Aqueous Biphasic Systems Composed of Ionic Liquids and Sodium Carbonate as Enhanced Routes for the Extraction of Tetracycline

Carlos F. C. Marques, Teresa Mourão, and Catarina M. S. S. Neves
Departamento de Química, CICECO, Universidade de Aveiro, 3810-193 Aveiro, Portugal

Álvaro S. Lima
Programa de Pós-Graduação em Engenharia de Processos, Universidade Tiradentes, Farolândia, Aracaju-SE, Brazil

Isabel Boal-Palheiros, João A. P. Coutinho, and Mara G. Freire
Departamento de Química, CICECO, Universidade de Aveiro, 3810-193 Aveiro, Portugal

Aqueous biphasic systems (ABS) using ionic liquids (ILs) offer an alternative approach for the extraction, recovery, and purification of biomolecules through their partitioning between two aqueous liquid phases. In this work, the ability of a wide range of ILs to form ABS with aqueous solutions of Na_2CO_3 was evaluated. The ABS formed by IL + water + Na_2CO_3 were determined at 25°C, and the respective solubility curves, tie-lines, and tie-line lengths are reported. The studied ILs share the common chloride anion, allowing the IL cation core, the cation isomerism, the presence of functionalized groups, and alkyl side chain length effects to be evaluated. An increase in the cation side alkyl chain length leads to a higher ability for liquid–liquid demixing whereas different positional isomers and the presence of an allyl group have no major influence in the phase diagrams behavior. Quaternary phosphonium- and ammonium-based fluids are more able to form an ABS when compared with imidazolium-, pyridinium-, pyrrolidinium-, and piperidium-based ILs. Moreover, the presence of an aromatic cation core has no major contribution to the formation of ABS when compared to the respective nonaromatic counterparts. Finally, to appraise on the systems applicability in downstream processing, selected systems were used for the partitioning of tetracyclines (neutral and salt forms) — a class of antibiotics produced by bacteria fermentation. Single-step extraction efficiencies for the IL-rich phase were always higher than 99% and confirm the great potential of ILs to be applied in the biotechnological field. © 2013 American Institute of Chemical Engineers *Biotechnol. Prog.*, 29:645–654, 2013
Keywords: aqueous two-phase system, ionic liquid, sodium carbonate, extraction, tetracycline

Introduction

The development of low-cost, recyclable, and biocompatible aqueous biphasic systems (ABS) capable of offering few stages in the separation and purification of added-value products is in high demand in the biotechnological industry. Some of these systems are formed when two substances, mutually incompatible though both miscible in water, are used. Above a critical concentration of those components, spontaneous phase separation takes place and a macroscopic biphasic system is formed. The extraction and purification of biomolecules using ABS was originally proposed by Albertsson in 1958[1] and, in the last decades, has been extended to the separation of cells, membranes, viruses, proteins, nucleic acids, enzymes, and other added-value biomolecules.[2] A recent overview of the developments in ABS[3] has evidenced that various components besides the conventional polymer/polymer and polymer/salt combinations are also capable of forming ABS, namely ionic liquids (ILs). In fact, IL-based ABS can be formed by the addition of aqueous solutions of inorganic/organic salts, amino acids, carbohydrates, or polymers.[4–13]

ILs emerged as a valuable option in extraction processes, and both hydrophobic and hydrophilic ionic fluids have been used to create IL–water biphasic systems, that is, working either with an IL that immediately forms two phases with water or by the addition of salting-out species when dealing with fluids miscible with water.[14] ILs are salts with melting temperatures below 100°C, negligible volatility and flammability, high thermal and chemical stabilities, high ionic conductivity, and also a strong solubilization capability for a large variety of organic and inorganic compounds.[15–17] These characteristics, together with the possibility of adjusting many of their physicochemical properties, bestow ILs a
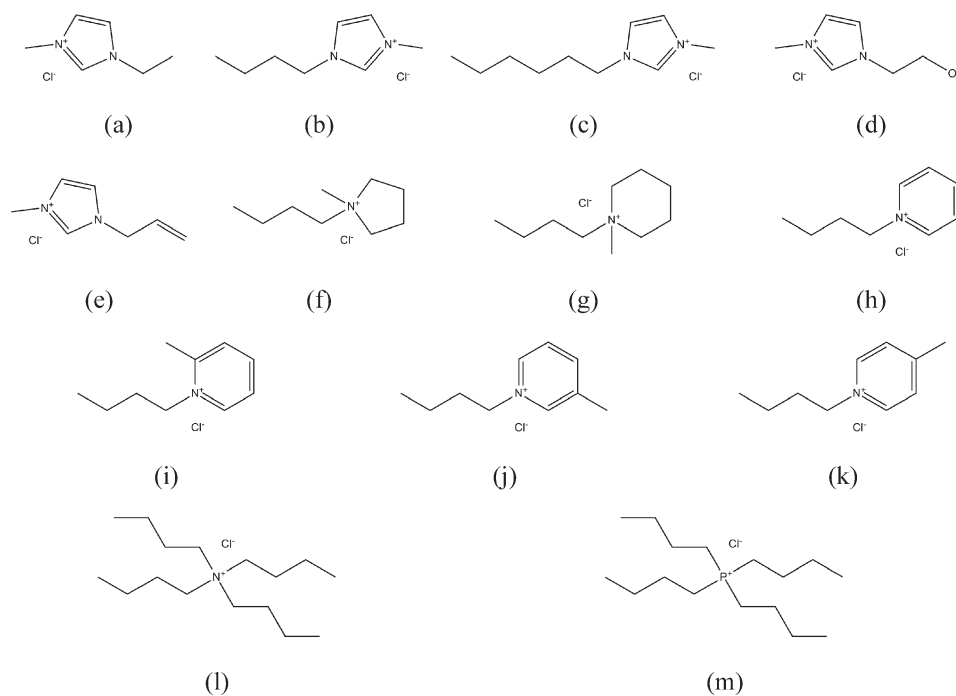
Figure 1. **Chemical structures of the studied ILs: (a) [C₂mim]Cl; (b) [C₄mim]Cl; (c) [C₆mim]Cl; (d) [OHC₂mim]Cl; (e) [amim]Cl; (f) [C₄mpyr]Cl; (g) [C₄mpip]Cl; (h) [C₄py]Cl; (i) *o*-[C₄mpy]Cl; (j) *m*-[C₄mpy]Cl; (k) *p*-[C₄mpy]Cl; (l) [N₄₄₄₄]Cl; and (m) [P₄₄₄₄]Cl.**

broad range of potential applications, and in particular make them promising extractive fluids.[18,19]

Besides the direct use of hydrophobic ILs as a second liquid phase, the application of IL-based ABS represents a cheaper, more benign, and efficient approach for extractive purposes.[20–23] IL-based ABS were already used in product recovery and in the separation/extraction of different substances, such as alkaloids,[21,24] antioxidant compounds,[25,26] amino acids,[9,23,27] proteins,[28] enzymes,[29,30] endocrine disruptors,[31] and drugs.[32–34]

In biotechnology, to effectively use IL-based ABS, beforehand experimental work is required to optimize the system parameters so as to ensure a selective extraction of the products of biotechnological interest. Among added-value metabolites are pharmaceutical drugs, namely antibiotics, and of which tetracyclines (TC) comprise a successful class.[35] TC are a family of polyketide antibiotics produced by *Streptomyces* genus of actinobacteria.[36] TC was first isolated in 1948[37] and differed from the existing antibiotics due to the broad spectrum for antimicrobial activity and low toxicity. TC are produced by fermentation and further require separation and purification steps. Diverse separation methods such as liquid–liquid extraction (either by organic solvents or by polymer-based ABS), ion-exchange, reverse micellar extraction, chromatography, crystallization, or the combination of some of these methods have been used.[38] Given all the benefits of IL-based ABS and their applicability in biotechnological routes, namely in downstream processing, this work aims at characterizing novel ABS formed by a vast number of chloride-based ILs and Na₂CO₃ as novel extractive systems. Sodium carbonate was chosen due its effectiveness in promoting ABS and based on its domestically well known use and applications as food additive; besides, carbonate is a common species already present in the broth, as calcium carbonate is usually added to the fermentative media as it proved to enhance TC production.[39] To study the influence

of the cation structure of the IL in the formation of ABS, the ternary phase diagrams were determined at 25°C for 13 chloride-based ILs. From the gathered phase diagrams, the most representative systems were selected and screened through their efficiencies in the partitioning of TC and TC.HCl.

