When the solute is non ionic the identity

$$\log D_{ow} = \log K_{ow} \quad \text{Eq. 4.5}$$

is valid at any pH value, otherwise the two values are different and log D_{ow} is inferior to log K_{ow}.

D_{ow} should not be confused with the true K_{ow}, since it does not represent a true equilibrium constant of a single species between two phases. Turner and Williamson [39] presented an interesting work on the relationship between D_{ow} and K_{ow} while Scherrer and Howard [40] discussed the dependency of these coefficients on pK_a and pH for acids and bases.

When the dissociation of the ILs is taken into account, as it must be at low IL concentrations, a series of chemical equilibria must be considered in the determination of these partition or distribution coefficients. The chemical equilibria considered in the current study are presented below, where I^+L^- is the ion pair of the ionic liquid, I^+ corresponds to the imidazolium cation, L^- is the anion, subscripts *w* and *oct* denote the water and the 1-octanol rich phases, respectively. The first equilibrium to be considered is the dissociation of the ionic liquid in the aqueous phase, with an equilibrium constant k_1:

$$\left[I^+L^-\right]_w \xleftrightarrow{k_1} \left[I^+\right]_w + \left[L^-\right]_w \qquad \text{Eq. 4.6}$$

The second equilibrium refers to the distribution of the ion pair IL between both phases, being the respective equilibrium constant, the true K_{ow}.

$$\left[I^+L^-\right]_w \xleftrightarrow{K_{ow}} \left[I^+L^-\right]_{oct} \qquad \text{Eq. 4.7}$$

Besides the distribution of the ion pair between both phases, also one must also take into account the distribution of the ions between the two phases as described by Eq. 4.8 below. To maintain the electroneutrality of the phases it is assumed that both ions have the same distribution constant, $\sqrt{k_2}$

$$\left[I^+\right]_w + \left[L^-\right]_w \xleftrightarrow{k_2} \left[I^+\right]_{oct} + \left[L^-\right]_{oct} \qquad \text{Eq. 4.8}$$

Finally, the formation of the ion pair in the 1-octanol phase is considered with an equilibrium constant k_3.

$$\left[I^+\right]_{oct} + \left[L^-\right]_{oct} \xleftrightarrow{k_3} \left[I^+L^-\right]_{oct} \qquad \text{Eq. 4.9}$$

From these equilibria the following expression for D_{ow} can be derived

$$D_{ow} = \frac{K_{ow}\left[I^+\right]_w + k_1\sqrt{k_2}}{k_1 + \left[I^+\right]_w} \qquad \text{Eq. 4.10}$$

If the IL in the aqueous phase is very diluted, it is possible to assume that the ionic liquid is completely dissociated. If the ionic liquid concentration is lower than k_1 then the D_{ow} is described as

$$D_{ow} = (K_2 K_3)[I^+]_w + \sqrt{k_2} = E[I^+]_w + \sqrt{k_2} \quad \text{Eq. 4.11}$$

where, for the present system D_{ow} and E, were described by Eqs. 4.12 and 4.13 as:

$$D_{ow} = \frac{[I^+L^-]_{oct} + [I^+]_{oct}}{[I^+L^-]_w + [I^+]_w} \quad \text{Eq. 4.12}$$

$$E = \frac{[I^+L^-]_{oct}}{[I^+]_w [L^-]_w} \quad \text{Eq. 4.13}$$

If the distribution coefficients, D_{ow}, are measured in a broad concentration range it can be observed that for low concentrations they follow a linear relationship with the ionic liquid concentration as predicted by Eq. 4.11 and shown in Figure 5.4.2, and also observed in the work by Lee and Lee. [34] For high concentrations this relationship deviates from the linear dependency. [34] If data at various concentrations of ionic liquid in the linear region is measured it is possible to use these values to access the pair extraction constant E, defined by Eq. 4.13, and also the equilibrium constants, k_2 and k_3 of Eqs. 4.8 and 4.9.

In the present work, the octanol-water distribution coefficient (D_{ow}) of several imidazolium-based ionic liquids at room temperature were measured by the slow-stirring method [41], for very dilute solutions. From the dependency of this data on the IL concentration in the aqueous phase, the equilibrium constants k_2 and k_3 and the ion pair extraction constant (E) were estimated. In addition, some of these ionic liquids were tested using the Microtox® procedure and the respective EC_{50} data (15 minutes) were estimated.

4.4.3. EXPERIMENTAL SECTION

4.4.3.1. Material

The 1-octanol was acquired from Fluka with a purity ≥ 99.5 % (established by GC analysis). The water used was double distilled, passed by a reverse osmosis system and further treated with a Milli-Q plus 185 water purification apparatus. It has a resistivity

of 18.2 MΩ·cm^{-1}, a TOC smaller than 5μg·L^{-1} and it is free of particles greater than 0.22 μm. The ILs used in this work, based on the imidazolium family, 1-hexyl-3-methylimidazolium hexafluorophosphate - [C_6mim][PF_6], 3-methyl-1-octylimidazolium hexafluorophosphate - [C_8mim][PF_6], 1-ethyl-3-methylimidazolium bis(trifluoromethylsulfonyl)imide - [C_2mim][NTf_2], 1-butyl-3-methylimidazolium bis(trifluoromethylsulfonyl)imide - [C_4mim][NTf_2] were acquired at Iolitec with purities ≥ 99% - Figure 4.4.1. The purities of all the ionic liquids used were checked by ^{1}H, ^{13}C and ^{19}F NMR spectroscopy.

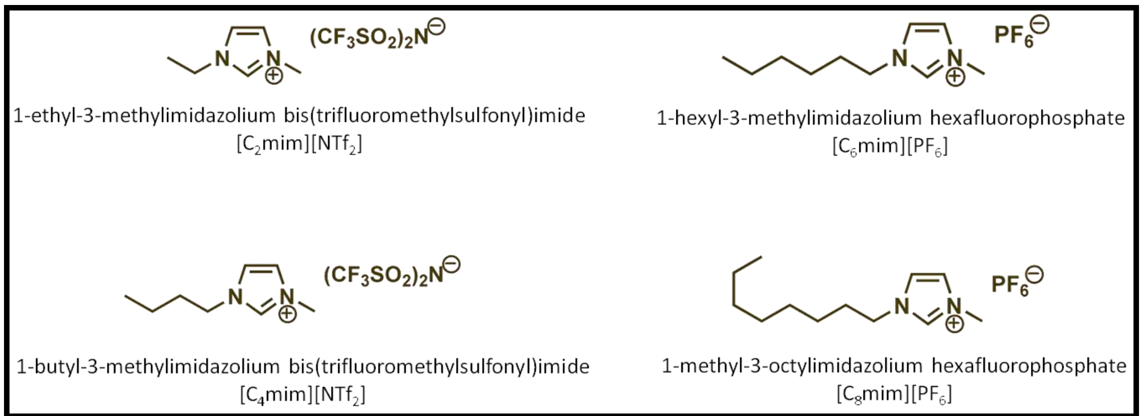

Figure 4.4.1. Chemical structures of all ILs used in this study.

4.4.3.2. Slow-Stirring measurements of D_{ow}

The 1-octanol and ultrapure water were mutually saturated prior to the experiment by equilibrating them for 48 hours. Some 1-octanol saturated with ultrapure water is then used to dissolve the IL and form the stock solution (with defined concentration) used to prepare all the IL concentrations studied. All the solutions prepared to determine the D_{ow} have concentrations lower than 10^{-3} M, to guarantee that a complete dissociation of the IL in the aqueous phase is achieved. The slow-stirring equilibrium cell consists of a 120 mL glass vial containing a 1 cm Teflon coated magnetic stirrer, built for these determinations. Approximately 45 mL of double distilled, deionized water pre-saturated with 1-octanol was introduced in the vial and an equal volume of 1-octanol saturated with water containing a known amount of IL from the stock solution, was carefully

added to the vial to avoid emulsification. The water rich phase in the vials was slowly stirred to prevent the emulsification of the system. These vials were kept at room temperature, 298 ± 1 K.

To obtain the dependency of the measured D_{ow} with the concentration of the IL cation on the aqueous phase, multiple samples with different initial IL concentrations were used. Three vials with the same initial concentration of IL were used for each measurement to control the precision and repeatability of the experiment. The IL concentrations in each phase were measured at 211 nm using UV-VIS spectroscopy (SHIMADZU UV-1700 Pharma-Spec Spectrometer) with a detection limit of 0.01 in absorbance. The same stock of solvents (both saturated 1-octanol and water) used in the preparation of the sample were used in the reference cell of the UV-VIS. The extinction coefficients (ε) of the ILs in 1-octanol and water rich phase were determined, since they are required to determine the concentration of IL in each phase by interpolation with calibration curves. Samples were collected from the 1-octanol and water-rich phases from all vials during at least five sampling events occurring over a 20 to 40 day period to follow the evolution of the partitioning and ensure full equilibration. Sampling ceased when the concentrations in both phases were stable and constant. It is considered that the equilibrium is attained when the distribution coefficient values do not vary more than 0.3, between measurements. The D_{ow} values at room temperature were determined for several imidazolium-based ILs, being the validation of this method done using the $[C_4mim][NTf_2]$.

Though it is known that fluorinated anions such as $[PF_6]$ are not water stable we have previously established that under the conditions used on this study the hydrolysis extent is very small [42].

4.4.3.3. Microtox® tests

The ILs toxicities were determined using the Microtox® Acute Toxicity Test [43]. The toxicity was evaluated and a 50 % reduction in luminescence was computed using the Microtox® Omni™ Software, version 4.3.0.1. [43] The EC_{50} results were determined accordingly to the Basic Test **- Section 4.1.3.2**.

4.4.4. RESULTS AND DISCUSSION

The values of extinction coefficients (ε) used for the determination of the IL concentrations in each phase are reported in Table 4.4.1. All the ILs studied have a pronounced hydrophobic character and their extinction coefficients have approximately the same value of about 4500 $L.mol^{-1}.cm^{-1}$. A more careful examination of the data shows that the most hydrophilic ILs have higher extinction coefficients for octanol and water-rich phases.

Table 4.4.1. Extinction coefficients, ε ($L.mol^{-1}.cm^{-1}$) for imidazolium-based ILs in water- and octanol-rich phases.

Ionic Liquid	ε_w / $L.mol^{-1}.cm^{-1}$	ε_{oct} / $L.mol^{-1}.cm^{-1}$
[C_2mim][NTf_2]	4526 ± 18	4498 ± 74
[C_4mim][NTf_2]	4239 ± 13	4729 ± 60
[C_6mim][PF_6]	4228 ± 19	4769 ± 92
[C_8mim][PF_6]	4322 ± 32	4220 ± 24

For the studied ILs, the range of D_{ow} values at equilibrium are reported in Tables 4.4.2-4.4.5, along with the concentration of IL in octanol-rich phase at the initial stage, and in the water rich phase at equilibrium.

Table 4.4.2. D_{ow} results for $[C_2mim][NTf_2]$.

Ionic Liquid	D_{ow}	$[IL]_{oct}$ mol.L^{-1}	$[IL]_w$ mol.L^{-1}
$[C_2mim][NTf_2]$	0.007	7.14×10^{-8}	1.02×10^{-5}
	0.009	1.80×10^{-7}	2.04×10^{-5}
	0.011	3.40×10^{-7}	3.05×10^{-5}
	0.013	5.30×10^{-7}	4.06×10^{-5}
	0.015	7.60×10^{-7}	5.06×10^{-5}
	0.019	1.44×10^{-6}	7.57×10^{-5}
	0.035	8.30×10^{-6}	2.35×10^{-4}
	0.046	2.14×10^{-5}	4.66×10^{-4}
	0.071	4.86×10^{-5}	6.82×10^{-4}
	0.093	8.29×10^{-5}	8.92×10^{-4}
	0.102	1.12×10^{-4}	1.11×10^{-3}
	0.107	1.42×10^{-4}	1.32×10^{-3}

Table 4.4.3. D_{ow} results for $[C_4mim][NTf_2]$.

Ionic Liquid	D_{ow}	$[IL]_{oct}$ mol.L^{-1}	$[IL]_w$ mol.L^{-1}
$[C_4mim][NTf_2]$	0.021	1.97×10^{-7}	9.44×10^{-6}
	0.029	8.26×10^{-7}	2.81×10^{-5}
	0.034	1.89×10^{-6}	5.59×10^{-5}
	0.038	3.19×10^{-6}	8.35×10^{-5}
	0.052	5.66×10^{-6}	1.10×10^{-4}
	0.069	9.32×10^{-6}	1.35×10^{-4}
	0.090	2.18×10^{-5}	2.41×10^{-4}
	0.169	7.33×10^{-5}	4.32×10^{-4}
	0.220	1.27×10^{-4}	5.79×10^{-4}
	0.280	2.36×10^{-4}	8.42×10^{-4}
	0.336	3.55×10^{-4}	1.06×10^{-3}
	0.435	5.86×10^{-2}	1.35×10^{-3}
	0.467	7.82×10^{-4}	1.67×10^{-3}
	0.540	1.05×10^{-3}	1.94×10^{-3}
	0.608	1.33×10^{-3}	2.18×10^{-3}

Table 4.4.4. D_{ow} results for $[C_6mim][PF_6]$.

Ionic Liquid	D_{ow}	$[IL]_{oct}$ mol.L^{-1}	$[IL]_w$ mol.L^{-1}
$[C_6mim][PF_6]$	0.139	4.63×10^{-6}	3.33×10^{-5}
	0.146	9.67×10^{-6}	6.62×10^{-5}
	0.153	1.51×10^{-5}	9.87×10^{-5}
	0.160	2.10×10^{-5}	1.31×10^{-4}
	0.169	2.74×10^{-5}	1.62×10^{-4}
	0.185	4.44×10^{-5}	2.40×10^{-4}
	0.201	6.36×10^{-5}	3.16×10^{-4}
	0.225	1.04×10^{-4}	4.65×10^{-4}
	0.276	2.05×10^{-4}	7.44×10^{-4}
	0.324	3.25×10^{-4}	1.00×10^{-3}
	0.373	4.64×10^{-4}	1.24×10^{-3}
	0.429	6.84×10^{-4}	1.59×10^{-3}

Table 4.4.5. D_{ow} results for $[C_8mim][PF_6]$.

