Separation and Purification Technology 108 (2013) 174–180

Contents lists available at SciVerse ScienceDirect

Separation and Purification Technology

journal homepage: www.elsevier.com/locate/seppur

The impact of self-aggregation on the extraction of biomolecules in ionic-liquid-based aqueous two-phase systems

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ARTICLE INFO

Article history:
Received 19 December 2012
Received in revised form 5 February 2013
Accepted 9 February 2013
Available online 20 February 2013

Keywords:
Aqueous two-phase system
Ionic liquid
Phase diagram
Partition coefficient
Alkaloid
Trendshift

ABSTRACT

On aqueous two-phase systems (ATPS) composed of ionic liquids (ILs) it is often observed that the increase in the cation alkyl side chain length enhances the biomolecules extraction for the IL-rich phase. However, mounting evidences suggest that very long alkyl side chains are unfavorable to the separation. This change on the extraction trends was previously suggested to be due to micelle formation; yet, no evidence was provided and an evaluation of the effect of the pH and of the hydrophobicity of the biomolecules on the partition coefficients for a homologous family of ILs was never carried out. In this work a systematic study of the cation alkyl side chain length effect on the partitioning of a series of alkaloids of variable hydrophobicity in ATPS constituted by 1-alkyl-3-methylimidazolium chloride ILs ([$C_n$mim]Cl) and potassium citrate (at controlled pH) was conducted. The results here reported show that the alkaloids partition coefficients increase with the cation alkyl chain length until the formation of micelles on the systems with cations larger than [C_6mim]Cl. Cations with longer aliphatic chains are not favorable for the extraction of alkaloids, inducing a trendshift on the system by decreasing the partition coefficients or the preferential migration for the IL-rich phase. The pH of the media, and consequently the charged/non-charged state of the alkaloids, as well as the hydrophobicity of the molecules, do not alter the extraction pattern observed. These results indicate that the IL ability to form micelles in ATPS is the interfacial phenomenon that creates the trendshift observed in the partition coefficients.

1. Introduction

Separation and purification stages in biotechnological processes usually require numerous steps with high energy and chemical consumption, representing typically 20–60% of the final cost of the product, but this value may reach 90% in some particular cases [1,2]. There have been considerable efforts addressing the development of fast, efficient and cost-effective separation techniques. Liquid–liquid extraction offers some advantages, such as high yields, improved purification, better selectivity and a good combination between the recovery and purification steps, while keeping the technological simplicity and a lower associated cost [1,3]. The liquid–liquid extraction of biomolecules is typically carried out using volatile organic compounds (VOCs) due to their immiscibility with the aqueous media where the biomolecules are present [4]. However, these organic compounds present a high volatility, often a significant toxicity, and are poorly biocompatible, denaturing the biomolecules to be recovered [1].

In 1958 Albertson introduced the aqueous two-phase systems (ATPS) aiming at the separation of biomolecules by their partitioning between two aqueous liquid phases [5]. These systems consist in two aqueous-rich phases and are classified into three main types: polymer/polymer, polymer/salt and salt/salt. Due to their biocompatibility, liquid–liquid extraction by ATPS has been intensively explored and used to separate, recover and purify several biomaterials, such as enzymes [6,7], proteins [8,9], nucleic acids [10] and antibodies [11].

Recently, a new salt/salt type of ATPS based on ionic liquids (ILs) was proposed [12]. It presents additional advantages over typical polymer-based systems, namely a low viscosity [13,14], quick phase separation and high extraction efficiency [15–18]. Moreover, the possibility of tuning their properties, such as the phase's hydrophobicity and solution behavior, by the tailoring of the phase constituents to develop specific interactions with the target molecule, and controlling the biodegradability and toxicity by design through the judicious choice of the type and structure of the IL ions, are the most interesting characteristics of IL-based ATPS [19–22]. These unique characteristics made these systems successful in the extraction of biomolecules from real systems such as fermentation broths [23,24].

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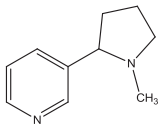
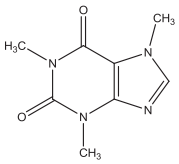
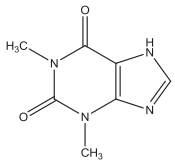
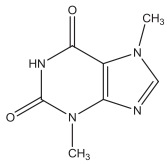
http://dx.doi.org/10.1016/j.seppur.2013.02.008

Table 1
Chemical structures and properties of the studied alkaloids.