Materials and Methods

Materials

The ILs used in this work were: 1-ethyl-3-methylimidazolium chloride, [C₂mim]Cl; 1-butyl-3-methylimidazolium chloride, [C₄mim]Cl; 1-hexyl-3-methylimidazolium chloride, [C₆mim]Cl; 1-(2-hydroxyethyl)−3-methylimidazolium chloride, [OHC₂mim]Cl; 1-allyl-3-methylimidazolium chloride, [amim]Cl; 1-butyl-1-methylpyrrolidinium chloride, [C₄mpyr]Cl; 1-butyl-1-methylpiperidinium chloride, [C₄mpip]Cl; 1-butylpyridinium chloride, [C₄py]Cl; 1-butyl-2-methylpyridinium chloride, *o*-[C₄mpy]Cl; 1-butyl-3-methylpyridinium chloride, *m*-[C₄mpy]Cl; 1-butyl-4-methylpyridinium chloride, *p*-[C₄mpy]Cl; tetrabutylammonium chloride, [N₄₄₄₄]Cl, and tetrabutylphosphonium chloride, [P₄₄₄₄]Cl. All the ILs' structures are presented in Figure 1.

The imidazolium-, pyridinium-, pyrrolidinium-, and piperidinium-based ILs were purchased from Iolitec (Germany). The tetrabutylphosphonium chloride was kindly supplied by Cytec Industries Inc. (USA) The tetrabutylammonium chloride was from Aldrich (Germany). Individual samples of IL were kept at constant agitation under vacuum and at a moderate temperature (50°C), for a minimum of 24 h, to reduce the volatile impurities content, particularly water, to negligible values. After this purification step, the purity of each IL was further checked by ¹H and ¹³C NMR spectra and found to be >98 wt % for all samples; the water content in all samples is < 0.5 wt %. Anhydrous sodium carbonate, Na₂CO₃ (>99.9 wt %) was from BDH Prolabo (UK). TC

(>98 wt %) was acquired from Fluka (Germany) and its hydrochloric adduct, TC.HCl (> 95 wt %), was from Sigma-Aldrich (Germany). The synthetic fermentation medium was prepared using: $H_3C_6H_5O_7 \cdot H_2O$ (100 wt %) from Fisher Scientific (UK), $(NH_4)_2SO_4$ (> 99.0 wt %), KH_2PO_4 (> 99.5 wt %), and $CaCO_3$ (>99.0 wt %) from Sigma-Aldrich (Germany), and $MgSO_4 \cdot 7H_2O$ (> 98.0 wt %), $MnSO_4 \cdot H_2O$ (98.0 wt %), $ZnSO_4 \cdot 7H_2O$ (> 99.5 wt %), and $K_2Cr_2O_7$ (>99.5 wt %) from Panreac (Spain).

The buffers used in the calibration of the pH meter equipment were the citric acid/sodium hydroxide/sodium chloride solution with a pH value of 4.00 (± 0.02), and the potassium dihydrogenphosphate/disodium hydrogenphosphate solution with a pH value of 7.00 (± 0.02), acquired from Fluka (Germany). Double distilled water, passed through a reverse osmosis system, and further treated with a Milli-Q plus 185 water purification equipment, was used in all experiments.

Methods

Phase Diagrams, Tie-Lines, and Tie-Line Lengths. The phase diagrams of the systems composed of IL, water, and sodium carbonate were determined through the cloud point titration method at 25°C (±1°C). The experimental procedure adopted in this work follows the method already validated by us for ABS constituted by other ILs and inorganic salts.[7–9,12,13] Aqueous solutions of Na_2CO_3 (≈20 wt %) and aqueous solutions of each IL (≈80 wt %) were prepared and used for the determination of the corresponding solubility curves. The aqueous salt solution was drop-wise added to the IL aqueous solution until the detection of a cloudy solution (the biphasic region); then drop-wise addition of water followed until the formation of a clear and limpid solution (the monophasic region). To complete the phase diagrams, the opposite addition of the IL aqueous solutions to the aqueous salt solution was also carried out. The additions were performed under constant stirring. The ternary systems' compositions were determined by weight quantification of all components within ± 10^{-4} g.

The experimental binodal curves were fitted by least-squares regression, according to a method originally proposed by Merchuk et al.[40] to describe polymer-based ABS, and defined by,

$$[IL] = A \exp \left[\left(B \times [Salt]^{0.5} \right) - \left(C \times [Salt]^3 \right) \right] \quad (1)$$

where [IL] and [Salt] are the IL and the sodium carbonate mass fraction percentages and the parameters A, B, and C are fitted constants obtained by the regression of the experimental data.

For the determination of each tie-line (TL), a ternary mixture composed of sodium carbonate + water + IL at the biphasic region was gravimetrically prepared (± 10^{-4} g), vigorously stirred, and left to equilibrate for at least 12 h at 25°C (± 1°C), so as to obtain a complete separation of the coexisting phases. The phases were further separated and individually weighed within ± 10^{-4} g. Each TL was then calculated according to the method proposed by Merchuk et al.[40] and previously used by us and others.[2–9,12,13,41–44] It should be noted that this mass balance approach already shown to be applicable to the calculation of TLs in other IL-based ABS and where analytical methods were also used to validate the technique.[4,42] The relationship between the weight of the top phase (or the bottom one) and the overall weight, by the lever-arm rule, allows to obtain the four unknown compositions ([IL]$_T$ [IL]$_B$, [Salt]$_T$, and [Salt]$_B$) by solving the following system of four equations (Eqs. 2–5),

$$[IL]_T = A \exp \left[\left(B \times [Salt]_T^{0.5} \right) - \left(C \times [Salt]_T^3 \right) \right] \quad (2)$$

$$[IL]_B = A \exp \left[\left(B \times [Salt]_B^{0.5} \right) - \left(C \times [Salt]_B^3 \right) \right] \quad (3)$$

$$[IL]_T = \frac{[IL]_M}{\alpha} - \frac{1-\alpha}{\alpha} \times [IL]_B \quad (4)$$

$$[Salt]_T = \frac{[Salt]_M}{\alpha} - \frac{1-\alpha}{\alpha} \times [Salt]_B \quad (5)$$

where "T", "B", and "M" designate the top phase, the bottom phase, and the mixture, respectively, [Salt] and [IL] represent the weight fraction of inorganic salt and ionic liquid, and α is the ratio between the mass of the top phase and the total mass of the mixture. The system solution results in the mass fraction percentage of the ionic liquid and inorganic salt in the top and bottom phases and thus represents the TLs of each system. The tie-line length (TLL) corresponds to the distance between the point in the binodal curve at the bottom phase composition to that at the top phase.

pH Measurements. The pH values of the IL- and Na_2CO_3-rich aqueous phases were measured at 25°C (±1°C) using an HI 9321 Microprocessor pH meter (HANNA instruments) with an uncertainty of ± 0.02. The calibration of the pH meter was carried out with two buffers (pH values of 4.00 and 7.00). The ternary mixtures were prepared by weight, vigorously stirred, and further kept still in small ampoules for phase separation for at least 12 h, and as previously described for the determination of TLs. After the careful separation of the phases, the pH of each aqueous phase was measured. The compositions at which the pH was measured correspond to the same compositions adopted for the antibiotic partitioning studies for a fixed TLL.