Ionic Liquid	D_{ow}	$[IL]_{oct}$ mol.L^{-1}	$[IL]_w$ mol.L^{-1}
$[C_8mim][PF_6]$	0.401	2.43×10^{-5}	6.06×10^{-5}
	0.430	5.42×10^{-5}	1.26×10^{-4}
	0.470	1.09×10^{-4}	2.32×10^{-4}
	0.500	1.72×10^{-4}	3.44×10^{-4}
	0.540	2.44×10^{-4}	4.52×10^{-4}
	0.570	2.91×10^{-4}	5.10×10^{-4}
	1.210	2.00×10^{-3}	1.65×10^{-3}

As shown in Figure 4.4.2, the literature values of $[C_4mim][NTf_2]$ by Ropel et al. [30] are well reproduced by this study, which confirms the suitability of the apparatus and experimental technique used for the D_{ow} determination.

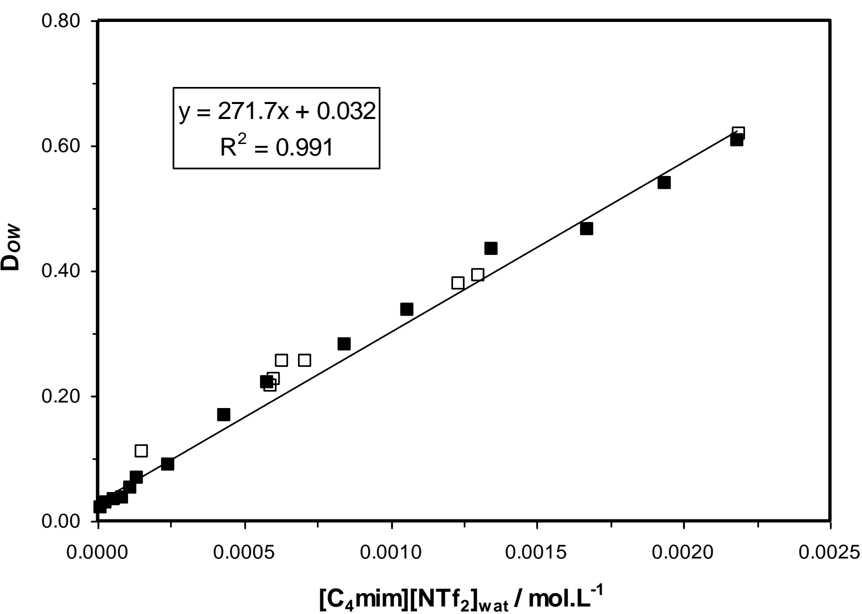

Figure 4.4.2. Experimental (■) and Ropel et al. [30] data (□) for octanol-water distribution coefficients at different [C_4mim][NTf_2] concentrations.

The data collected here are plotted in Figure 4.4.3. The linearity of the dependency of D_{ow} with the total cation concentration in the aqueous phase suggests that in the concentration range studied, the IL is completely dissociated.

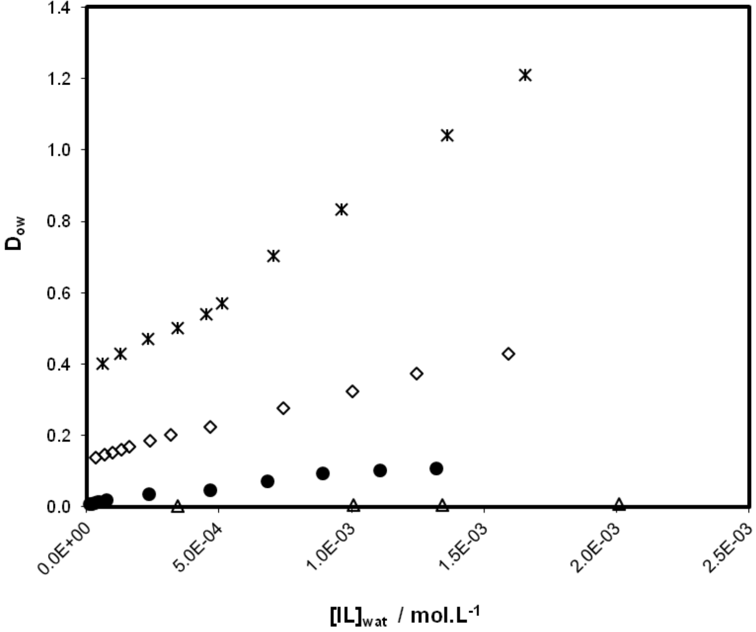

Figure 4.4.3. Experimental results of octanol-water distribution coefficients for various ILs, (◊) [C_6mim][PF_6]; (*) [C_8mim[PF_6]; (●) [C_2mim][NTf_2]; (Δ) [C_4mim][PF_6] [34] and different concentrations.

At higher IL concentrations the partition coefficient is no longer linear, as shown in the data reported by Lee and Lee [34]. At these low concentrations Eq. 4.11 can be used to correlate the experimental data and extract the values for the equilibrium constants k_2, k_3 and E. The values of these constants are reported on Table 4.4.6 for the ionic liquids studied. They show that, in contrast to observations in the aqueous phase, the dissociation on the organic phase is very small.

Table 4.4.6. Reports of k_2, k_3 and E values of the studied ILs.

Ionic Liquid	k_2	k_3/ $mol^{-1}.L$	E/ $mol^{-1}.L$
$[C_2mim][NTf_2]$	0.00012 ± 0.000036	698742.4 ± 202511.7	81.0 ± 3.6
$[C_4mim][NTf_2]$	0.0010 ± 0.0003	268184.3 ± 87574.6	271.7 ± 7.2
$[C_6mim][PF_6]$	0.019 ± 0.003	9965.6 ± 180.4	186.7 ± 2.0
$[C_8mim][PF_6]$	0.116 ± 0.006	4448.2 ± 254.1	514.9 ± 14.6
$[C_4mim][PF_6]$ [a]	0.0000044 ± 0.00000082	573680.1 ± 115342.3	2.55 ± 0.21

[a]Results from Lee and Lee [34].

Under these conditions the values of E are the true partition constants of the ILs between the two phases that are relevant for the analysis of the partition of ionic liquids between water and 1-octanol. An inspection to Eqs. 4.3 and 4.13 shows, however, the different nature of these two equilibrium constants and that their numerical values cannot be directly compared. Qualitatively their behavior will also present important differences. While K_{ow} will presuppose a constant relation between the concentrations in the two phases, E suggests that at concentrations below 1/E the compound will be preferentially present in the aqueous phase; above that concentration it will partition preferentially to the organic phase. This peculiar behavior can be observed in the data of Lee and Lee [34] for the $[C_4mim][PF_6]$ where the E value here reported correctly estimates the concentration at which inversion on the partition between the two phases is observed. The E values measured show, with no surprise, that E increases with the hydrophobicity, represented as the ionic liquid water solubility in Figure 4.4.4. This behavior follows the expected trends with the increase of the cation alkyl chain length and the anion hydrophobicity.

For analysis of the influence of the anions, a comparison between the D_{ow} values for $[C_4mim][PF_6]$ determined Lee and Lee [34] with $[C_4mim][NTf_2]$ determined by our group was carried. In this case, it is possible to observe that the D_{ow} value is higher for the $[NTf_2]$-based IL. This could easily be explained by the solubilities of these two ILs in water and 1-octanol, which, according to Lee and Lee [34], were 71.0 and 16.0 $mmol.L^{-1}$ in water and, 6.7 and 143.2 $mmol.L^{-1}$ in 1-octanol, for the $[PF_6]$ and $[NTf_2]$, respectively. Since the solubility of the IL in 1-octanol increases from $[PF_6]$ to $[NTf_2]$, is expected that the latest has a greater affinity for 1-octanol, thus increasing the D_{ow}.
In this work, the pair extraction constant E values were compared with EC_{50} values measured by Microtox® and the IL aqueous solubility reported in Table 4.4.7.

Table 4.4.7. E results of some ILs and EC_{50} ($mg.L^{-1}$) values after 15 minutes of exposure to the luminescent marine bacteria *Vibrio fischeri*.

Ionic Liquid	E / $mol^{-1}.L$	Water solubility mol fraction × 10^4	EC_{50} / $mg.L^{-1}$ (lower limit; upper limit)
$[C_2mim][NTf_2]$	81.0 ± 3.6	8.38 [b]	145.08 (101.63; 283.82)
$[C_4mim][NTf_2]$	271.7 ± 7.2	1.96 [b]	141.99 (70.99; 141.99)
$[C_4mim][PF_6]$ [a]	2.55 ± 0.21	12.10 [c]	333.88 (300.34; 403.55)
$[C_6mim][PF_6]$	186.7 ± 2.0	4.30 [c]	40.22 (20.45; 60.86)
$[C_8mim][PF_6]$	514.9 ± 14.6	1.30 [c]	1.71 (0.00; 1.71)

[a]Results from Lee and Lee [34]
[b]Results from Freire et al. [44]
[c]Results from Freire et al. [45]

Very good correlation between the pair extraction constant E and the IL aqueous solubility was observed and is shown in Figure 4.4.4. This result suggests that the partition between the aqueous and organic phases is controlled by the ionic liquid solubility in water.

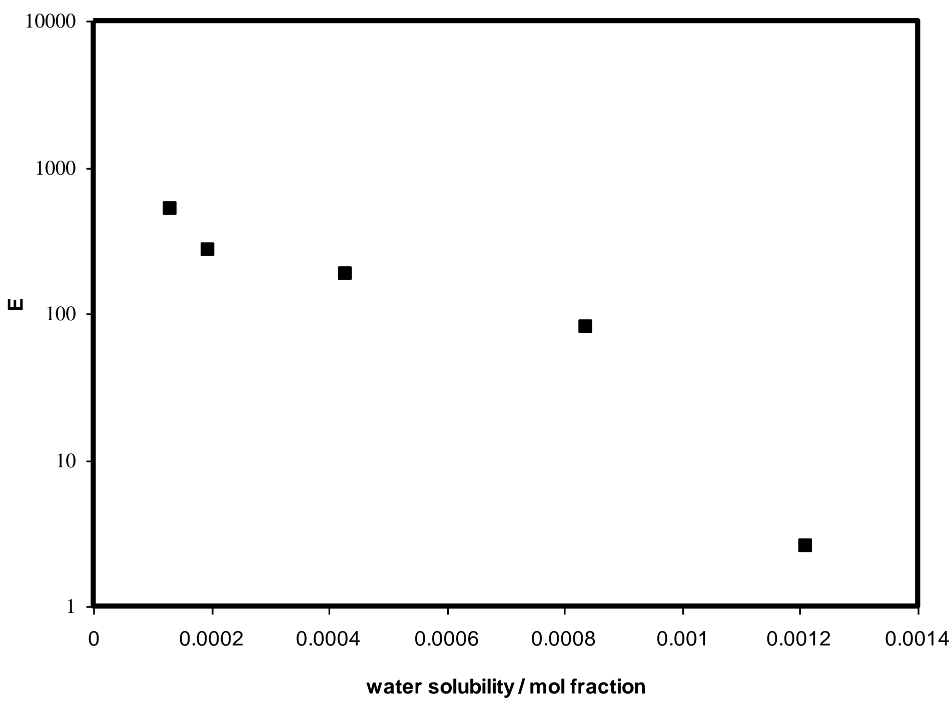

Figure 4.4.4. Experimental results of the ion pair extraction constant (E) and the water solubility for the ILs studied described for Freire et al. [44, 45].

The trend of the EC_{50} dependency on the pair extraction constant E, reported in Figure 4.4.5, is expectable with the toxicity increasing with increasing affinity of the ionic liquid for the organic phase. A higher affinity for the 1-octanol would imply a higher adherence of the IL to the living tissues, resulting in a higher toxicity. However, this correlation is not as smooth as that observed for the aqueous solubility suggesting that other effects besides the affinity of the ionic liquid for the different phases plays a role on the ionic liquid toxicity as discussed by us in a previous work [26].

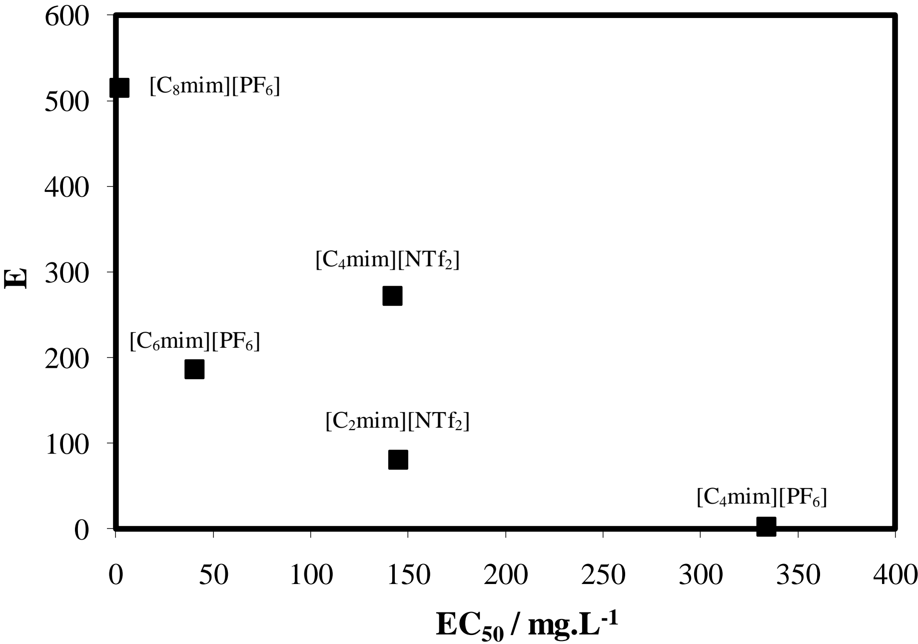

Figure 4.4.5. Experimental results of the ion pair extraction constant (E) and the EC_{50} ($mg.L^{-1}$) results for the ILs studied.

4.4.5. CONCLUSIONS

The slow-stirring method was used to measure the octanol-water distribution coefficient (D_{ow}) for different ionic liquids, it being confirmed that an approach based on the ion pair extraction constant, E, is more consistent for the description of the partition. The D_{ow} results of the imidazolium-based ILs investigated are concentration dependent and are much lower than many commonly used industrial solvents. An increase in the alkyl chain length on the cation or substitution of hydrogen atoms with methyl groups on the cation ring increases the distribution coefficient. The results indicate that for concentrations below 1/E the ionic liquid will partition preferentially towards the aqueous phase.

Combining the toxicological and partition information, results suggest that the hydrophobic imidazolium-based ionic liquids studied do not represent a serious environmental threat at low concentrations. However, more data is required for a full understanding of the environmental risk profile of ionic liquids.

4.4.6. REFERENCES

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5. GENERAL CONCLUSIONS

A new pathway for the quantification of ILs solubility in water using the ESI-MS method was here proposed, and original data for pyrrolidinium- and piperidinium-based ILs, in the temperature range of 288.15 K and 318.15 K, were presented. The ILs solubility in water is dependent of the cation core and the length of the alkyl chains. Indeed, the ILs solubility in water increases with the decrease in the alkyl side chain length and from piperidinium to pyridinium to pyrrolidinium to imidazolium-based ILs. Thus, the solubility of ILs in water is primarily defined by the cation size. Contrary to what was observed for the IL-rich phase, the solubility of water in ILs largely depends on the IL availability of electrons for privileged interactions. Finally, the standard molar thermodynamic functions of solution, derived from experimental solubility data, indicate that the ILs dissolution in water is an endothermic and entropic driven process.

The ILs ability to form Aqueous Two-Phase Systems (ATPS) allows for hydrophilic ILs to be used in liquid-liquid extractions, and new phase equilibrium data for several systems involving hydrophilic ILs + inorganic salts + water were presented (at atmospheric pressure and ambient temperature). K_3PO_4 was one of the inorganic salts studied, using diverse ILs structures. The results obtained indicate that the IL cation has a significant influence in the behaviour of the binodal curves and in the promotion of ATPS. Increasing the alkyl chain length increases the phase separation ability, while the insertion of a double bond, a benzyl or an hydroxyl group leads to the decrease of the capacity for the ATPS formation. Moreover, the ability of imidazolium-based ILs for aqueous phase separation was shown to closely follow the hydrogen bond accepting strength decrease of the anions composing the IL. The results indicate that IL-based ATPS can be obtained over a large range of concentration of both the inorganic salt and the IL, and that such systems can be fine tuned by the adjustment of the IL anion.
The capacity of those IL-based ATPS as extraction media was demonstrated using L-tryptophan as a model biomolecule. The L-tryptophan partitions closely follow the Hofmeister series, since they preferentially migrate for IL-rich phases composed by halogenated ions such as Cl or Br or to the most hydrophobic anions (higher hydrogen-bonding accepting strength (β)). The influence of the IL imidazolium-based cation on the extraction capacity of ATPS seems to be more important than the influence of the anion. Indeed, in the study of the cation influence the K_{Trp} was found to vary between 10 and 120 (at approximately the same mass fraction compositions of IL plus inorganic salt). The presence of benzyl groups and double bounds at the imidazolium side chain

increased substantially the partition coefficients. Finally, in what concerns the influence of the alkyl chain, increasing the IL cation alkyl chain decreases the L-tryptophan partitioning in the di-substituted imidazolium-based ILs. On the other hand, the highest partition coefficient occurs for the [amim]Cl followed by [OHC$_2$mim]Cl due to the possibility of to form more hydrogen-bonds between the alkyl chain of the imidazolium and the amino-acid. These results indicate that ILs are a novel option for the purification and separation of biomolecules with much larger partition coefficients than conventional ATPS such as polymer-polysaccharide ($K_{\mathrm{Trp}} \cong 1$) and polymer-inorganic salt ($K_{\mathrm{Trp}} \cong$ 1-7).

Despite the higher solubility in water and thus higher ability to promote the phase separation, the aqueous solutions formed by K_3PO_4 and K_2HPO_4 present higher pH values around 13.7 and 9.1, which is undesirable when the objective is their application to enzymatic processes, due to the deleterious effect of the pH in some enzyme structures, causing the decrease of their activity or even their inactivation. Thus, those inorganic salts were here replaced by a buffer solution composed of K_2HPO_4/KH_2PO_4, to achieve pH 7.0. It was shown that this inorganic salt show a lower capacity for the ATPS formation, described by the following series: $K_3PO_4 > K_2HPO_4 > K_2HPO_4/KH_2PO_4$, which is accordance with the Hofmeister series. Strong salting-out inducing anions, PO_4^{3-} and HPO_4^{2-}, exhibit a better capability for creating ion-hydration complexes by excluding water from the IL-rich phase, favoring the formation of ATPS.

The formation of ATPS based in K_2HPO_4/KH_2PO_4 + IL + water were also investigated. The effects of IL cation core, length of the alkyl side chain, positional isomerism and anion were investigated. An increase in the length of the aliphatic chain at the cation leads to an increase on the IL ability to form ATPS, for C_2 to C_6 alkyl chains. However, when the number of carbons at the alkyl side chain is higher than 6, there is an inversion on the ILs sequence, due to the self-aggregation of ILs in aqueous phases, and consequent micelle formation. Moreover, it was observed that, in general, the ability of ILs to form ATPS is more dependent on the size or molecular volume of the ions, and consequently more dependent on steric contributions. Finally, in what respects the anion influence, it was shown that the lower the hydrogen bond basicity of the IL, the higher is the ability of the IL to form ATPS. The ATPS based in the phosphate buffer solution were used to investigate their ability to extract the enzyme *Candida antarctica* lipase B (CaLB), at a defined pH 7.0, considering the use of those different IL structural features (anion, cation and alkyl chain length). The results obtained indicate that the higher

purification factor values were found for the octyl side chain, with the dicyanamide $[N(CN)_2]$ anion and the pyridinium $[C_4mpyr]$ cation, as isolated effects. However, the additive properties were not verified for the extraction parameter, since the tailored $[C_8pyr][N(CN)_2]$ was further tested, and its purification capacity was found to be almost inexistent.

The best IL features, optimized in this work (C_8 as the alkyl chain, pyridinium and dicyanamide as the cation core and anion, respectively) were used to test the production and extraction of a lipolytic enzyme produced by the bacteria *Bacillus* sp. ITP-001. In this case, the separation and purification steps were performed on the fermentation broth after the end of the production phase. The data suggests that the purification of the enzyme was controlled mainly by the alkyl chain length, followed by the cation core and finally, by the anion moiety. It was observed that, as for CaLB, the changes on the hydrophobic nature of the IL and the consequent interactions established between the enzyme, contaminant proteins and each one of the phases (salt- and IL-rich phases) were the conditions responsible for the separation/purification profiles. Finally, it was possible to show that the ILs-based ATPS are more efficient and significantly contribute for the improvement of the extraction capacity when compared with the commonly used polymers-based ATPS. Despite the variable (sometimes higher) separation capacity, the biocompatibility of these ionic structures is one of the main concerns.

In this work, a study of the IL effect on the enzymatic activity was carried, using the enzyme CaLB and different ionic liquids (varying the alkyl chain length and anion) and system conditions (ILs' molar concentration). It was shown that the ionic liquid concentration is one of the main factors with negative impact on the enzymatic activity. Moreover, it was observed that the negative impact of the longest alkyl chains is related with the increase in the hydrophobic nature of the IL, promoting the ability of the alkyl chain to obstruct the non-polar active site of the enzyme. It was concluded that the impact of the IL can be explained by the water activity of the system and the strength of the interactions established between the enzyme and the different anions, represented by dispersion forces and Hydrogen bonding.

Despite these good separation and purification results here described, there are some important concerns to take into account when addressing the design of a sustainable process. For that purpose it is also important to evaluate the potential impact of ILs on human health and on the environment. Furthermore, one of the principles of green chemistry aims at the reduction of the toxicity of the chemical compounds used in

industrial processes or product formulations. Legislation concerning this subject is nowadays more stringent as REACH (Registration, Evaluation, Authorization and Restriction of Chemicals), requires the registration of new commercial chemicals and holds the suppliers responsible for their products. Therefore, in the development of new products, the optimization of technical performance must run in parallel with the minimization of hazard potentials. Although ILs can lessen the risk of air pollution due to their insignificant vapor pressures, they may have significant solubility in water, as was also shown. Considering this picture, the determination of the ILs' ecotoxicological profiles is of utmost interest and importance. It is in this context that this work shows a large study addressing the effect of different ILs towards different biological model organisms (marine bacterium, freshwater green algae and cladocerans), at various trophic levels (primary producers and primary consumers) and species at the same trophic level (*Pseudokirchenriella subcapitata* and *Chlorella vulgaris* as first producers and *Daphnia magna* and *Dapnhia longispina* as first consumers). Finally, it was also determined the bioaccumulation capacity of some hydrophobic ILs through the determination of the 1-octanol-water distribution coefficient (D_{ow}). The first conclusion determined is that the study of the toxicological profiles of ILs is extremely important, because their toxicity can be significantly varied between trophic levels and even between species at the same trophic level. In what concerns the influence of the IL features, it was confirmed that the elongation of the side chain is responsible for the increase on the toxicity, which is normally called by the "side chain effect", being this effect extinct after a given number of carbons. This number of carbons depends of the species under study. This effect was normally entitled in literature as the "cut-off" effect. However, when the side chain is functionalized, for example with ether and ester groups, it was possible to decrease significantly their toxicity in comparison with the respective non-functionalized alkyl chains. In what concerns the effect of different cations cores, it was shown that they can have a significant influence in the toxicity, for example in the phosphonium and guanidinium cases, when compared with the imidazolium cation core. It was concluded that, despite the general idea reported in literature that, increasing the hydrophobicity of the IL increases the toxicity, it is possible to design ILs by enhancing their hydrophobic character while simultaneously decreasing their toxicity, in a paradigmatic example of the "designer solvent" character of the ionic liquids. The observed effects of different anionic moieties on the toxicity were also assessed in this work. It was seen that the influence of the anions was

characteristically varied but less pronounced when compared with the cation influence, which can be totally inhibited by the biggest effects of the alkyl chain lengths.

Having in mind the possibility of to enlarge the ecotoxicological risk profile of ILs, it was here studied another risk indicator, the bioaccumulation. This study was performed through the experimental determination of the octanol-water distribution coefficient (D_{ow}) using the slow stirring method. The D_{ow} results of the investigated hydrophobic imidazolium-based ILs are concentration dependent and are much lower than many commonly used industrial solvents. An increase in the alkyl chain length on the cation or substitution of hydrogen atoms with methyl groups on the cation ring increases the distribution coefficient. The results indicate that for concentrations below 1/E the IL will partition preferentially towards the aqueous phase. Combining the toxicological and partition information the results suggest that the hydrophobic imidazolium-based ionic liquids here studied do not represent a serious environmental threat at low concentrations. Of course, it was needed to highlight that, as is usually mentioned in the literature, each IL is a particular case and the determination of their effects was not easy to predict. In this perspective, maybe with the exception of the alkyl side chain effect, the remaining conclusions here described require more data for a more generalized and full understanding of the environmental risk profile of ILs.

6. FINAL REMARKS AND FUTURE WORK

The present work addresses a profound study on the use of ILs as new alternative solvents for the extraction of biomolecules. The hydrophilic ILs are shown to be interesting compounds to use as biocompatible solvents of low toxicity. Besides all the investigations here developed, some new fields should be considered and new contributions can be performed for a most complete characterization of those systems. In fact, some of those tests are already ***in progress***:

- ✓ Determination of the performance of new conditions in what concerns the aqueous two phase systems formation and its applicability as extractant systems. Examples of these conditions are the pH and temperature and also the variation on the ILs and inorganic salt concentrations.

- ✓ Investigation of other parameters testing the biocompatibility of the ILs, such as the enzymatic stability of *Candida antarctica* lipase B and respective comparison with the data obtained for some organic solvents commonly used in industry.

- ✓ Measurement of the toxicity caused by different ILs towards enzyme producer microorganisms, generally known as antimicrobial activity tests.

Meanwhile, additional work in the environmental field must still be performed and should include:

- Determination of the modes of toxic action of different IL' features.

- Investigation of the ILs' biodegradability according to OECD standards protocols.

- Development and test of feasible modeling tools for ILs' environmental toxicity prediction based on the physico-chemical properties of ILs, using for example, a quantum-based methodology provided by COSMO-RS.