Partitioning of TC. The partitioning of TC, at 25°C (± 1°C), was evaluated in several biphasic mixtures composed of Na_2CO_3 and the following ILs: [C$_6$mim]Cl, [C$_4$mim]Cl, [C$_2$mim]Cl, [amim]Cl, [C$_4$mpyr]Cl, and [P$_{4444}$]Cl. For TC.HCl, the partitioning between the two aqueous phases was determined in ABS formed by [C$_4$mim]Cl, [P$_{4444}$]Cl, or [C$_4$mpyr]Cl and Na_2CO_3 and at the same temperature. Some of these systems (particularly those composed of the ILs [C$_4$mim]Cl, [P$_{4444}$]Cl, and [C$_4$mpyr]Cl) were further used to study the partition of TC.HCl between the IL phase and a complex aqueous medium whose composition mimics the fermentation broth. This synthetic fermentation medium was prepared according the description given by Darken et al.[39] and that it is composed of: $H_3C_6H_5O_7 \cdot H_2O$ (12.8 g L^{-1}); sucrose (40.0 g L^{-1}); $(NH_4)_2SO_4$ (6.0 g L^{-1}); $MgSO_4 \cdot 7H_2O$ (0.25 g L^{-1}); KH_2PO_4 (0.15 g L^{-1}); $CaCO_3$ (11.0 g L^{-1}); $MnSO_4 \cdot H_2O$ (0.010 g L^{-1}); $ZnSO_4 \cdot 7H_2O$ (0.04 g L^{-1}); and $K_2Cr_2O_7$ (1.6 × 10^{-5} g L^{-1}). Although sucrose should be present at the initial medium composition,[39] it was not used here as the extraction stage takes place at the end of the fermentation process when it is safe to admit that most of the sugar was already consumed by the microorganism. After the preparation of the synthetic medium, and its proper mixing, it was centrifuged and the upper aqueous solution was used as the aqueous phase which constitutes a given ABS. The initial mixtures were prepared by the addition of the antibiotic at 0.05 wt % to the required amount of each IL and salt/complex media aqueous solutions so that the TLL is
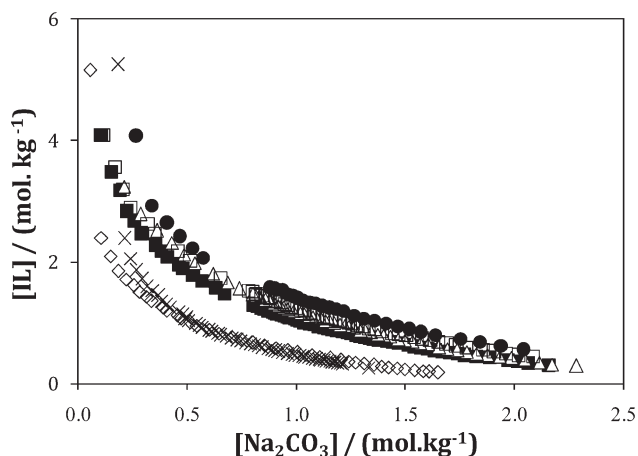
Figure 2. Binodal curves for ABS composed of IL + Na$_2$CO$_3$ + H$_2$O at 25°C (evaluation of the cation core): ●, [C$_4$mim]Cl; □, [C$_4$mpyr]Cl; △, *p*-[C$_4$mpy]Cl; ■, [C$_4$mpip]Cl; ×, [P$_{4444}$]Cl; ◇, [N$_{4444}$]Cl.

circa 40. The TC concentration value is similar to values usually found in fermentation broth media (0.2–2 g L^{-1}).[39,45]

The biphasic mixture was mechanically stirred (vortex) and left to equilibrate for 12 h to achieve a complete partitioning of the molecule between the two phases. The partitioning solute quantification, in both phases, was accomplished by UV spectroscopy using a SHIMADZU UV-1700, Pharma-Spec spectrometer, at the wavelength of 276 nm, using calibration curves previously established. Possible interferences of the IL, the salt, and the synthetic fermentation medium in the TC quantification were taken in account, and blank controls were used whenever necessary. The partition coefficient of TC, K, was determined as the ratio of the concentration of TC in the IL and in the sodium carbonate aqueous phases, according to Eq. (6):

$$K = \frac{[TC]_{IL}}{[TC]_{Salt}} \qquad (6)$$

where [TC]$_{IL}$ and [TC]$_{Salt}$ are the concentrations of TC (or TC.HCl) in the IL and in the Na$_2$CO$_3$ aqueous phases,
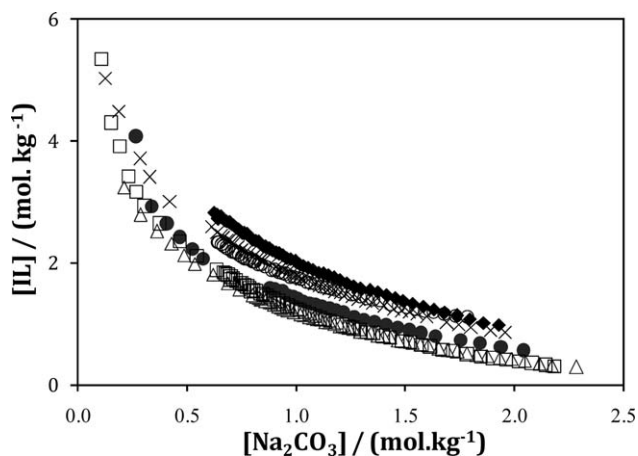
respectively. At least three isolated samples of each system were prepared to determine the average partition coefficient and the respective standard deviation. Both phases were also weighed so as to obtain the corresponding TLs as previously described.

The extraction efficiencies (%EE) of TC were determined according to Eq. (7),

$$\%EE = \frac{[TC]_{IL} \times w_{IL}}{[TC]_{IL} \times w_{IL} + [TC]_{Salt} \times w_{Salt}} \times 100 \qquad (7)$$

where w_{IL} and w_{Salt} are the weight values of the IL-rich phase and of the carbonate-rich phase, respectively, and [TC]$_{IL}$ and [TC]$_{Salt}$ are the concentrations of TC (or TC.HCl) in the IL-rich phase and in the sodium carbonate phase, respectively.

Results and Discussion

The addition of high charge density salts to aqueous solutions of ILs leads to liquid–liquid demixing due to a preferential hydration of the salt which further leads to the salting-out (exclusion) of the IL to the IL-rich phase. In the studied systems, the Na$_2$CO$_3$-rich solution forms the bottom phase whereas the IL solution is almost completely separated as the upper phase; this is a very useful advantage considering both the process efficiency and the IL recovery that may follow thereafter.

Effect of the IL on ABS formation

In this work, 13 ILs were studied to form ABS with Na$_2$CO$_3$. All the studied ILs are chloride-based and allowed the study of the influence of the cation characteristics in the two phase's formation. The same salt (sodium carbonate) was used in all ABS studied and the temperature (25°C) and pressure (1 bar) were also fixed in all experiments. Hence, the differences among the binodal curves are imputable to the IL cation identity alone. The influence of the cation core ([P$_{4444}$]Cl, [N$_{4444}$]Cl, [C$_4$mpip]Cl, [C$_4$mpy]Cl, [C$_4$mpyr]Cl, and [C$_4$mim]Cl), the effect of the methyl group position in the pyridinium ring (the *orto*, *meta*, and *para* methylbutyl-pyridinium chloride isomers), as well as the influence of the length and the presence of functionalized groups at the cation's alkyl side chain were evaluated. All solubility data are presented in Figures 2–4 and are depicted in molality units

Figure 3. Binodal curves for ABS composed of IL + Na$_2$CO$_3$ + H$_2$O at 25°C (evaluation of the presence of functionalized groups and alkyl chain length at the cation): ◆, [C$_2$mim]Cl; ●, [C$_4$mim]Cl; □, [C$_6$mim]Cl; ×, [amim]Cl; ○, [C$_4$py]Cl; △, *p*-[C$_4$mpy]Cl.
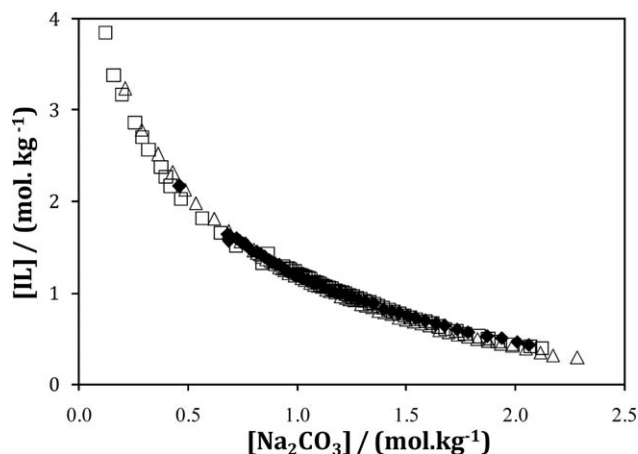

Figure 4. Binodal curves for ABS composed of IL + Na$_2$CO$_3$ + H$_2$O at 25°C (evaluation of the positional isomerism at the cation): ◆, *o*-[C$_4$mpy]Cl; □, *m*-[C$_4$-mpy]Cl; △, *p*-[C$_4$-mpy]Cl.