7. LIST OF PUBLICATIONS

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8. SUPPORTING INFORMATION

8.2. Solubility of ILs in water

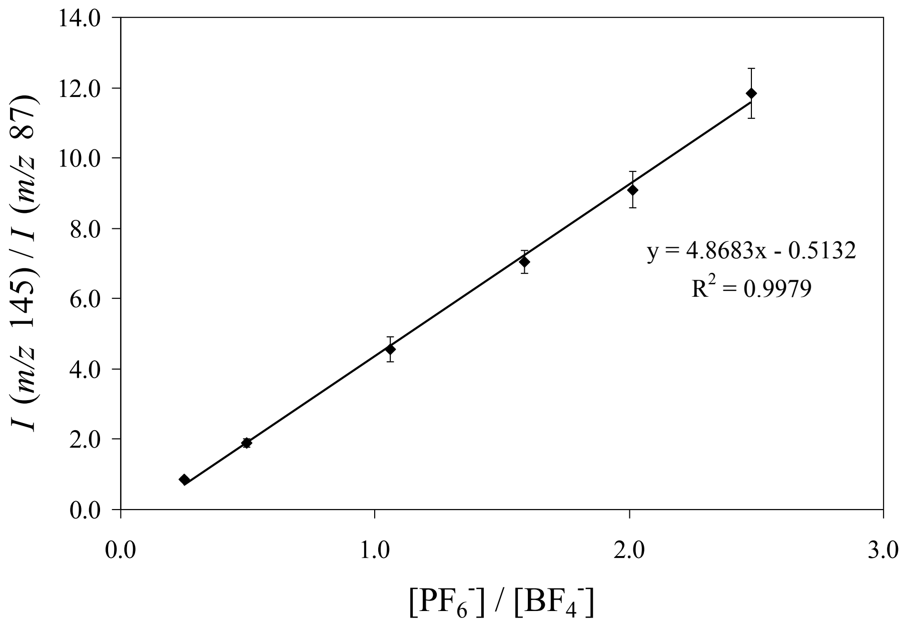

Figure S8.2.1. Calibration curve for [C_4mim][PF_6] using [C_4mim][BF_4] as internal standard (peaks abundances ratio as function of the concentrations ratio).

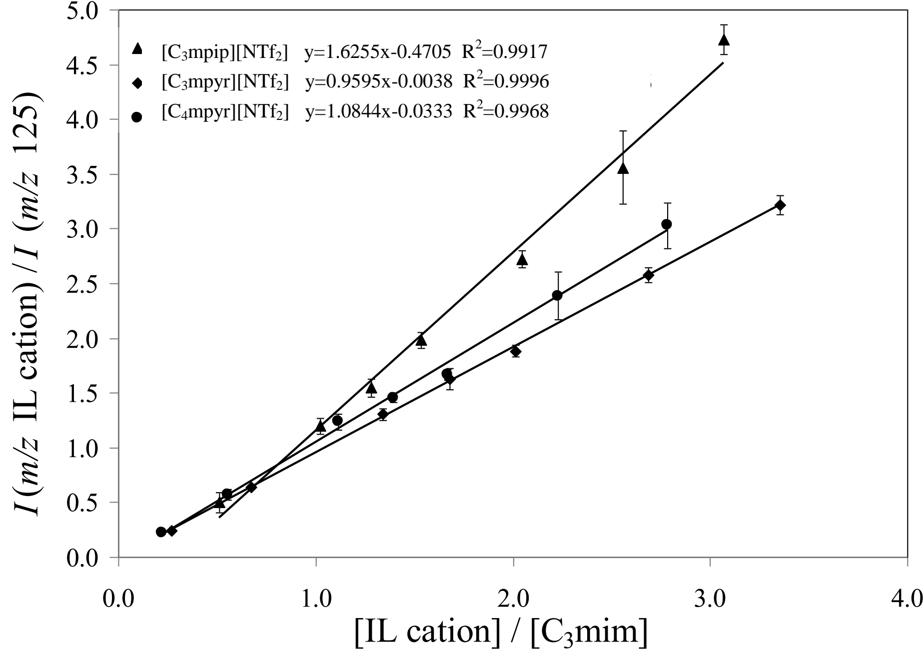

Figure S8.2.2. Calibration curve for [C_3mpip][NTf_2], [C_3mpyr][NTf_2] and [C_4mpyr][NTf_2] using [C_3mim][NTf_2] as internal standard (peaks abundances ratio as function of the concentrations ratio).

8.3. Aqueous Two-Phase Systems as New Separation Techniques

8.3.1. Synthesis of 1,3-dimethylimidazolium chloride synthesis. 1,3-dimethylimidazolium chloride ([C_1im]Cl) has been synthesized by means of Carbonate Based Ionic Liquid Synthesis (CBILS©) of proionic / Sigma Aldrich.[1] 1,3-dimethylimidazolium hydrogenocarbonate (the CBIL precursor as a 45.91 % solution in H_2O : Methanol (3 : 2)) has been treated with an exact stoichiometric amount of hydrochloric acid (FIXANAL / Riedel-de Haën). After the evolution of CO_2 ceased, the solvents have been removed in vacuum, leaving white crystals of [C_1im]Cl.

^{1}H NMR Chemical Shifts (in $CDCl_3$, δ / ppm relative to TMS): 10.56 (s, 1H), 7.47 (d, 2H), 4.08 (s, 6H).

References

[1]http://www.sigmaaldrich.com/Area_of_Interest/Chemistry/Chemical_Synthesis/Product_Highlights/CBILS.html

Table S8.3.1 Experimental binodal curve mass fraction data for the system IL (1) + K_3PO_4 (2) + H_2O (3) at 298.15 K.

[im]Cl		[C_1mim]Cl		[OHC_2mim]Cl	
100 w_1 ± 0.01	**100 w_2 ± 0.01**	**100 w_1 ± 0.01**	**100 w_2 ± 0.01**	**100 w_1 ± 0.01**	**100 w_2 ± 0.01**
14.07	26.44	40.22	7.72	67.86	2.32
13.36	26.27	36.70	8.39	64.17	2.77
12.89	26.43	33.90	8.94	54.22	3.72
12.33	26.35	30.42	11.31	49.93	4.55
11.91	26.47	27.79	12.33	45.32	6.92
11.47	26.55	25.36	14.07	38.31	10.59
10.95	26.78	22.86	15.97	34.71	12.74
10.50	27.03	21.37	17.09	30.19	15.75
7.48	30.41	19.79	18.48	28.26	17.12
7.01	30.79	18.40	19.69	21.47	22.29
6.30	31.44	16.67	21.22	67.86	2.32
5.58	32.21	15.17	22.49	64.17	2.77
		13.81	23.77	54.22	3.72
		12.58	24.93	49.93	4.55
		11.89	25.62	45.32	6.92
		10.79	26.69	38.31	10.59
		9.21	28.40	34.71	12.74
		7.59	30.29	30.19	15.75
				28.26	17.12
				21.47	22.29

Table S8.3.2. Experimental binodal curve mass fraction data for the system [amim]Cl (1) + K_3PO_4 (2) + H_2O (3) at 298.15 K.

[amim]Cl			
100 w_1 ± 0.01	**100 w_2 ± 0.01**	**100 w_1 ± 0.01**	**100 w_2 ± 0.01**
44.18	2.72	14.93	19.27
39.55	4.18	14.46	19.63
37.57	5.12	13.86	20.17
34.55	5.98	13.11	20.91
33.07	6.91	12.53	21.43
31.57	7.61	12.04	21.82
30.24	8.29	11.61	22.22
29.01	9.00	11.11	22.72
27.21	10.19	10.65	23.18
26.09	10.71	10.28	23.53
24.57	11.89	9.96	23.82
23.03	12.96	9.57	24.20
21.72	13.90	9.20	24.59
20.63	14.59	8.86	24.93
19.42	15.56	8.56	25.23
18.31	16.49	8.26	25.52
17.58	16.96	7.80	26.11
16.62	17.80	7.47	26.47
15.72	18.58	6.94	27.06

Table S8.3.3. Experimental binodal curve mass fraction data for the system $[C_6mim]Cl$ (1) + K_3PO_4 (2) + H_2O (3) at 298.15 K.

$[C_6mim]Cl$							
100 w_1 ± 0.01	100 w_2 ± 0.01	100 w_1 ± 0.01	100 w_2 ± 0.01	100 w_1 ± 0.01	100 w_2 ± 0.01	100 w_1 ± 0.01	100 w_2 ± 0.01
42.38	3.90	11.32	19.94	6.19	24.83	3.67	28.21
35.98	5.40	11.07	20.01	6.07	24.96	3.58	28.36
33.61	6.21	10.84	20.14	6.01	24.99	3.50	28.48
31.27	7.30	10.63	20.27	5.90	25.13	3.39	28.69
29.17	8.25	10.28	20.65	5.70	25.39	3.34	28.77
27.42	9.05	10.09	20.78	5.55	25.54	3.20	29.03
25.95	9.81	9.77	21.11	5.46	25.68	3.11	29.19
24.57	10.49	9.53	21.28	5.37	25.79	3.03	29.35
23.57	11.20	9.44	21.41	5.29	25.91	2.95	29.52
22.43	11.79	9.22	21.66	5.21	26.02	2.85	29.65
21.48	12.34	9.07	21.73	5.12	26.13	2.80	29.76
20.72	12.89	8.96	21.88	5.05	26.23	2.75	29.86
20.09	13.47	8.82	21.98	4.97	26.33	2.69	29.99
19.24	13.87	8.60	22.24	4.89	26.42	2.63	30.12
18.58	14.34	8.36	22.46	4.86	26.47	2.54	30.30
17.97	14.73	8.24	22.54	4.79	26.55	2.48	30.43
17.37	15.14	8.04	22.79	4.69	26.71	2.41	30.57
16.85	15.53	7.93	22.86	4.60	26.85	2.36	30.68
16.37	15.89	7.74	23.09	4.53	26.92	2.32	30.79
15.91	16.23	7.64	23.14	4.44	27.07	2.24	30.97
15.49	16.58	7.45	23.37	4.33	27.23	2.17	31.11
14.76	17.25	7.37	23.47	4.25	27.35	2.11	31.29
14.38	17.49	7.20	23.62	4.19	27.42	2.05	31.43
14.02	17.74	7.05	23.81	4.12	27.55	1.96	31.65
13.66	17.96	6.91	24.01	4.05	27.68	1.91	31.79
13.34	18.20	6.82	24.04	3.96	27.82	1.80	32.07
12.83	18.76	6.68	24.22	3.91	27.88	1.71	32.33
12.51	18.91	6.55	24.38	3.86	27.91	1.61	32.63
12.05	19.41	6.43	24.54	3.80	28.01	1.49	32.98
11.77	19.57	6.30	24.69	3.73	28.10	1.39	33.33
11.54	19.76						

Table S8.3.4. Experimental binodal curve mass fraction data for the system $[C_1im]Cl$ (1) + K_3PO_4 (2) + H_2O (3) at 298.15 K.

$[C_1im]Cl$					
100 w_1 ± 0.01	**100 w_2 ± 0.01**	**100 w_1 ± 0.01**	**100 w_2 ± 0.01**	**100 w_1 ± 0.01**	**100 w_2 ± 0.01**
19.99	22.21	8.54	21.34	3.83	24.05
18.15	22.15	8.32	21.51	3.75	24.17
17.57	22.18	7.93	21.79	3.67	24.27
16.84	22.02	7.61	21.84	3.59	24.37
16.31	22.00	7.38	21.84	3.52	24.44
15.70	21.84	7.19	21.90	3.45	24.55
15.23	21.80	7.04	22.06	3.38	24.66
14.67	21.60	6.86	22.10	3.25	24.87
14.25	21.58	6.69	22.17	3.18	24.93
13.79	21.46	6.52	22.17	3.11	25.05
13.42	21.45	6.32	22.30	3.05	25.10
12.97	21.29	6.13	22.40	2.98	25.20
12.58	21.17	5.98	22.39	2.91	25.35
12.24	21.15	5.86	22.43	2.85	25.44
11.89	21.05	5.75	22.52	2.79	25.54
11.62	21.06	5.53	22.62	2.73	25.59
11.30	20.97	5.36	22.87	2.68	25.67
11.05	20.97	5.12	22.81	2.61	25.80
10.77	20.92	4.96	23.18	2.54	25.91
10.54	20.92	4.74	23.17	2.48	26.05
10.31	20.93	4.58	23.41	2.39	26.27
10.09	20.92	4.38	23.40	2.29	26.43
9.93	20.98	4.26	23.70	2.22	26.63
9.69	20.90	4.08	23.73	2.12	26.82
9.48	21.23	3.97	23.89	2.01	27.09
9.11	21.18	3.90	23.97	1.90	27.40
8.83	21.30				

Table S8.3.5. Experimental binodal curve mass fraction data for the system $[C_2im]Cl$ (1) + K_3PO_4 (2) + H_2O (3) at 298.15 K.

$[C_2im]Cl$							
100 w_1 ± 0.01	**100 w_2 ± 0.01**	**100 w_1 ± 0.01**	**100 w_2 ± 0.01**	**100 w_1 ± 0.01**	**100 w_2 ± 0.01**	**100 w_1 ± 0.01**	**100 w_2 ± 0.01**
25.97	19.52	9.74	16.51	5.36	17.99	2.02	21.87
24.38	19.55	9.54	16.46	5.24	18.08	1.94	22.08
23.33	19.26	9.41	16.54	5.12	18.16	1.81	22.43
22.66	19.29	9.27	16.63	5.00	18.25	1.73	22.62
22.06	19.19	9.09	16.61	4.89	18.32	0.85	23.49
21.09	19.02	8.93	16.60	4.78	18.40	0.83	23.65
20.27	18.77	8.81	16.67	4.68	18.50	0.81	23.78
19.62	18.87	8.70	16.75	4.58	18.56	0.80	23.98
18.86	18.74	8.54	16.73	4.49	18.63	0.78	24.08
18.13	18.61	8.40	16.72	4.38	18.78	0.77	24.24
17.41	18.50	8.30	16.79	4.27	18.84	0.75	24.31
16.79	18.36	8.20	16.87	4.15	18.99	0.73	24.54
16.03	18.08	8.06	16.82	3.99	19.09	0.71	24.78
15.47	17.95	7.86	16.97	3.79	19.35	0.69	24.96
14.98	17.89	7.65	17.03	3.32	20.01	0.67	25.12
14.35	17.60	7.47	17.15	3.18	19.97	0.66	25.26
13.91	17.51	7.27	17.18	3.07	20.11	0.64	25.39
13.50	17.45	7.10	17.29	2.96	20.33	0.63	25.59
13.10	17.39	6.92	17.34	2.87	20.48	0.61	25.83
12.63	17.21	6.78	17.45	2.76	20.52	0.59	25.97
12.28	17.12	6.59	17.35	2.67	20.63	0.56	26.29
11.97	17.06	6.46	17.43	2.60	20.82	0.55	26.42
11.57	16.88	6.35	17.50	2.52	20.92	0.53	26.72
11.28	16.83	6.16	17.67	2.46	21.03	0.51	26.94
11.02	16.80	5.96	17.73	2.39	21.12	0.49	27.08
10.68	16.62	5.83	17.69	2.34	21.24	0.48	27.32
10.43	16.62	5.71	17.86	2.28	21.36	0.46	27.61
10.20	16.59	5.59	17.84	2.23	21.49	0.43	27.96
9.96	16.55	5.50	17.91	2.14	21.67		

Table S8.3.6. Experimental binodal curve mass fraction data for the system $[C_7H_7mim]Cl$ (1) + K_3PO_4 (2) + H_2O (3) at 298.15 K.

$[C_7H_7mim]Cl$									
100 w_1 ± 0.01	**100 w_2 ± 0.01**	**100 w_1 ± 0.01**	**100 w_2 ± 0.01**	**100 w_1 ± 0.01**	**100 w_2 ± 0.01**	**100 w_1 ± 0.01**	**100 w_2 ± 0.01**	**100 w_1 ± 0.01**	**100 w_2 ± 0.01**
43.69	4.76	24.13	12.44	14.72	18.04	9.31	21.96	5.70	25.53
40.14	5.65	23.33	12.79	13.96	18.52	8.84	22.39	5.41	25.83
37.63	6.46	22.10	13.58	13.08	19.18	8.45	22.75	5.04	26.24
35.69	7.15	21.45	13.80	12.36	19.67	8.08	23.07	4.78	26.62
33.97	7.88	20.49	14.43	12.17	19.72	7.80	23.33	4.39	27.22
32.34	8.53	19.62	15.01	11.85	20.02	7.49	23.63	4.04	27.81
30.91	9.22	18.78	15.55	11.37	20.36	7.22	23.91	3.78	28.14
29.58	9.84	17.74	16.07	11.13	20.51	6.96	24.15	3.64	28.32
28.36	10.34	17.17	16.41	10.88	20.76	6.71	24.39	3.42	28.66
27.45	10.81	16.64	16.79	10.46	21.05	6.48	24.63	3.18	29.01
26.44	11.15	16.02	17.27	10.23	21.24	6.26	24.87	2.74	29.79
25.54	11.54	15.23	17.67	9.75	21.60	6.03	25.15	2.22	30.87

Table S8.3.7. Experimental binodal curve mass fraction data for the system IL (1) + K_3PO_4 (2) + H_2O (3) at 298.15 K.

[C_2mim]Cl		[C_4mim]Cl			
100 w_1 ± 0.01	**100 w_2 ± 0.01**	**100 w_1 ± 0.01**	**100 w_2 ± 0.01**	**100 w_1 ± 0.01**	**100 w_2 ± 0.01**
5.60	30.04	2.91	32.16	14.34	18.40
6.54	28.65	3.80	30.52	14.94	17.86
7.37	27.50	4.51	29.35	15.41	17.49
8.23	26.41	4.98	28.61	16.06	16.87
9.16	25.24	5.31	28.11	16.65	16.39
10.06	24.24	5.64	27.67	17.26	15.90
10.78	23.44	5.99	27.22	17.90	15.37
11.82	22.34	6.33	26.81	18.63	14.79
12.69	21.43	6.74	26.32	19.30	14.30
13.55	20.57	7.15	25.84	20.13	13.63
14.58	19.56	7.65	25.27	20.95	13.01
15.76	18.40	8.02	24.89	21.69	12.52
17.05	17.15	8.53	24.41	22.51	11.95
18.42	15.88	9.11	23.80	23.52	11.18
20.02	14.42	9.79	23.11	24.37	10.66
21.55	13.05	10.43	22.56	25.29	10.05
23.37	11.51	11.08	22.02	27.45	8.73
25.03	10.29	12.30	20.15	30.18	7.54
26.80	8.79	12.68	19.83	32.27	6.53
31.37	7.14	13.02	19.56	33.88	5.68
34.75	6.37	13.53	19.05	37.15	4.57
38.73	4.18	13.93	18.71	39.32	3.48

Table S8.3.8 Experimental binodal curve mass fraction data for the system $[C_2mim][CF_3SO_3]$ (1) + K_3PO_4 (2) + H_2O (3) at 298.15 K.

$[C_2mim][CF_3SO_3]$					
100 w_1 ± 0.01	**100 w_2 ± 0.01**	**100 w_1 ± 0.01**	**100 w_2 ± 0.01**	**100 w_1 ± 0.01**	**100 w_2 ± 0.01**
0.64	32.89	6.06	19.65	15.68	12.85
0.71	32.19	6.30	19.21	16.34	12.60
0.76	31.84	6.60	18.86	16.96	12.29
0.82	31.22	6.88	18.35	17.73	12.02
0.87	30.71	7.34	18.16	18.56	11.69
0.91	30.43	7.78	17.75	19.56	11.40
1.37	29.67	8.26	17.28	20.64	11.05
1.59	28.73	8.80	16.76	27.65	8.82
1.82	27.85	9.29	16.36	29.87	8.06
1.99	27.36	9.75	15.77	32.63	7.30
2.17	26.65	10.24	15.53	36.12	6.41
2.43	25.95	10.75	15.22	39.54	5.38
2.63	25.42	11.20	15.05	45.77	3.91
2.82	24.93	11.43	14.81		
3.03	24.42	11.78	14.70		
3.23	24.02	12.07	14.49		
3.54	23.39	12.43	14.34		
3.79	22.84	12.84	14.21		
4.25	21.95	13.60	13.75		
4.53	21.35	13.98	13.47		
5.08	20.90	14.51	13.30		
5.49	20.20	15.07	13.09		

Table S8.3.9. Experimental binodal curve mass fraction data for the system $[C_2mim][MeSO_4]$ (1) + K_3PO_4 (2) + H_2O (3) at 298.15 K.

$[C_2mim][MeSO_4]$			
100 w_1 ± 0.01	**100 w_2 ± 0.01**	**100 w_1 ± 0.01**	**100 w_2 ± 0.01**
3.08	30.38	14.06	20.79
4.89	29.30	14.63	20.37
5.04	29.30	14.95	20.13
5.74	28.39	15.30	19.93
6.25	27.77	15.98	19.43
6.72	27.15	16.32	19.16
7.17	26.71	14.75	18.95
7.67	26.15	17.14	18.64
8.00	25.82	17.56	18.35
8.35	25.42	18.08	18.05
8.74	25.05	18.60	17.77
9.20	24.72	19.06	17.40
9.69	24.33	19.60	17.05
10.21	23.74	20.12	16.63
10.67	23.36	20.75	16.24
11.18	22.98	21.45	15.85
11.53	22.65	22.08	15.37
11.92	22.35	22.84	14.92
12.35	22.11	23.64	14.47
12.78	21.75	44.34	5.63
13.24	21.37		
13.76	20.96		

Table S8.3.10. Experimental binodal curve mass fraction data for the system $[C_2mim][EtSO_4]$ (1) + K_3PO_4 (2) + H_2O (3) at 298.15 K.

$[C_2mim][EtSO_4]$					
100 w_1 ± 0.01	**100 w_2 ± 0.01**	**100 w_1 ± 0.01**	**100 w_2 ± 0.01**	**100 w_1 ± 0.01**	**100 w_2 ± 0.01**
2.17	32.59	8.43	23.59	15.75	18.37
2.52	31.65	8.66	23.41	16.66	17.83
2.77	31.17	8.92	23.25	17.51	17.06
3.05	30.53	9.15	23.02	18.19	16.87
3.28	30.11	9.41	22.78	19.23	16.02
3.58	29.51	9.57	22.70	16.66	17.83
4.16	28.67	9.74	22.61	17.51	17.06
4.57	27.97	10.02	22.22	18.19	16.87
4.98	27.53	10.19	22.12	19.23	16.02
5.41	26.82	10.37	22.04	19.85	15.67
5.79	26.46	10.89	21.63	20.56	15.24
6.02	26.05	11.26	21.30	21.24	14.79
6.32	25.79	11.56	21.32	22.03	14.32
6.65	25.46	12.16	20.62	22.90	13.83
6.94	25.23	12.46	20.53	23.75	13.26
7.21	24.83	12.93	20.10	24.96	12.69
7.47	24.64	13.22	19.93	26.13	12.06
7.73	24.27	13.55	19.75	27.51	11.46
7.92	24.07	13.87	19.55	28.86	10.67
8.11	23.88	14.22	19.37		
8.22	23.81	14.95	18.86		

Table S8.3.11. Experimental binodal curve mass fraction data for the system $[C_2mim]Br$ (1) + K_3PO_4 (2) + H_2O (3) at 298.15 K.

$[C_2mim]Br$					
100 w_1 ± 0.01	100 w_2 ± 0.01	100 w_1 ± 0.01	100 w_2 ± 0.01	100 w_1 ± 0.01	100 w_2 ± 0.01
3.54	31.89	9.95	23.60	16.30	18.73
3.97	31.09	10.25	23.40	16.65	18.33
4.46	30.22	10.55	23.12	17.13	18.10
4.81	29.63	10.69	22.97	17.57	17.81
5.23	28.99	10.87	22.87	18.04	17.44
5.61	28.45	11.01	22.67	18.57	17.12
5.79	28.18	11.20	22.55	19.16	16.81
6.06	27.78	11.54	22.19	19.76	16.45
6.27	27.54	11.74	22.04	20.27	15.90
6.48	27.23	11.96	21.92	20.97	15.43
6.74	27.01	12.17	21.76	21.73	14.99
6.99	26.71	12.38	21.53	22.51	14.48
7.20	26.48	12.66	21.42	23.31	13.90
7.50	26.18	13.10	20.99	24.30	13.45
7.81	25.80	13.34	20.77	25.39	12.84
7.99	25.61	13.60	20.58	26.53	12.12
8.27	25.35	13.90	20.40	27.53	11.32
8.55	25.01	14.16	20.16	28.86	10.62
8.86	24.69	14.52	20.02	30.49	9.86
9.08	24.45	14.83	19.82	32.43	8.91
9.20	24.35	15.14	19.52	34.57	7.92
9.44	24.13	15.49	19.27	38.25	7.01
9.83	23.77	15.82	18.96	45.59	3.82

Table S8.3.12. Experimental binodal curve mass fraction data for the system IL (1) + K_3PO_4 (2) + H_2O (3) at 298.15 K.

$[C_2mim][CH_3SO_3]$		$[C_2mim][CH_3COO]$	
100 w_1 ± 0.01	**100 w_2 ± 0.01**	**100 w_1 ± 0.01**	**100 w_2 ± 0.01**
3.86	38.05	6.42	30.52
4.25	37.58	7.74	28.76
4.92	36.15	7.80	28.74
5.70	34.73	8.35	28.06
6.21	34.12	8.78	27.47
6.98	33.16	9.01	27.28
8.38	30.90	9.73	26.43
18.69	22.74	9.75	26.37
29.15	18.25	10.63	25.42
32.06	18.14	10.94	24.97
		11.42	24.51
		12.06	23.79
		12.14	23.75
		13.45	22.23
		15.02	20.52
		16.77	18.65
		18.46	16.96
		20.38	15.10
		22.06	13.61
		24.16	11.72
		26.44	9.74

Table S8.3.13. Experimental binodal curve mass fraction data for the system $[C_4mim][N(CN)_2]$ (1) + K_3PO_4 (2) at 298.15 K and atmospheric pressure at pH 7.0.