(mole of ionic liquid per kg of salt + water vs. mole of Na_2CO_3 per kg of IL + water). The detailed experimental weight fraction data are provided in the Supporting Information.

Cation core effect

The solubility curves of the ILs combining the chloride ion with different cation cores are shown in Figure 2. The closer the curve is to the origin, the less IL and/or salt is needed to induce the phase splitting and the wider is the biphasic region.

From the comparison of the binodal curves depicted in Figure 2, an order for the ABS forming ability of ILs can be established: $[P_{4444}]Cl \approx [N_{4444}]Cl > [C_4mpip]Cl \geq p$-$[C_4mpy]Cl \approx [C_4mpyr]Cl \geq [C_4mim]Cl$. These results agree well with the ones from the first investigation on the effect of the cation core through the formation of ABS conducted by Bridges et al. in 2007.[5] The authors evaluated the phase behavior of imidazolium-, pyridinium-, quaternary ammonium-, and phosphonium-based chloride ILs salted-out by several salts. In the presence of potassium carbonate they concluded that the order of the ionic liquids' ability to undergo liquid–liquid demixing is as follows: $[P_{4444}]Cl > [N_{4444}]Cl > [C_4mim]Cl$.[5] Similar results were obtained using potassium phosphate[44] and potassium citrate[9] as the salting-out agents; the phosphonium-based ILs are more effective in promoting ABS when compared to their imidazolium-based counterparts with similar anions. In general, the two quaternary salts ($[P_{4444}]Cl$ and $[N_{4444}]Cl$) exhibit the highest ability for ABS formation: they have highly shielded charges, located mostly on the heteroatom that is surrounded by four alkyl chains, and hence possess a low affinity for water. The smaller the affinity for water and/or the more extensive hydrophobic nature of the IL, the more prone it is to be salted-out.

From the data depicted in Figure 2, it can be seen that the discrepancies among the solubility curves of $[C_4mpip]Cl$, p-$[C_4mpy]Cl$, and $[C_4mpyr]Cl$ are very small; also the proximity between the curves of $[C_4mim]Cl$ and $[C_4mpyr]Cl$ shows that aromaticity does not play a significant role, as previously pointed out by us.[9,46] From the cations studied in this work, imidazolium, followed by pyrrolidinium, displays the most reduced ability for liquid–liquid demixing. In fact, these cations have five-atom rings, whereas pyridinium and piperidinium have six-atom rings. Therefore, as observed before with different salts,[9,46] the solubility of ILs in water, and thus the formation of their ABS, is mainly ruled by steric and entropic contributions where the size of the cation plays a dominant role.

Alkyl side chain length effect

To evaluate the alkyl side chain effect, the solubility curves of ethyl, butyl, and hexyl methylimidazolium chlorides with aqueous solutions of Na_2CO_3 are shown in Figure 3. From those curves it is evident that molecules bearing longer aliphatic chains require a lower salt concentration for being salted-out and to be excluded from the salt-rich phase to the IL-rich phase. The successive replacement of a C_2 group by C_4 and by C_6 leads to a stronger ability for ABS formation due to the increased hydrophobic character of the IL.[46–49] This behavior, similar to the dissolution of *n*-alkanes in water, is most likely to be driven by entropy (larger cations dissolution leads to greater entropy changes) as the enthalpy of dissolution is reasonably independent of the alkyl side chain length.[50] Nevertheless, it should be pointed out that when alkyl side chains longer than hexyl are considered more complex phenomena take place due to the ILs' ability to self-aggregate in aqueous media.[8]

Figure 3 also depicts the solubility curves of $[C_4py]Cl$ and p-$[C_4mpy]Cl$, where an additional methyl group is added in the former IL. Similarly to the alkyl side chain length influence, an increase or addition of an "extra" aliphatic moiety increases the hydrophobicity of the IL, and hence, improves its ability for liquid–liquid demixing.

Functionalized chain effect

The hydrophobicity of an IL cation is modified by the presence of functionalized groups and/or double bonds in the alkyl side chain, and this may affect the cation's affinity for water. To address this effect, the systems composed of 1-allyl-3-methylimidazolium and 1-hydroxyethyl-3-methylimidazolium chlorides were studied. However, for the latter, instead of two coexisting liquid aqueous phases, solid–liquid equilibrium was observed in the overall composition range. The solid phase was identified by ^{1}H and ^{13}C NMR spectra and corresponds to the IL. Due to the salt's higher affinity for water there is the preferential exclusion of the IL from the aqueous solution and the formation of solid–liquid equilibrium instead of liquid–liquid equilibrium. This behavior is also a consequence of the higher melting temperature of $[OHC_2mim]Cl$ (86°C) when compared with the remaining ionic liquids.[51] For [amim]Cl, the liquid–liquid equilibrium was observed and the correspondent solubility curve is depicted in Figure 3 together with the data gathered for $[C_nmim]Cl$ for comparison purposes. As can be appreciated, the presence of a double bond at the longer alkyl side chain of the cation has a minor effect in the ABS ability formation, and as also observed when using K_3PO_4.[52] If the allyl group is considered similar to a C_3 side chain, and estimating that the $[C_3mim]Cl$ would present a curve between those from $[C_2mim]Cl$ and $[C_4mim]Cl$, then it may be inferred that the presence of the double bond does not have a significant effect in the solubility of the IL in water.

Structural isomerism effect

The influence of positional isomerism of the methyl moiety in the butylpyridinium cation when forming ABS can be inferred from the comparison of the solubility curves of o-$[C_4$-mpy]Cl, m-$[C_4$-mpy]Cl, and p-$[C_4$-mpy]Cl depicted in Figure 4. From the inspection of the solubility curves, there are no significant differences among the three positional isomers regarding their phase separation ability. A similar result has already been achieved by Ventura et al.[46] in a study using the same ILs but a different salt, a K_2HPO_4/KH_2PO_4 mixture to provide a buffered aqueous solution (pH = 7).

Considering the whole IL series studied in this work, size emerges as the most meaningful characteristic related to the ABS inducing ability. Ventura et al.[46] already suggested that for ILs composed of the same anions, the most important characteristic ruling the salting-out phenomenon for ABS formation is the cationic molar volume. This pattern is in close agreement with the solubility behavior of ILs (constituted by similar anions) in water.[53]

Table 1. Parameters *A*, *B*, and *C* Obtained from the Regression of the Experimental Binodal Data by Eq. (1) (and Respective Standard Deviations, *σ*, and Correlation Coefficients, *R*^{2}) for the IL + Na$_2$CO$_3$+ H$_2$O Systems at 25°C

IL + Na$_2$CO$_3$ + H$_2$O ABS	A ± σ	B ± σ	10^5 (C ± σ)	R^2
[C$_2$mim]Cl	58.1 ± 0.6	−0.271 ± 0.004	9.4 ± 0.3	0.997
[C$_4$mim]Cl	67.4 ± 1.1	−0.372 ± 0.007	8.1 ± 0.4	0.999
[C$_6$mim]Cl	77.6 ± 0.5	−0.398 ± 0.003	1.0 ± 0.3	0.999
[amim]Cl	65.3 ± 0.5	−0.328 ± 0.003	7.5 ± 0.3	0.999
[C$_4$py]Cl	64.1 ± 0.4	−0.321 ± 0.003	3.2 ± 0.2	0.999
o-[C$_4$mpy]Cl	83.4 ± 0.4	−0.456 ± 0.003	6.5 ± 0.3	0.999
m-[C$_4$mpy]Cl	62.6 ± 0.5	−0.367 ± 0.003	1.0 ± 0.2	0.999
p-[C$_4$mpy]Cl	67.6 ± 0.5	−0.391 ± 0.003	1.0 ± 0.2	0.999
[C$_4$mpyr]Cl	63.8 ± 0.3	−0.382 ± 0.002	9.2 ± 0.2	0.999
[C$_4$mpip]Cl	75.4 ± 1.2	−0.468 ± 0.009	7.9 ± 0.1	0.994
[N$_{4444}$]Cl	65.7 ± 0.3	−0.467 ± 0.003	2.0 ± 0.4	0.999
[P$_{4444}$]Cl	106.9 ± 1.7	−0.655 ± 0.008	2.0 ± 0.1	0.999

TLs and TLLs

For the studied systems, the experimental binodal data were further fitted by the empirical relationship described by Eq. (1), using nonlinear least-squares regression. The regression parameter values and corresponding standard deviations (*σ*) are provided in Table 1. In general, good correlation coefficients were obtained for all systems. The calculated TLs, that is, the compositions of the coexisting phases, along with their respective length, are reported in Table 2. An example of the fitted data and the TLs obtained for this type of systems are illustrated in Figure 5.