$[C_4mim][N(CN)_2]$											
100 w_1 ± 0.01	100 w_2 ± 0.01	100 w_1 ± 0.01	100 w_2 ± 0.01	100 w_1 ± 0.01	100 w_2 ± 0.01	100 w_1 ± 0.01	100 w_2 ± 0.01	100 w_1 ± 0.01	100 w_2 ± 0.01	100 w_1 ± 0.01	100 w_2 ± 0.01
47.15	2.13	11.81	11.71	7.25	14.40	4.90	16.98	3.25	19.44	1.76	23.36
42.28	2.85	11.53	11.75	7.18	14.46	4.83	17.02	3.21	19.50	1.72	23.49
38.09	3.44	11.36	11.85	7.12	14.54	4.76	17.07	3.17	19.58	1.66	23.68
35.26	4.36	11.20	11.99	7.02	14.72	4.71	17.11	3.14	19.65	1.58	23.92
32.31	4.81	10.98	12.05	6.93	14.70	4.66	17.22	3.10	19.71	1.07	24.55
30.29	5.50	10.82	12.18	6.87	14.76	4.59	17.28	3.06	19.79	1.04	24.78
28.63	5.99	10.64	12.18	6.81	14.82	4.55	17.37	3.03	19.86	1.02	24.92
27.07	6.33	10.50	12.31	6.76	14.85	4.49	17.43	3.00	19.93	1.00	25.09
25.70	6.75	10.37	12.40	6.70	14.90	4.45	17.55	2.96	20.03	0.99	25.22
24.52	7.06	10.21	12.42	6.65	14.97	4.40	17.60	2.92	20.12	0.97	25.38
23.44	7.36	10.10	12.50	6.59	15.01	4.35	17.67	2.87	20.24	0.95	25.58
22.43	7.61	9.98	12.61	6.54	15.05	4.30	17.71	2.82	20.32	0.93	25.76
21.52	7.90	9.81	12.62	6.49	15.10	4.26	17.79	2.78	20.42	0.91	25.90
20.92	8.21	9.64	12.85	6.43	15.13	4.21	17.91	2.74	20.52	0.88	26.11
20.12	8.44	9.48	12.92	6.39	15.22	4.15	17.92	2.70	20.61	0.86	26.32
19.47	8.56	9.32	12.95	6.32	15.23	4.10	17.96	2.66	20.71	0.84	26.47
19.00	8.83	9.21	13.04	6.27	15.26	4.03	18.12	2.62	20.84	0.83	26.62
18.31	9.03	9.10	13.12	6.22	15.30	3.98	18.18	2.58	20.98	0.81	26.81
17.91	9.23	8.99	13.23	6.18	15.33	3.95	18.27	2.54	21.10	0.79	26.97
17.34	9.36	8.85	13.24	6.11	15.46	3.90	18.30	2.50	21.15	0.75	27.24
16.96	9.61	8.75	13.32	6.02	15.55	3.86	18.35	2.46	21.27	0.73	27.53
16.44	9.74	8.65	13.42	5.96	15.67	3.83	18.42	2.41	21.34	0.71	27.75
15.98	9.88	8.45	13.61	5.88	15.74	3.79	18.46	2.37	21.43	0.69	27.93
15.67	10.07	8.36	13.70	5.78	15.83	3.76	18.51	2.31	21.67	0.66	28.31
15.24	10.16	8.22	13.71	5.69	15.92	3.73	18.59	2.26	21.81	0.52	28.49
14.80	10.28	8.13	13.80	5.63	16.07	3.69	18.63	2.20	21.95	0.51	28.58
14.54	10.46	8.06	13.86	5.54	16.15	3.66	18.69	2.16	22.09	0.50	28.76
14.18	10.54	7.98	13.94	5.46	16.22	3.62	18.73	2.12	22.18	0.49	29.00

13.82	10.64	7.87	13.96	5.40	16.27	3.59	18.80	2.07	22.30	0.47	29.37
13.57	10.81	7.79	14.02	5.34	16.37	3.54	18.89	2.03	22.43	0.44	29.80
13.25	10.92	7.71	14.09	5.27	16.43	3.50	18.97	1.99	22.58	0.42	30.20
13.03	11.08	7.64	14.15	5.21	16.55	3.45	19.04	1.95	22.72		
12.71	11.14	7.52	14.16	5.14	16.62	3.41	19.13	1.91	22.80		
12.50	11.30	7.45	14.22	5.07	16.70	3.37	19.22	1.88	22.96		
12.30	11.46	7.39	14.29	5.02	16.79	3.33	19.30	1.84	23.07		
12.02	11.53	7.32	14.35	4.96	16.91	3.29	19.36	1.80	23.20		

Table S8.3.14. Experimental binodal curve mass fraction data for the system $[C_4mim][CF_3SO_3]$ (1) + K_3PO_4 (2) + H_2O (3) at 298.15 K.

$[C_4mim][CF_3SO_3]$					
100 w_1 ± 0.01	100 w_2 ± 0.01	100 w_1 ± 0.01	100 w_2 ± 0.01	100 w_1 ± 0.01	100 w_2 ± 0.01
47.97	1.92	11.30	8.07	7.71	10.93
32.60	4.05	10.94	8.32	7.35	11.16
26.88	4.48	10.55	8.50	7.08	11.43
22.88	4.85	10.21	8.72	6.83	11.70
20.30	5.28	9.92	8.95	6.64	11.98
18.37	5.57	9.61	9.12	6.41	12.20
17.08	5.94	9.39	9.34	6.24	12.45
15.87	6.21	9.22	9.57	6.06	12.68
14.81	6.48	8.94	9.68	5.89	12.90
14.09	6.78	8.74	9.87	5.69	13.03
13.38	7.06	8.52	10.02	5.53	13.39
12.69	7.28	8.33	10.18	5.28	13.89
12.17	7.53	8.16	10.36	5.09	14.10
11.78	7.88	7.95	10.47	4.92	14.30
				4.77	14.55

Table S8.3.15. Experimental binodal curve mass fraction data for the system $[C_4mim]Br$ (1) + K_3PO_4 (2) + H_2O (3) at 298.15 K.

$[C_4mim]Br$									
100 w_1 ± 0.01	100 w_2 ± 0.01	100 w_1 ± 0.01	100 w_2 ± 0.01	100 w_1 ± 0.01	100 w_2 ± 0.01	100 w_1 ± 0.01	100 w_2 ± 0.01	100 w_1 ± 0.01	100 w_2 ± 0.01
1.54	33.63	3.69	28.63	6.17	24.49	10.48	20.44	18.73	14.44
1.72	33.05	3.86	28.34	6.38	24.24	10.96	19.94	19.24	14.05
1.88	32.55	3.95	28.19	6.56	24.07	11.28	19.59	19.85	13.71
2.07	32.01	4.03	28.07	6.80	23.77	11.69	19.31	20.48	13.31
2.20	31.68	4.09	27.96	6.98	23.54	12.13	19.00	21.07	12.90
2.28	31.47	4.23	27.78	7.20	23.33	12.59	18.65	21.49	12.86
2.37	31.24	4.30	27.69	7.34	23.18	13.09	18.29	23.03	11.87
2.46	31.03	4.40	27.54	7.58	22.94	13.55	17.79	23.80	11.33
2.58	30.76	4.62	27.08	7.83	22.67	13.82	17.63	24.66	10.78
2.77	30.36	4.80	26.84	8.20	22.31	14.16	17.51	26.01	10.34
2.94	29.93	4.92	26.68	8.38	22.05	14.47	17.30	27.05	9.84
3.05	29.81	5.08	26.48	8.63	21.94	14.82	17.09	28.31	9.12
3.16	29.57	5.32	26.15	8.83	21.71	15.16	16.85	30.08	8.46
3.24	29.41	5.43	26.00	9.05	21.45	15.87	16.43	31.56	7.58
3.31	29.30	5.55	25.87	9.18	21.38	16.79	15.69	33.64	6.69
3.39	29.15	5.61	25.25	9.55	21.02	17.22	15.40	35.87	5.77
3.48	28.97	5.84	24.92	9.83	20.88	17.61	15.00	41.91	5.02
3.58	28.81	6.02	24.68	10.10	20.55	18.24	14.80	53.04	2.21

Table S8.3.16. Experimental binodal curve mass fraction data for the system $[C_4mim][CF_3COO]$ (1) + K_3PO_4 (2) + H_2O (3) at 298.15 K.

$[C_4mim][CF_3COO]$							
100 w_1 ± 0.01	100 w_2 ± 0.01	100 w_1 ± 0.01	100 w_2 ± 0.01	100 w_1 ± 0.01	100 w_2 ± 0.01	100 w_1 ± 0.01	100 w_2 ± 0.01
0.40	34.33	6.04	21.26	11.39	17.34	19.29	13.08
0.47	33.71	6.23	21.00	11.73	17.00	19.83	12.83
0.63	32.86	6.48	20.87	12.06	16.69	20.44	12.53
0.81	31.29	6.75	20.66	12.28	16.57	21.11	12.19
1.93	28.01	6.94	20.48	12.51	16.45	21.77	11.82
2.30	27.06	7.14	20.31	12.76	16.31	22.43	11.57
2.60	26.42	7.35	20.12	13.04	16.16	23.19	11.24
3.00	25.60	7.55	19.94	13.32	16.06	23.93	10.83
3.23	25.07	7.79	19.73	13.69	15.76	24.60	10.32
3.41	24.70	8.06	19.63	14.01	15.55	25.36	9.93
3.62	24.48	8.32	19.39	14.48	15.52	26.24	9.57
3.84	24.08	8.62	19.11	15.01	15.01	27.24	9.12
4.12	23.83	8.96	18.75	15.36	14.85	28.38	8.60
4.41	23.30	9.22	18.65	15.70	14.68	29.56	8.12
4.64	22.94	9.42	18.44	16.07	14.52	30.79	7.58
4.88	22.85	9.72	18.30	16.44	14.35	32.43	6.94
5.25	22.28	10.02	18.17	16.84	14.19	33.86	6.28
5.52	21.83	10.28	17.90	17.27	14.03	35.55	5.42
5.69	21.72	10.63	17.77	17.78	13.76	40.97	4.63
5.86	21.48	11.00	17.54	18.25	13.54	44.51	3.98

Table S8.3.17. Experimental binodal curve mass fraction data for the system IL (1) + K_3PO_4 (2) + H_2O (3) at 298.15 K.

$[C_4mim][CH_3COO]$		$[C_4mim][CH_3SO_3]$			
100 w_1 ± 0.01	**100 w_2 ± 0.01**	**100 w_1 ± 0.01**	**100 w_2 ± 0.01**	**100 w_1 ± 0.01**	**100 w_2 ± 0.01**
6.42	30.52	2.50	34.38	14.24	21.26
7.74	28.76	3.43	32.63	14.98	20.55
7.80	28.74	4.07	31.60	15.91	19.89
8.35	28.06	4.53	30.87	16.66	19.36
8.78	27.47	4.85	30.61	17.46	18.66
9.01	27.28	5.40	29.87	18.51	17.78
9.73	26.43	6.11	28.86	19.54	17.08
9.75	26.37	6.72	28.13	20.23	16.50
10.63	25.42	7.45	27.35	21.44	15.70
10.94	24.97	7.93	26.85	22.17	15.10
11.42	24.52	8.34	26.41	23.72	14.09
12.06	23.79	8.79	25.94	24.19	13.79
12.14	23.75	9.28	25.50	25.80	12.67
13.45	22.23	9.81	24.95	27.28	11.76
15.02	20.52	10.27	24.59	27.99	11.26
16.77	18.65	10.78	24.16	29.69	10.31
18.46	16.96	11.32	23.72	31.84	9.09
20.38	15.10	11.79	23.28	34.33	7.76
22.06	13.61	12.36	22.86	37.06	6.35
24.16	11.72	12.89	22.36	40.19	4.95
26.44	9.74	13.53	21.84	46.00	2.59

Table S8.3.18. Experimental binodal curve mass fraction data for the system $[C_4mim][HSO_4]$ (1) + K_3PO_4 (2) + H_2O (3) at 298.15 K.

$[C_4mim][HSO_4]$			
100 w_1 ± 0.01	100 w_2 ± 0.01	100 w_1 ± 0.01	100 w_2 ± 0.01
4.09	24.01	22.74	5.57
8.87	15.74	25.74	4.79
11.28	12.68	35.88	2.60
16.72	8.00	48.25	1.32
18.34	6.56	59.38	0.55

Table S8.3.19. Experimental binodal curve mass fraction data for the system $[C_2mim]Cl$ (1) + K_2HPO_4 (2) at 298.15 K and atmospheric pressure.

$[C_2mim]Cl$									
100 w_1 ± 0.01	100 w_2 ± 0.01	100 w_1 ± 0.01	100 w_2 ± 0.01	100 w_1 ± 0.01	100 w_2 ± 0.01	100 w_1 ± 0.01	100 w_2 ± 0.01	100 w_1 ± 0.01	100 w_2 ± 0.01
36.90	6.44	19.85	18.03	13.49	24.81	9.91	29.05	7.34	32.52
33.42	8.24	18.84	19.30	13.01	25.49	9.64	29.45	7.17	32.74
30.40	9.74	18.23	19.72	12.70	25.71	9.37	29.82	6.82	33.41
28.68	10.95	17.43	20.68	12.23	26.30	9.09	30.14	6.67	33.59
27.30	12.11	16.89	21.05	11.81	26.88	8.85	30.50	6.40	33.99
25.51	12.81	16.09	22.05	11.55	27.02	8.61	30.85	6.05	34.62
23.67	14.65	15.39	22.93	11.16	27.53	8.35	31.07	5.78	35.07
22.71	15.39	14.73	23.67	10.81	27.99	8.07	31.57	5.47	35.46
21.79	16.10	14.36	23.92	10.48	28.37	7.87	31.80	5.01	36.37
20.61	17.51	13.81	24.62	10.17	28.74	7.51	32.29		

Table S8.3.20. Experimental binodal curve mass fraction data for the system [C_4mim]Cl (1) + K_2HPO_4 (2) at 298.15 K and atmospheric pressure.

[C_4mim]Cl									
100 w_1 ± 0.01	100 w_2 ± 0.01	100 w_1 ± 0.01	100 w_2 ± 0.01	100 w_1 ± 0.01	100 w_2 ± 0.01	100 w_1 ± 0.01	100 w_2 ± 0.01	100 w_1 ± 0.01	100 w_2 ± 0.01
34.97	7.34	13.96	21.80	8.35	28.08	5.61	31.60	3.11	36.17
31.75	8.61	13.56	22.18	8.20	28.19	5.51	31.77	3.01	36.44
28.85	9.86	13.00	23.05	8.05	28.30	5.30	32.19	2.89	36.66
27.28	11.06	12.58	23.18	7.83	28.73	5.16	32.35	2.82	36.90
25.32	11.75	12.28	23.46	7.68	28.76	5.00	32.59	2.68	37.45
24.18	12.61	11.83	24.14	7.55	28.87	4.80	32.95	2.61	37.53
23.00	13.52	11.54	24.38	7.42	28.96	4.66	33.13	2.51	37.84
22.08	14.13	11.29	24.61	7.30	29.07	4.50	33.47	2.45	37.94
20.83	15.56	11.03	24.85	7.12	29.44	4.39	33.59	2.37	38.22
20.08	16.07	10.67	25.38	6.97	29.65	4.25	33.83	2.30	38.44
19.43	16.62	10.46	25.49	6.88	29.71	4.13	34.09	2.22	38.70
18.39	17.72	10.23	25.68	6.73	30.00	4.00	34.30	2.14	38.86
17.87	18.20	10.06	25.94	6.63	30.05	3.89	34.58	2.08	39.00
17.30	18.61	9.76	26.42	6.49	30.28	3.80	34.77	2.00	39.24
16.74	19.03	9.57	26.55	6.34	30.58	3.64	35.12	1.94	39.46
16.00	19.90	9.38	26.69	6.25	30.62	3.55	35.25	1.87	39.66
15.49	20.31	9.09	27.20	6.12	30.91	3.46	35.46	1,.823	39.87
15.02	20.69	8.91	27.34	5.99	31.16	3.35	35.66	1.77	40.00
14.66	21.04	8.73	27.48	5.82	31.24	3.28	35.80	1.71	40.20
14.27	21.38	8.58	27.66	5.73	31.37	3.21	35.95	1.66	40.44

Table S8.3.21. Experimental binodal curve mass fraction data for the system $[C_6mim]Cl$ (1) + K_2HPO_4 (2) at 298.15 K and atmospheric pressure.