Partitioning of TC

To evaluate the potential of the studied systems for downstream processing, the partitioning of TC was explored as a model antibiotic produced in fermentative media. Only the most representative systems, namely those composed of

[C$_6$mim]Cl, [C$_4$mim]Cl, [C$_2$mim]Cl, [amim]Cl, [C$_4$mpyr]Cl, and [P$_{4444}$]Cl, were chosen. Those systems allow us to infer on the alkyl chain length and cation core effects toward the preferential partition of the biomolecule. To gain a better insight in the parameters that rule the partition behavior between the aqueous phases, some of those systems, [C$_4$mim]Cl, [C$_4$mpyr]Cl, and [P$_{4444}$]Cl, were further used to extract the TC.HCl adduct, a more polar and water soluble formulation of the antibiotic.

The mixture composition of each selected system was chosen so as to correspond to a similar TLL ($\approx$ 40), aiming at ensuring equivalent differences in the composition of both phases in the diverse systems. The initial mixture compositions and pH values of each phase are presented in Table 3. The corresponding TLs are presented in Table 2.

TC is chemically characterized by containing an octahydronaphthacene ring skeleton, consisting of four fused rings structure with a carboxyamide functional group (Figure 6). The complex functionality of the TC molecules is reflected in three ionization constants (pK_a): 3.32, 7.78, 9.58[54]; the extent of these equilibria and hence the medium pH determines the charge of TC. In liquid–liquid extraction processes, the preferential migration of a molecule is dependent on its interaction with both liquid phases and for ion containing liquid phases the solute charge is a relevant property. Differences between pH values of both phases should be kept within narrow limits or else partition disturbances may arise from differences in the charge of TC. The alkaline pH values for both phases of all the systems investigated were comprised within less than 1 pH unit (11.4 ± 0.4), hence the TC molecule is negatively charged.

The partition coefficients of the TC molecule, *K*, were obtained according to Eq. (7), and the corresponding extraction efficiencies were also determined through Eq. 8. Their average values and associated standard deviations are

Table 2. Calculated Phase Equilibrium Compositions for the IL + Na$_2$CO$_3$ + Water ABS at 25°C and Respective Values of TLL

IL + Na$_2$CO$_3$ + H$_2$O ABS	100.(Weight Fraction Composition ± 2^{-4})*						
	[IL]$_T$	[Salt]$_T$	[IL]$_M$	[Salt]$_M$	[IL]$_B$	[Salt]$_B$	TLL
[C$_2$mim]Cl	30.35	5.45	24.11	11.18	0.43	32.91	40.61
	33.27	4.11	25.04	11.06	0.64	31.68	42.72
	43.36	1.16	29.97	12.93	0.04	39.26	57.69
[C$_4$mim]Cl	28.98	5.01	20.23	11.93	2.44	25.98	33.82
	33.80	3.40	21.12	12.77	2.03	26.88	39.50
	34.39	3.24	21.61	12.68	1.87	27.27	40.44
[C$_6$mim]Cl	35.26	3.87	25.64	9.96	2.40	24.66	38.89
	38.75	3.02	26.06	10.81	1.92	25.63	43.22
[amim]Cl	34.22	3.83	25.58	9.94	3.65	25.45	37.45
	35.21	3.83	25.12	10.97	1.67	29.37	41.37
[C$_4$py]Cl	32.88	4.31	26.19	10.01	6.83	26.51	34.22
	39.28	2.33	29.59	12.22	0.84	41.57	54.93
o-[C$_4$mpy]Cl	27.48	5.79	21.94	9.97	3.92	23.57	29.72
	31.15	4.61	22.27	11.08	3.06	25.10	34.62
m-[C$_4$mpy]Cl	27.01	5.08	20.80	9.95	2.39	24.39	31.29
	30.54	3.77	21.18	10.91	1.77	25.73	36.20
p-[C$_4$mpy]Cl	26.71	5.43	20.32	9.87	4.65	20.74	26.85
	29.55	4.37	19.92	11.19	2.94	23.20	32.59
[C$_4$mpyr]Cl	31.87	3.27	25.87	8.18	0.97	28.60	39.95
	33.19	2.90	26.13	9.09	0.42	31.59	43.56
[C$_4$mpip]Cl	32.95	3.11	26.86	7.31	2.53	24.06	39.36
	36.09	2.47	25.89	9.86	1.18	27.77	43.11
[N$_{4444}$]Cl	30.15	2.76	20.86	8.25	1.91	19.47	32.81
	31.68	2.42	20.32	9.22	1.41	20.52	35.27
[P$_{4444}$]Cl	30.73	3.57	25.20	6.06	3.57	15.82	29.79
	35.11	2.86	25.70	7.15	2.14	17.87	36.23
	42.09	2.02	25.29	8.96	1.69	18.73	43.72

*Expanded uncertainty at the 0.95 confidence level evaluated from the standard deviation and applying a coverage factor *k* = 2.
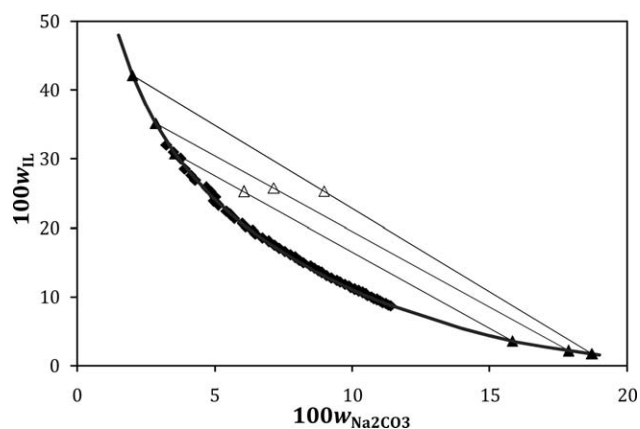
Figure 5. Binodal curves for the ABS composed of [P₄₄₄₄]Cl + Na₂CO₃ + water at 25°C: ◆, experimental solubility data; △, TL data, —, fitting by Eq. (1).

presented in Table 3. As the results show, the partition coefficients are, in every case, much larger than unity, varying from 34 to 105, and evidencing the TC's higher affinity for the IL-rich phase. Albeit TC presents higher affinity for more hydrophilic phases ($\log K_{ow} = -1.19$),[55] the trend here observed is a preferential migration of the antibiotic for the more hydrophobic IL-rich phase. This behavior, taking into account the TC's negative charge, suggests that the partition process is not dominated by electrostatic interactions between the charged antibiotic and the inorganic salt cation. On the other hand, Na₂CO₃, a strong salting-out agent and ABS promoter, seems to force the migration of the antibiotic to the IL-rich phase. Indeed, the affinity of the biomolecule for the IL phase follows closely the two phase formation ability.

The correspondent partition coefficients for TC.HCl are also presented in Table 3 and show a pattern similar to TC ones with a slight enhanced affinity for [P₄₄₄₄]Cl. Nonetheless, the partition coefficients are always greater than 70 for all the extractions, unequivocally revealing the considerable affinity of both TC formulations for the IL-rich phase. In fact, both forms of the antibiotic are negatively charged at the pH values attained in the ABS studied. In Figure 7, a comparison of the extraction efficiency values obtained for

both antibiotics forms is depicted. Outstandingly, the extraction of both forms of the antibiotic from the sodium carbonate solution is nearly complete, with values around or above 99% for all the studied systems.

To further test these systems envisaging their use in down-streaming processing, the partitioning of TC.HCl was studied using a synthetic solution reproducing the fermentation media composition at the stage of the extraction, that is, when depleted of sucrose. The results obtained for the ILs studied show extraction efficiency values that are identical to those obtained using the simple model solution. As it may be observed in Table 3, the partition coefficients are close to 45 and 70 for [C₄mim]Cl and [C₄mpyr]Cl, respectively, and higher than 150 for [P₄₄₄₄]Cl. For all the studied systems, regardless of the antibiotic form or the media composition, the extraction efficiencies are above 99% and no major differences are observed within the same system, taking into account the standard deviations associated to the extraction efficiency values.

Enhanced values were also previously reported for the extraction of other antibiotics, for instance penicillin G (maximum extraction yield of 93%) with ABS composed of NaH₂PO₄·2H₂O and [C₄mim]Cl;[56] chloramphenicol (extraction efficiency of 90.1% under the optimal conditions) with an ABS constituted by Na₃C₆H₅O₇ and [C₄mim][BF₄];[57] and acetylspiramycin (extraction efficiency of 90.14% with the optimized conditions) with an ABS formed by [C₄mim][BF₄] and NaH₂PO₄.[58] However, these extractions required higher amounts of inorganic salt and most of the systems used a nonstable fluorinated ionic liquid.[59] Hence, the systems now proposed emerge as valuable alternatives to extract TC from fermentative media and may play a significant role in down-stream processing, taking into account that chloride ILs of low cost and low toxicity can be properly selected. When compared with traditional extraction techniques, using for instance, conventional polymer–salt ABS,[60–62] reverse micelle mediated extractions[63] or hollow fiber renewal liquid membranes,[64] the results achieved in this work validate the ionic liquid + Na₂CO₃ aqueous systems as promising media for the extraction of antibiotics directly from the fermentation broth.

The nearly complete extraction of TC achieved with every IL-based system studied in this work, independently of the

Table 3. Partition Coefficients (K) and Extraction Efficiencies (%EE), and Respective Standard Deviations (σ), for TC and TC.HCl, Initial Compositions of the System, and pH Values of Both Phases at 25°C

	100.(Weight Fraction Composition ± 2⁻⁴)*		pH ± 0.02			
IL + Na₂CO₃ + H₂O ABS	Ionic Liquid	Na₂CO₃	IL-rich Phase	Na₂CO₃-Rich Phase	$K \pm \sigma$	%EE ± σ
TC						
[C₂mim]Cl	24.11	11.18	11.28	11.41	34 ± 3	99.02 ± 0.04
[C₄mim]Cl	21.61	12.68	11.39	11.47	74 ± 4	99.04 ± 0.14
[C₆mim]Cl	25.64	9.96	11.16	11.46	73 ± 3	99.28 ± 0.06
[amim]Cl	25.12	10.97	11.66	11.47	53 ± 5	99.13 ± 0.18
[C₄mpyr]Cl	25.87	8.18	11.49	10.71	105 ± 6	99.63 ± 0.07
[P₄₄₄₄]Cl	25.29	8.96	11.59	11.21	77 ± 4	98.97 ± 0.05
TC.HCl						
[C₄mim]Cl	21.61	12.68	11.49	11.36	73 ± 5	99.09 ± 0.62
[C₄mpyr]Cl	25.87	8.18	11.03	11.77	82 ± 1	99.69 ± 0.02
[P₄₄₄₄]Cl	25.29	8.96	11.41	11.30	165 ± 7	99.59 ± 0.01
TC.HCl in the synthetic fermentative medium						
[C₄mim]Cl					70 ± 5	99.07 ± 0.60
[C₄mpyr]Cl					45 ± 2	99.41 ± 0.03
[P₄₄₄₄]Cl					152 ± 7	99.53 ± 0.02

*Expanded uncertainty at the 0.95 confidence level evaluated from the standard deviation and applying a coverage factor $k = 2$.
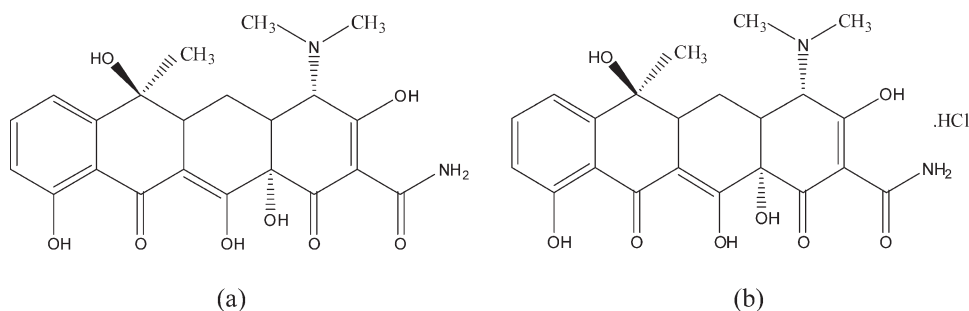
Figure 6. Molecular structure of (a) TC and (b) TC.HCl.

ionic liquid characteristics, that is partly a consequence of the very high ability of the sodium carbonate salt for salting-out the antibiotic, would allow an optimization of the downstream process based on the wide range of ILs that can be tailored according to the required needs — biotechnological, environmental, or economical ones.

Conclusion

Novel phase diagrams for ABS composed of Na_2CO_3 and several chloride-based ILs constituted by different cations (positional isomers, distinct cores and alkyl side chains, and functionalized groups) were determined at 25°C. From the gathered results, the ability of the different IL cations in the two-phase formation was evaluated and discussed. The IL cation properties have a significant role in ABS forming, with size and hydrophobicity emerging as the dominant parameters favoring liquid–liquid splitting. Our data, obtained using Na_2CO_3 as ABS promoter, together with literature data using different salts, show that the IL ability to promote ABS follows approximately the same order independently of the salt, as long as this contains high charge density ions and the media pH values are neutral or preferably alkaline.

The ABS systems were further used in the extraction of TC, an antibiotic representative of pharmaceuticals produced in fermentative media. The results demonstrated the very high efficacy of IL-based ABS in the extraction of the antibiotic, assessing the potentiality of those systems for extracting added-value compounds directly from the fermentation broth
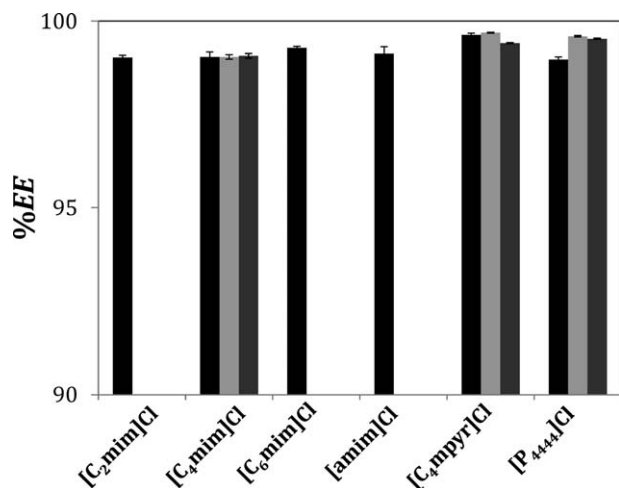
in a single-step procedure. For all the studied systems the extraction efficiencies for both antibiotic forms were around or above 99%. The fact that every IL, independently of its properties, was able to extract the TC molecules nearly to completion making use of a common salt, allows that other industrially and environmentally relevant parameters, such as cost and toxicity, may dictate the choice of the IL to be employed in the extraction.