$[C_6mim]Cl$									
100 w_1 ± 0.01	100 w_2 ± 0.01	100 w_1 ± 0.01	100 w_2 ± 0.01	100 w_1 ± 0.01	100 w_2 ± 0.01	100 w_1 ± 0.01	100 w_2 ± 0.01	100 w_1 ± 0.01	100 w_2 ± 0.01
34.59	7.84	12.43	22.53	7.61	27.66	5.40	30.26	4.02	32.63
31.33	9.54	12.18	22.77	7.51	27.74	5.35	30.28	3.99	32.65
29.55	10.55	11.95	23.00	7.42	27.83	5.28	30.42	3.91	32.80
27.22	11.57	11.70	23.25	7.33	27.89	5.19	30.67	3.86	32.89
25.77	12.70	11.47	23.53	7.26	28.04	5.14	30.69	3.82	33.05
24.34	13.46	11.25	23.72	7.17	28.11	5.09	30.73	3.79	33.06
23.30	14.29	11.05	23.88	7.08	28.18	5.02	30.91	3.74	33.17
21.81	14.97	10.86	24.07	6.94	28.51	4.97	30.96	3.72	33.21
20.97	15.61	10.65	24.31	6.86	28.56	4.90	31.14	3.67	33.32
20.20	16.25	10.45	24.52	6785.00	28.66	4.85	31.17	3.64	33.34
19.44	16.90	10.25	24.64	6707.00	28.74	4.81	31.20	3.62	33.40
18.76	17.46	10.08	24.84	6.62	28.80	4.77	31.26	3.57	33.49
18.17	17.94	9.91	25.01	6.42	29.16	4.70	31.39	3.53	33.59
17.55	18.29	9.73	25.21	6.34	29.18	4.64	31.54	3.51	33.59
16.90	18.60	9.56	25.35	6.24	29.42	4.60	31.56	3.45	33.80
16.45	19.01	9.41	25.50	6.16	29.52	4.54	31.73	3.40	33.81
16.09	19.50	9.29	25.71	6.09	29.62	4.47	31.80	3.37	33.79
15.71	19.89	9.15	25.87	6.02	29.61	4.44	31.84	3.31	34.24
15.31	20.21	9.01	26.00	5.96	29.66	4.40	31.88	3.25	34.41
14.93	20.49	8.78	26.40	5.91	29.75	4.35	32.05	3.22	34.52
14.57	20.78	8.66	26.53	5.86	29.83	4.29	32.19	3.19	34.51
14.24	21.08	8.53	26.62	5.77	30.03	4.25	32.18	3.16	34.60
13.91	21.35	8.41	26.71	5.72	30.06	4.20	32.32	3.06	34.89
13.52	21.48	8.30	26.82	5.62	30.29	4.16	32.34	2.96	34.97
13.23	21.77	8.19	26.94	5.52	30.00	4.11	32.48	2.90	35.16
12.96	22.05	7.97	27.19	5.44	30.18	4.07	32.51	2.85	35.31
12.70	22.31	7.80	27.48						

Table S8.3.22. Experimental binodal mass fraction data for the system [1-C_2-3-C_1im]Cl (1) + K_2HPO_4/KH_2PO_4 (2) at 298 K.

[1-C_2-3-C_1im]Cl			
100 w_1 ± 0.01	100 w_2 ± 0.01	100 w_1 ± 0.01	100 w_2 ± 0.01
57.77	1.08	38.73	5.68
53.76	1.73	36.48	6.40
50.34	2.98	33.78	7.75
45.31	4.08	32.52	8.25
40.84	4.82		

Table S8.3.23. Experimental binodal mass fraction data for the system [1-C_6-3-C_1im]Cl (1) + K_2HPO_4/KH_2PO_4 (2) at 298 K.

[1-C_6-3-C_1im]Cl					
100 w_1 ± 0.01	100 w_2 ± 0.01	100 w_1 ± 0.01	100 w_2 ± 0.01	100 w_1 ± 0.01	100 w_2 ± 0.01
59.42	0.87	35.98	7.26	23.57	14.40
54.62	1.89	35.11	7.55	22.34	15.40
51.06	3.22	33.83	8.24	21.34	16.18
47.43	3.58	32.54	8.97	20.53	16.81
45.19	4.02	31.79	9.25	19.73	17.40
43.76	4.51	30.63	9.86	18.83	18.12
42.43	5.00	29.60	10.50	18.09	18.76
40.88	5.38	28.64	11.04	17.05	19.69
39.69	5.81	27.29	12.02	16.15	20.57
38.84	5.94	25.25	13.30	15.21	21.59
37.34	6.80	24.45	13.78	14.40	22.36

Table S8.3.24. Experimental binodal mass fraction data for the system [1-C_7-3-C_1im]Cl (1) + K_2HPO_4/KH_2PO_4 (2) at 298 K.

[1-C_7-3-C_1im]Cl			
100 w_1 ± 0.01	**100 w_2 ± 0.01**	**100 w_1 ± 0.01**	**100 w_2 ± 0.01**
56.53	3.19	41.15	7.71
53.31	4.12	39.75	8.34
50.72	4.56	38.02	9.13
47.90	5.44	36.24	10.11
46.61	5.81	34.42	11.19
45.60	6.08	32.01	12.68
43.94	6.82	29.86	14.14
42.84	7.14	24.65	18.29

Table S8.3.25. Experimental binodal mass fraction data for the system [1-C_8-3-C_1im]Cl (1) + K_2HPO_4/KH_2PO_4 (2) at 298 K.

[1-C_8-3-C_1im]Cl			
100 w_1 ± 0.01	**100 w_2 ± 0.01**	**100 w_1 ± 0.01**	**100 w_2 ± 0.01**
54.78	3.19	26.53	15.23
50.07	4.29	24.40	16.77
45.62	5.71	22.95	17.83
41.32	7.30	21.71	18.73
37.29	8.91	19.53	20.42
35.46	9.91	17.62	22.01
32.89	11.45	15.01	24.35
31.08	12.44	12.10	27.13
28.47	14.07		

Table S8.3.26. Experimental binodal mass fraction data for the system [1-C_7H_7-3-C_1im]Cl (1) + K_2HPO_4/KH_2PO_4 (2) at 298 K.

[1-C_7H_7-3-C_1im]Cl			
100 w_1 ± 0.01	100 w_2 ± 0.01	100 w_1 ± 0.01	100 w_2 ± 0.01
55.86	3.39	34.78	9.80
51.22	4.78	33.25	10.68
44.99	5.55	31.71	11.65
41.87	6.87	29.92	12.85
38.89	7.77	27.92	14.28
36.18	9.03	25.28	16.44

Table S8.3.27. Experimental binodal mass fraction data for the system [1-C_4-3-C_1im]Cl (1) + K_2HPO_4/KH_2PO_4 (2) at 298 K.

[1-C_4-3-C_1im]Cl			
100 w_1 ± 0.01	100 w_2 ± 0.01	100 w_1 ± 0.01	100 w_2 ± 0.01
57.71	1.10	39.98	5.33
54.17	1.82	37.76	6.53
50.68	2.43	35.05	7.86
48.33	3.02	32.50	9.15
46.07	3.75	30.92	10.15
43.20	4.27	27.55	12.93
41.37	4.74		

Table S8.3.28. Experimental binodal mass fraction data for the system $[1\text{-}C_4\text{-}1\text{-}C_1pip]Cl$ (1) + K_2HPO_4/KH_2PO_4 (2) at 298 K.

$[1\text{-}C_4\text{-}1\text{-}C_1pip]Cl$	
100 w_1 ± 0.01	**100 w_2 ± 0.01**
57.23	1.17
50.03	2.21
44.50	3.22
40.23	4.29
34.53	6.58
28.00	10.61
21.27	16.43

Table S8.3.29. Experimental binodal mass fraction data for the system $[1\text{-}C_4\text{-}1\text{-}C_1pyrr]Cl$ (1) + K_2HPO_4/KH_2PO_4 (2) at 298 K.

$[1\text{-}C_4\text{-}1\text{-}C_1pyrr]Cl$	
100 w_1 ± 0.01	**100 w_2 ± 0.01**
53.75	3.41
47.35	4.23
34.67	7.96
23.44	15.43

Table S8.3.30. Experimental binodal mass fraction data for the system [1-C_4-3-C_1pyr]Cl (1) + K_2HPO_4/KH_2PO_4 (2) at 298 K.

[1-C_4-3-C_1pyr]Cl					
100 w_1 ± 0.01	**100 w_2 ± 0.01**	**100 w_1 ± 0.01**	**100 w_2 ± 0.01**	**100 w_1 ± 0.01**	**100 w_2 ± 0.01**
56.59	0.80	28.43	8.14	17.16	16.88
49.22	1.83	26.52	9.26	16.34	17.71
42.57	2.61	24.47	10.60	14.84	19.28
39.39	3.33	22.65	11.92	13.69	20.66
37.00	4.22	20.93	13.29	12.67	21.69
34.80	5.05	20.11	14.15	10.34	24.85
32.48	6.11	19.18	14.86	8.15	27.33
30.42	7.11	18.43	15.53	6.13	30.03

Table S8.3.31. Experimental binodal mass fraction data for the system [1-C_4-2-C_1pyr]Cl (1) + K_2HPO_4/KH_2PO_4 (2) at 298 K.

[1-C_4-2-C_1pyr]Cl					
100 w_1 ± 0.01	**100 w_2 ± 0.01**	**100 w_1 ± 0.01**	**100 w_2 ± 0.01**	**100 w_1 ± 0.01**	**100 w_2 ± 0.01**
58.36	0.68	32.64	6.19	20.50	14.80
51.56	1.67	31.11	7.20	18.85	16.28
44.91	2.52	28.91	8.49	17.62	17.56
41.45	3.24	26.28	10.37	15.24	20.04
38.98	3.95	24.83	11.13	13.71	21.80
36.63	4.76	23.26	12.55	11.64	24.37
34.48	5.52	22.08	13.25	8.93	27.69

Table S8.3.32. Experimental binodal mass fraction data for the system [1-C_4-4-C_1pyr]Cl (1) + K_2HPO_4/KH_2PO_4 (2) at 298 K.

[1-C_4-4-C_1pyr]Cl					
100 w_1 ± 0.01	100 w_2 ± 0.01	100 w_1 ± 0.01	100 w_2 ± 0.01	100 w_1 ± 0.01	100 w_2 ± 0.01
57.84	0.77	31.17	6.82	21.75	12.59
52.99	1.30	29.42	7.61	20.30	14.07
45.61	2.29	27.48	8.71	19.07	14.97
40.70	3.52	25.81	9.64	15.02	19.98
37.81	4.16	24.93	10.24	10.35	24.90
35.78	4.96	24.02	10.89	8.01	27.51
33.02	5.92	22.63	12.21	6.03	30.09

Table S8.3.33. Experimental binodal mass fraction data for the system [C_2mim][CH_3COO] (1) + K_2HPO_4/KH_2PO_4 (2) at 298 K.

[C_2mim][CH_3COO]	
100 w_1 ± 0.01	100 w_2 ± 0.01
42.42	11.85
31.29	17.16
25.64	20.04
13.60	27.39

Table S8.3.34. Experimental binodal mass fraction data for the system [C_2mim][$MeSO_4$] (1) + K_2HPO_4/KH_2PO_4 (2) at 298 K.

[C_2mim][$MeSO_4$]	
100 w_1 ± 0.01	100 w_2 ± 0.01
56.47	2.49
40.93	10.17
27.04	18.95
20.99	24.77

Table S8.3.35. Experimental binodal mass fraction data for the system $[C_2mim][EtSO_4]$ (1) + K_2HPO_4/KH_2PO_4 (2) at 298 K.

$[C_2mim][EtSO_4]$			
100 w_1 ± 0.01	**100 w_2 ± 0.01**	**100 w_1 ± 0.01**	**100 w_2 ± 0.01**
52.80	4.81	24.02	16.39
41.61	7.67	19.29	19.76
36.50	9.48	15.39	23.09
29.81	13.80		

Table S8.3.36. Experimental binodal mass fraction data for the system $[C_4mim][SCN]$ (1) + K_2HPO_4/KH_2PO_4 (2) at 298 K.