Antibiotics recovery with IL-based ABS can be an effective, cost-reducing, and greener alternative to the classic extraction methods with volatile organic solvents. The results gathered in this work are a significant contribution to expand the knowledge of the influence of the IL characteristics in ABS formation and emphasize their promising role in the biotechnological field. One advantage of the use of Na_2CO_3 in ABS envisaging downstream processes is the fact that the IL is almost completely segregated in the upper phase which has positive implications in the efficacy of the process and in the IL recovery that may subsequently be accomplished. Also the removal of carbonate whenever needed can be achieved by adding hydrochloric acid to the aqueous phase, releasing carbon dioxide and leaving sodium chloride in solution. The high efficacy, versatility, and amenability to regeneration and recovery of the proposed systems represent a further input to the prerequisites of greener and sustainable technological processes.

Figure 7. Extraction efficiencies (%EE) in ABS composed of IL + Na_2CO_3 + H_2O of TC (black) and TC.HCl (light gray) and of TC.HCl in the synthetic fermentative medium (dark gray), at 25°C.

Literature Cited

1. Albertsson PA. Partitioning of proteins in liquid polymer–polymer two-phase systems. *Nature*. 1958;182:709–711.

2. Hatti-Kaul R. Aqueous two-phase systems. A general overview. *Mol Biotechnol*. 2001;19:269–277.

3. Freire MG, Cláudio AFM, Araújo JMM, Coutinho JAP, Marrucho IM, Canongia Lopes JN, Rebelo LPN. Aqueous biphasic systems: a boost brought about by using ionic liquids. *Chem Soc Rev*. 2012;41:4966–4995.

4. Gutowski KE, Broker GA, Willauer HD, Huddleston JG, Swatloski RP, Holbrey JD, Rogers RD. Controlling the aqueous miscibility of ionic liquids: aqueous biphasic systems of water-miscible ionic liquids and water-structuring salts for recycle, metathesis, and separations. *J Am Chem Soc*. 2003;125:6632–6633.

5. Bridges NJ, Gutowski KE, Rogers RD. Investigation of aqueous biphasic systems formed from solutions of chaotropic salts with kosmotropic salts (salt-salt ABS). *Green Chem.* 2007;9:177–183.

6. Sadeghi R, Golabiazar R, Shekaari H. The salting-out effect and phase separation in aqueous solutions of tri-sodium citrate and 1-butyl-3-methylimidazolium bromide. *J Chem Thermodyn.* 2010;42:441–453.

7. Mourão T, Cláudio AFM, Boal-Palheiros I, Freire MG, Coutinho JAP. Evaluation of the impact of phosphate salts on the formation of ionic-liquid-based aqueous biphasic systems. *J Chem Thermodyn.* 2012;54:398–405.

8. Freire MG, Neves CMSS, Canongia Lopes JN, Marrucho IM, Coutinho JAP, Rebelo LPN. Impact of self-aggregation on the formation of ionic-liquid-based aqueous biphasic systems. *J Phys Chem B.* 2012;116:7660–7668.

9. Passos H, Ferreira AR, Cláudio AFM, Coutinho JAP, Freire MG. Characterization of aqueous biphasic systems composed of ionic liquids and a citrate-based biodegradable salt. *Biochem Eng J.* 2012;67:68–72.

10. Freire MG, Pereira, JFB, Francisco M, Rodríguez H, Rebelo LPN, Rogers RD, Coutinho JAP. Insight into the interactions that control the phase behaviour of new aqueous biphasic systems composed of polyethylene glycol polymers and ionic liquids. *Chem Eur J.* 2012;18:1831–1839.

11. Zafarani-Moattar MT, Hamzehzadeh S, Nasiri S. A new aqueous biphasic system containing polypropylene glycol and a water-miscible ionic liquid. *Biotechnol Prog.* 2012;28:146–156.

12. Domínguez-Pérez M, Tomé LIN, Freire MG, Marrucho IM, Cabeza O, Coutinho JAP. Extraction of biomolecules using aqueous biphasic systems formed by ionic liquids and aminoacids. *Sep Purif Technol.* 2010;72:85–91.

13. Freire MG, Louros CLS, Rebelo LPN, Coutinho JAP. Aqueous biphasic systems composed of a water-stable ionic liquid + carbohydrates and their applications. *Green Chem.* 2011;13:1536–1545.

14. Abraham MH, Zissimos AM, Huddleston JG, Willauer HD, Rogers RD, Acree WE Jr. Some novel liquid partitioning systems: water-ionic liquids and aqueous biphasic systems. *Ind Eng Chem Res.* 2003;42:413–418.

15. Oliveira FS, Freire MG, Pratas MJ, Pauly J, Daridon JL, Marrucho IM, Coutinho JAP. Solubility of adamantane in phosphonium-based ionic liquids. *J Chem Eng Data.* 2010;55:662–665.

16. Zakrzewska ME, Bogel-Łukasik E, Bogel-Łukasik R. Solubility of carbohydrates in ionic liquids. *Energy Fuel.* 2010;24:737–745.

17. Seddon KR. Ionic liquids: a taste of the future. *Nat Mater.* 2003;2:363–365.

18. Huddleston JG, Rogers RD. Room temperature ionic liquids as novel media for 'clean' liquid–liquid extraction. *Chem Commun.* 1998;1765–1766.

19. Zhao H, Xia S, Ma P. Use of ionic liquids as 'green' solvents for extractions. *J Chem Technol Biotechnol.* 2005;80:1089–1096.

20. Freire MG, Teles ARR, Canongia Lopes JN, Rebelo LPN, Marrucho IM, Coutinho JAP, Partition coefficients of alkaloids in biphasic ionic-liquid-aqueous systems and their dependence on the Hofmeister series. *Sep Sci Technol.* 2012;47:284–291.

21. Freire MG, Neves CMSS, Marrucho IM, Canongia Lopes JN, Rebelo LPN, Coutinho JAP. High-performance extraction of alkaloids using aqueous two-phase systems with ionic liquids. *Green Chem.* 2010;2:1715–1718.

22. Tomé LIN, Catambas VR, Teles RR, Freire MG, Marrucho IM, Coutinho JAP. Tryptophan extraction using hydrophobic ionic liquids. *Sep Purif Technol.* 2010;72:167–173.

23. Ventura SPM, Neves CMSS, Freire MG, Marrucho IM, Oliveira J, Coutinho JAP. Evaluation of anion influence on the formation and extraction capacity of ionic-liquid-based aqueous biphasic systems. *J Phys Chem B.* 2009;113:9304–9310.

24. Li S, He C, Liu H, Li K, Liu F. Ionic liquid-based aqueous two-phase system, a sample pretreatment procedure prior to high-performance liquid chromatography of opium alkaloids. *J Chromatogr B.* 2005;826:58–62.

25. Cláudio AFM, Freire MG, Freire CSR, Silvestre AJD, Coutinho JAP. Extraction of vanillin using ionic-liquid-based aqueous two-phase systems. *Sep Purif Technol.* 2010;75:39–47.

26. Cláudio AFM, Ferreira AM, Freire CSR, Silvestre AJD, Freire MG, Coutinho JAP. Optimization of the gallic acid extraction using ionic-liquid-based aqueous two-phase systems. *Sep Purif Technol.* 2012;97:142–149.

27. Zafarani-Moattar MT, Hamzehzadeh S. Partitioning of amino acids in the aqueous biphasic system containing the water-miscible ionic liquid 1-butyl-3-methylimidazolium bromide and the water-structuring salt potassium citrate. *Biotechnol Prog.* 2011;27:986–997.

28. Du Z, Yu YL, Wang JH. Extraction of proteins from biological fluids by use of an ionic liquid/aqueous two-phase system. *Chem Eur J.* 2007;13:2130–2137.

29. Tzeng YP, Shen CW, Yu T. Liquid–liquid extraction of lysozyme using a dye-modified ionic liquid. *J Chromatogr A.* 2008;1193:1–6.

30. Ventura SPM, Barros RLF, Barbosa JMP, Soares CMF, Lima AS, Coutinho JAP. Production and purification of an extracellular lipolytic enzyme using ionic liquid-based aqueous two-phase systems. *Green Chem.* 2012;14:734–740.