$[C_4mim][SCN]$									
100 w_1 ± 0.01	**100 w_2 ± 0.01**	**100 w_1 ± 0.01**	**100 w_2 ± 0.01**	**100 w_1 ± 0.01**	**100 w_2 ± 0.01**	**100 w_1 ± 0.01**	**100 w_2 ± 0.01**	**100 w_1 ± 0.01**	**100 w_2 ± 0.01**
58.67	0.91	30.70	5.92	23.30	8.55	17.81	10.57	14.39	12.14
53.62	1.55	29.90	6.17	22.49	8.90	17.57	10.71	13.81	12.59
48.42	2.04	29.25	6.47	21.93	9.00	17.32	10.84	13.14	12.93
45.43	2.52	28.35	6.67	21.20	9.33	17.09	10.94	12.48	13.33
42.41	3.01	27.78	6.92	20.84	9.48	16.85	11.05	11.76	13.81
40.37	3.38	26.65	7.37	20.15	9.78	16.40	11.26	11.05	14.23
38.07	4.18	25.86	7.51	19.38	10.09	15.93	11.50	10.38	14.84
36.09	4.54	25.37	7.71	18.93	10.14	15.51	11.66	9.23	15.71
34.65	4.80	24.43	8.22	18.65	10.25	15.11	11.86	7.51	17.84
32.88	5.46	23.74	8.35	18.09	10.49	14.73	12.01	6.18	18.63
31.64	5.67								

Table S8.3.37. Experimental binodal mass fraction data for the system $[C_4mim][TOS]$ (1) + K_2HPO_4/KH_2PO_4 (2) at 298 K.

$[C_4mim][TOS]$									
100 w_1 ± 0.01	100 w_2 ± 0.01	100 w_1 ± 0.01	100 w_2 ± 0.01	100 w_1 ± 0.01	100 w_2 ± 0.01	100 w_1 ± 0.01	100 w_2 ± 0.01	100 w_1 ± 0.01	100 w_2 ± 0.01
57.16	1.82	37.51	5.70	27.82	9.11	19.64	12.77	13.27	15.83
55.56	2.15	36.46	6.17	27.16	9.44	19.08	13.00	12.84	16.08
53.15	2.48	35.81	6.30	26.53	9.75	18.56	13.18	12.42	16.33
50.90	2.76	35.21	6.44	26.17	9.79	17.99	13.44	12.18	16.38
49.59	3.08	34.28	6.85	25.65	10.02	17.57	13.59	12.03	16.40
47.56	3.32	33.72	6.96	24.65	10.47	17.31	13.75	11.72	16.63
46.46	3.58	32.89	7.29	24.10	10.73	16.86	13.95	11.34	17.04
45.45	3.81	32.06	7.65	23.39	10.97	16.38	14.17	10.71	17.54
44.37	4.08	31.55	7.75	22.96	11.22	15.67	14.60	10.02	17.72
43.41	4.30	31.09	7.86	22.56	11.39	15.30	14.73	9.61	18.11
41.55	4.76	30.30	8.28	21.91	11.68	15.03	14.85	8.85	18.91
39.81	5.17	29.64	8.59	21.23	11.96	14.58	15.15	8.13	19.31
38.98	5.34	29.06	8.63	20.61	12.27	14.13	15.35	7.51	19.69
38.19	5.56	28.32	8.99	20.05	12.52	13.66	15.62		

Table S8.3.38. Experimental binodal mass fraction data for the system $[C_4mim][CH_3COO]$ (1) + K_2HPO_4/KH_2PO_4 (2) at 298 K.

$[C_4mim][CH_3COO]$			
100 w_1 ± 0.01	100 w_2 ± 0.01	100 w_1 ± 0.01	100 w_2 ± 0.01
56.95	2.12	26.46	11.21
41.00	6.47	21.56	13.45
35.67	8.49	19.65	15.59
28.15	10.42	17.75	16.70

Table S8.2.39. Experimental binodal mass fraction data for the system $[C_4mim][CF_3SO_3]$ (1) + K_2HPO_4/KH_2PO_4 (2) at 298 K.

$[C_4mim][CF_3SO_3]$							
100 w_1 ± 0.01	100 w_2 ± 0.01	100 w_1 ± 0.01	100 w_2 ± 0.01	100 w_1 ± 0.01	100 w_2 ± 0.01	100 w_1 ± 0.01	100 w_2 ± 0.01
57.38	2.03	31.89	5.00	24.28	6.37	20.43	7.44
52.13	2.49	30.70	5.16	23.76	6.52	20.09	7.58
47.88	2.88	29.58	5.35	23.19	6.63	19.86	7.73
44.78	3.22	28.58	5.49	22.70	6.74	19.52	7.81
42.05	3.55	27.66	5.61	22.22	6.88	18.99	8.13
39.81	3.81	26.80	5.75	21.87	7.01	18.39	8.38
37.97	4.08	26.15	5.94	21.61	7.14	17.85	8.57
36.32	4.30	25.54	6.11	21.16	7.24	17.45	8.77
34.91	4.58	24.99	6.27	20.83	7.36	17.17	8.85
33.22	4.79						

Table S8.3.40. Experimental binodal mass fraction data for the system $[C_4mim][EtSO_4]$ (1) + K_2HPO_4/KH_2PO_4 (2) at 298 K.

$[C_4mim][EtSO_4]$							
100 w_1 ± 0.01	100 w_2 ± 0.01	100 w_1 ± 0.01	100 w_2 ± 0.01	100 w_1 ± 0.01	100 w_2 ± 0.01	100 w_1 ± 0.01	100 w_2 ± 0.01
57.16	1.69	23.01	15.47	18.60	18.69	15.59	21.03
43.79	4.12	22.19	16.03	17.88	19.28	14.65	21.78
27.98	12.55	21.40	16.60	17.36	19.65	14.22	22.10
25.85	13.72	20.74	17.05	16.69	20178.00	13.58	22.72
24.55	14.45	20.13	17.50	16.13	20.60	12.82	23.22
23.60	15.14	19.35	18.11				

Table S8.3.41. Experimental binodal mass fraction data for the system $[C_4mim][N(CN)_2]$ (1) + K_2HPO_4/KH_2PO_4 (2) at 298 K.

$[C_4mim][N(CN_2)]$							
100 w_1 ± 0.01	**100 w_2 ± 0.01**	**100 w_1 ± 0.01**	**100 w_2 ± 0.01**	**100 w_1 ± 0.01**	**100 w_2 ± 0.01**	**100 w_1 ± 0.01**	**100 w_2 ± 0.01**
56.89	1.18	32.92	6.55	24.39	10.46	18.09	13.88
54.32	1.59	32.35	6.74	24.04	10.57	17.85	14.04
49.70	2.09	31.79	6.89	23.54	10.88	17.56	14.22
46.96	2.48	31.08	7.33	23.08	11.18	17.27	14.40
45.26	2.92	30.54	7.53	22.24	11.63	17.08	14.47
42.35	3.56	30.01	7.68	21.68	11.85	16.82	14.69
40.73	3.83	29.56	7.89	21.22	12.11	16.56	14.83
39.91	4.13	29.11	8.01	20.50	12.50	16.33	15.03
39.08	4.43	28.46	8.37	20.12	12.76	16.09	15.19
38.19	4.75	27.97	8.56	19.78	12.97	15.80	15.43
37.30	5.02	27.29	8.99	19.56	13.07	15.62	15.51
36.11	5.29	26.89	9.16	19.26	13.24	15.16	15.80
35.32	5.56	26.44	9.36	18.98	13.38	14.81	15.98
34.56	5.83	25.92	9.61	18.72	13.50	14.46	16.19
33.82	6.09	25.08	10.23	18.41	13.68	13.99	16.59

Table S8.3.42. Experimental binodal mass fraction data for the system $[C_4mim][CH_3SO_3]$ (1) + K_2HPO_4/KH_2PO_4 (2) at 298 K.

$[C_4mim][CH_3SO_3]$	
100 w_1 ± 0.01	**100 w_2 ± 0.01**
44.92	10.09
40.54	11.74
34.82	14.30
29.64	17.56

Table S8.3.43. Experimental binodal curve mass fraction data for the system $[C_8pyr][N(CN)_2]$ (1) + K_2HPO_4/KH_2PO_4 (2) at 25 ºC and atmospheric pressure.

$[C_8pyr][N(CN)_2]$									
100 w_1 ± 0.01	100 w_2 ± 0.01	100 w_1 ± 0.01	100 w_2 ± 0.01	100 w_1 ± 0.01	100 w_2 ± 0.01	100 w_1 ± 0.01	100 w_2 ± 0.01	100 w_1 ± 0.01	100 w_2 ± 0.01
57.96	1.50	25.77	4.15	18.17	5.94	14.10	7.14	11.50	8.20
50.43	1.94	24.71	4.71	17.64	6.02	13.89	7.21	11.05	8.44
42.77	2.38	23.42	4.82	17.15	6.14	13.65	7.26	10.45	8.83
38.28	2.98	22.49	4.97	16.74	6.23	13.45	7.33	9.98	9.05
33.71	3.16	21.90	5.01	16.43	6.35	13.23	7.38	9.59	9.32
31.54	3.51	21.25	5.15	16.07	6.50	13.12	7.48	9.12	9.56
29.74	3.58	20.75	5.32	15.65	6.57	12.92	7.56	8.55	10.11
28.55	3.76	20.06	5.40	15.01	6.94	12.59	7.72	8.16	10.28
27.32	4.09	19.40	5.71	14.60	6.98	12.24	7.86	7.53	10.78
		18.69	5.79	14.32	7.05	11.92	8.09	7.24	11.08

Table S8.3.44. Correlation parameters used in Merchuck's equation to describe the binodal data of the different ternary systems.

Ternary System		Merchuck Parameters		
Salt	**Ionic liquid**	**A**	**B**	**C**
PB	$[C_8pyr][N(CN)_2]$	189.1017	-0.9578	8.136×10^{-5}

Table S8.3.45. Mass fraction composition for the TLs and TLLs determination and compositions of the respective top (T) and bottom (B) phases for different ILs (Y) and salt solutions (X). The subscript M represents the mass composition of IL and salt in the initial aqueous two-phase system mixture.

Ternary System		Mass fraction / wt %						
Salt	**Ionic Liquid**	Y_M	X_M	Y_T	X_T	Y_B	X_B	**TLL**
PB	$[C_8pyr][N(CN)_2]$	25.05	29.97	78.01	0.85	0.00	43.74	89.02
		20.19	7.57	45.90	2.18	8.35	10.06	38.36
		15.09	15.00	62.43	1.34	1.71	18.85	63.19

8.4. Ecotoxicological Evaluation

Table S8.4.1. EC_{50} data for different pyrrolidinium- and imidazolium-based ionic liquids.

	Non-aromatic ILs		Aromatic ILs	
IPC-81 leukemia cells	1-butyl-1-methylpyrrolidinium chloride	3550	3-butyl-1-methylimidazolium chloride	626
Vibrio fischeri		7980		519
IPC-81 leukemia cells	1-butyl-1-methylpyrrolidinium cyanocyanamide	3560	1-butyl-1-methylimidazolium cyanocyanamide	292
IPC-81 leukemia cells	1-butyl-1-methylpyrrolidinium bis(trifluoromethylsulfunyl)imide	429	3-butyl-1-methyl-imidazolium bis(trifluoromethylsulfunyl)imide	202
Vibrio fischeri		148		124
IPC-81 leukemia cells	1-hexyl-1-methylpyrrolidinium chloride	174	3-hexyl-1-methyl-imidazolium chloride	134
IPC-81 leukemia cells	1-methyl-1-octylpyrrolidinium chloride	90.6	1-methyl-3-octyl-imidazolium chloride	23.9
IPC-81 leukemia cells	1-hexyl-1-methylpyrrolidinium bis(trifluoromethylsulfunyl)imide	162	3-hexyl-1-methyl-1H-imidazolium bis(trifluoromethylsulfunyl)imide	78.5
Vibrio fischeri		113		50.4
IPC-81 leukemia cells	1-butyl-1-methylpyrrolidinium bromide	1300	3-butyl-1-methyl-1H-imidazolium bromide	585
IPC-81 leukemia cells	1-(2-ethoxyethyl)-1-methylpyrrolidinium bromide	4760	3-(2-ethoxyethyl)-1-methyl-1H-imidazolium bromide	3240

Table S8.4.2. EC_{50} data for different piperidinium- and pyridinium-based ionic liquids.

	Non-aromatic ILs		Aromatic ILs	
IPC-81 leukemia cells	1-butyl-1-methylpiperidinium bromide	4930	1-butyl-3-methylpyridinium bromide	130
	1-(2-ethoxyethyl)-1-methylpiperidinium bromide	5100	1-(2-ethoxyethyl)pyridinium bromide	4060
	1-(3-hydroxypropyl)-1-methylpiperidinium chloride	5810	1-(3-hydroxypropyl)pyridinium chloride	3470
	1-(3-methoxypropyl)-1-methylpiperidinium chloride	5230	1-(3-methoxypropyl)pyridinium chloride	3750
	1-(ethoxymethyl)-1-methylpiperidinium chloride	3330	1-(ethoxymethyl)pyridinium chloride	365
	1-(3-hydroxypropyl)-1-methylpiperidinium chloride	5810	1-(3-hydroxypropyl)pyridinium chloride	3470

The EC_{50} data presented in these two tables was retrieved from the UFT / Merck Ionic Liquids Biological Effects Database available at http://www.il-eco.uft.uni-bremen.de/

Table S8.4.3. EC_{50} values ($mg.L^{-1}$) for two pyrrolidinium.- based ILs obtained from the UFT/Merck Ionic Liquids Biological Effects Database (http://www.il-eco.uft.uni-bremen.de).

Ionic Liquids	**EC_{50} ($mg.L^{-1}$) (lower limit; upper limit)**	
	AChE	IPC-81
1-(2-ethoxy-2-oxoethyl)-1-methylpyrrolidinium bis(trifluromethylsulfunyl)imide	≥ 452	1770 (1380 - 2320)
1-butyl-1-methylpyrrolidinium bis(trifluromethylsulfunyl)imide	56.6 (51.2 - 62.6)	429 (384 - 484)

AChE - *Acetylcholinesterase*, IPC-81 - promyelocytic leukaemia cells. NTf_2 – bis(trifluromethylsulfunyl)imide.