31. Passos H, Sousa ACA, Ramiro Pastorinho M, Nogueira AJA, Rebelo LPN, Coutinho JAP, Freire MG. Ionic-liquid-based aqueous biphasic systems for improved detection of bisphenol A in human fluids. *Anal Methods.* 2012;4:2664–2667.

32. Li C-X, Han J, Wang Y, Yan Y-S, Xu X-H, Pan J-M. Extraction and mechanism investigation of trace roxithromycin in real water samples by use of ionic liquid–salt aqueous two-phase system. *Anal Chim Acta.* 2009;653:178–183.

33. Jiang Y, Xia H, Guo C, Mahmood I, Liu H. Phenomena and mechanism for separation and recovery of penicillin in ionic liquids aqueous solution. *Ind Eng Chem Res.* 2007;46:6303–6312.

34. Han J, Wang Y, Kang W, Li C, Yan Y, Pan J, Xie X. Phase equilibrium and macrolide antibiotics partitioning in real water samples using a two-phase system composed of the ionic liquid 1-butyl-3-methylimidazolium tetrafluoroborate and an aqueous solution of an inorganic salt. *Microchim Acta.* 2010;169:15–22.

35. Bienkowski PR, Lee DD, Byers CH. Evaluation of separation and purification processes in the antibiotic industry. *Appl Biochem Biotechnol.* 1988;18:261–273.

36. Mahajan GB, Balachandran L. Antibacterial agents from actinomycetes—a review. *Front Biosci.* 2012;4:240–253.

37. Duggar BM, Aureomycin: a product of the continuing search for new antibiotics. *Ann NY Acad Sci.* 1948;51:177–181.

38. Neidleman SL. Tetracylines in antibiotics, isolation, separation, and purification. *J Chromatogr Library.* 1978;15:715–759.

39. Darken MA, Berenson H, Shirk RJ, Sjolander NO. Production of tetracycline by Streptomyces aureofaciens in synthetic media. *Appl Microbiol.* 1960;8:46–51.

40. Merchuk JC, Andrews BA, Asenjo JA. Aqueous two-phase systems for protein separation. Studies on phase inversion. *J Chromatogr B.* 1998;711:1–2.

41. Deive FJ, Rodriguez A, Marrucho IM, Rebelo LPN. Aqueous biphasic systems involving alkylsulfate-based ionic liquids. *J Chem Thermodyn.* 2011;43:1565–1572.

42. Neves CMSS, Freire MG, Coutinho JAP. Improved recovery of ionic liquids from contaminated aqueous streams using aluminium-based salts. *RSC Adv.* 2012;2:10882–10890.

43. Li Z, Pei Y, Liu L, Wan J. (Liquid+liquid) equilibria for (acetate-based ionic liquids+inorganic salts) aqueous two-phase systems. *J Chem Thermodyn.* 2010;42:932–937.

44. Louros CLS, Cláudio AFM, Neves CMSS, Freire MG, Marrucho IM, Pauly J, Coutinho JAP. Extraction of biomolecules using phosphonium-based ionic liquids + K3PO4 aqueous biphasic systems. *Int J Mol Sci.* 2010;11:1777–1791.

45. Pereira JFB, Vicente F, Santos-Ebinuma VC, Araújo JM, Pessoa A, Freire MG, Coutinho JAP. Extraction of tetracycline from fermentation broth using aqueous two-phase systems composed of polyethylene glycol and cholinium-based salts. *Proc Biochem.* 2013; DOI:10.1016/j.procbio.2013.02.025.

46. Ventura SPM, Sousa SG, Serafim LS, Lima AS, Freire MG, Coutinho JAP. Ionic liquid based aqueous biphasic systems with

controlled pH: the ionic liquid cation effect. *J Chem Eng Data*. 2011;56:4253–4260.

47. Pei Y, Wang J, Liu L, Wu K, Zhao Y. Liquid–liquid equilibria of aqueous biphasic systems containing selected imidazolium ionic liquids and salts. *J Chem Eng Data*. 2007;52:2026–2031.

48. Shill K, Padmanabhan S, Xin Q, Prausnitz JM, Clark DS, Blanch HW. Ionic liquid pretreatment of cellulosic biomass: enzymatic hydrolysis and ionic liquid recycle. *Biotechnol Bioeng*. 2011;108:511–520.

49. Yu C, Han J, Hu S, Yan Y, Li Y. Phase diagrams for aqueous two-phase systems containing the 1-ethyl-3-methylimidazolium tetrafluoroborate/1-propyl-3-methylimidazolium tetrafluoroborate and trisodium phosphate/sodium sulfite/sodium dihydrogen phosphate at 298.15 K: experiment and correlation. *J Chem Eng Data*. 2011;56:3577–3584.

50. Freire MG, Carvalho PJ, Gardas RL, Marrucho IM, Santos LMNBF, Coutinho JAP. Mutual solubilities of water and the [Cnmim][Tf2N] hydrophobic ionic liquids. *J Phys Chem B*. 2008;112:1604–1610.

51. Online database of chemicals from around the world. Available at: http://www.chemblink.com/. Accessed on July 20, 2012.

52. Neves CMSS, Ventura SPM, Freire MG, Marrucho IM, Coutinho JAP. Evaluation of cation influence on the formation and extraction capability of ionic-liquid-based aqueous biphasic systems. *J Phys Chem B*. 2009;113:5194–5199.

53. Freire MG, Neves CMSS, Shimizu K, Bernardes CES, Marrucho IM, Coutinho JAP, Canongia Lopes JN, Rebelo LPN. *J Phys Chem B*. 2010;114:15925–15934.

54. Qiang Z, Adams C. Potentiometric determination of acid dissociation constants (pKa) for human and veterinary antibiotics. *Water Res*. 2004;38:2874–2890.

55. Liu Q, Yu J, Li W, Hu X, Xia H, Liu H, Yang P. Partitioning behavior of penicillin G in aqueous two phase system formed by ionic liquids and phosphate. *Sep Purif Technol*. 2006;41:2849–2858.

56. Han J, Wang Y, Yu C-L, Yan Y-S, Xie X-Q. Extraction and determination of chloramphenicol in feed water, milk, and honey samples using an ionic liquid/sodium citrate aqueous two-phase system coupled with high-performance liquid chromatography. *Anal Bioanal Chem*. 2011;399:1295–1304.

57. Wang Y, Han J, Xie X-Q, Li C-X. Extraction of trace acetylspiramycin in real aqueous environments using aqueous two-phase system of ionic liquid 1-butyl-3-methylimidazolium tetrafluoroborate and phosphate. *Cent Eur J Chem*. 2010;8:1185–1191.

58. Freire MG, Neves CMSS, Marrucho IM, Coutinho JAP, Fernandes AM. Hydrolysis of tetrafluoroborate and hexafluorophosphate counter ions in imidazolium-based ionic liquids. *J Phys Chem A*. 2010;114:3744–3749.

59. Mokhtarani B, Karimzadeh R, Amini MH. Partitioning of ciprofloxacin in aqueous two-phase system of poly(ethylene glycol) and sodium sulphate. *Biochem Eng J*. 2008;38:241–247.

60. Stolls J. Sorption of veterinary pharmaceuticals in soils: a review. *Environ Sci Technol*. 2001;35:3397–3406.

61. Bora MM, Borthakur S, Rao PC, Dutta NN. Aqueous two-phase partitioning of cephalosporin antibiotics: effect of solute chemical nature. *Sep Purif Technol*. 2005;45:153–156.

62. Viana Marques DA, Pessoa-Junior A, Lima-Filho JL, Converti A, Perego P, Porto ALF. Extractive fermentation of clavulanic acid by Streptomyces DAUFPE 3060 using aqueous two-phase system. *Biotechnol Prog*. 2011;27:95–103.

63. Fadnavis NW, Satyavathi B, Deshpande AA. Reverse micellar extraction of antibiotics from aqueous solutions. *Biotechnol Prog*. 1997;13:503–505.

64. Ren Z, Zhang W, Lv Y, Li J. Simultaneous extraction and concentration of penicillin G by hollow fiber renewal liquid membrane. *Biotechnol Prog*. 2009;25:468–475.