	Nicotine	Caffeine	Theophylline	Theobromine
Chemical structure				
K_{OW} [26]	10.47	0.25	0.14	0.14
pK_a at 298 K [25]	3.12; 8.02	–	8.6	10

In previous works, Cláudio et al. [13,16] and Freire et al. [15] reported different patterns in the extraction of several (bio)molecules using IL-based ATPS with the increase of the cation alkyl side chain length of the IL. Albeit the changes on the partition trends were suggested to be due to micelle formation no further evidences were provided. Other effects such as the hydrophilicity/hydrophobicity of the biomolecules or the medium pH and its effect upon the biomolecules charge do play an important role on the partition coefficients and could not be ruled out as the cause of the change in the partition trends observed.

This work intends to carry out a systematic study of the cation alkyl side chain length effect on the partitioning of a series of alkaloids of variable hydrophobicity at different pH values. For that purpose the partition coefficients of four alkaloids – nicotine, caffeine, theophylline and theobromine – were investigated in ATPS composed of 1-alkyl-3-methylimidazolium, [$C_n$mim]Cl (n = 4, 6, 7, 8, and 10), and potassium citrate or potassium citrate – citric acid buffer to allow a variation of the pH values of the systems. This group of four alkaloids was selected because it allows the study of a large range of molecules' hydrophobicities, as well as the manipulation of their charged or non-charged forms at different pH values. The chemical structures and properties of the four studied alkaloids are reported in Table 1 [25,26].

2. Experimental section

2.1. Materials

The ATPS studied in this work were prepared using potassium citrate monohydrate, $C_6H_5K_3O_7{\cdot}H_2O$, ⩾99 wt% pure from Sigma–Aldrich, and citric acid monohydrate, $C_6H_8O_7{\cdot}H_2O$, 100 wt% pure from Fisher Scientific. The ILs studied were 1-butyl-3-methylimidazolium chloride, [C_4mim]Cl (99 wt%), 1-methyl-3-pentylimidazolium chloride, [C_5mim]Cl (>98 wt%), 1-hexyl-3-methylimidazolium chloride, [C_6mim]Cl (>98 wt%), 1-heptyl-3-methylimidazolium chloride, [C_7mim]Cl (>98 wt%), 1-methyl-3-octylimidazolium chloride, [C_8mim]Cl (>98 wt%) and 1-decyl-3-methylimidazolium chloride, [C_{10}mim]Cl (>98 wt%). All ILs were supplied by Iolitec. To reduce the impurities content to negligible values, ILs individual samples were dried under constant stirring at vacuum and moderate temperature (≈353 K) for a minimum of 24 h. After this procedure, the purity of each IL was checked by ^{1}H and ^{13}C NMR spectra. The nicotine, ⩾99.0 wt% pure, was supplied by Fluka, caffeine, >98.5 wt% pure, was acquired at Marsing & Co. Ltd. A/S., and theophylline, ⩾99 wt% pure, and theobromine, ⩾99.0 wt% pure, were from Sigma. 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.

2.2. Phase diagrams and tie-lines

The binodal curves of the phase diagrams were determined through the cloud point titration method [14] at (298 ± 1) K and atmospheric pressure. Aqueous solutions of $C_6H_5K_3O_7$ or $C_6H_5K_3O_7/C_6H_8O_7$ buffer (pH = 7 for a $C_6H_5K_3O_7/C_6H_8O_7$ weight ratio of 25 [27]) at *ca.* 50 wt%, and aqueous solutions of the different hydrophilic ILs at variable concentrations, were prepared gravimetrically. Repetitive drop-wise addition of the aqueous organic salt solution to each IL aqueous solution was carried out until the detection of a cloudy solution (biphasic region), followed by the drop-wise addition of ultra-pure water until the detection of a clear and limpid solution (monophasic region). Drop-wise additions were carried out 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 and (298 ± 1) K.

For the system composed of [C_{10}mim]Cl + organic salt + water the turbidimetric method was used [28]. Various mixtures of the system at the biphasic region were initially prepared. Under constant stirring, ultra-pure water was added until the detection of a clear and limpid solution (monophasic region). Each mixture corresponds to one point of the binodal curve. The mixture compositions were gravimetrically determined within $\pm 10^{-4}$ g and at (298 ± 1) K.

The tie-lines (TLs) were determined by a gravimetric method originally described by Merchuck et al. [29] and used by us in IL-based systems [30–32]. A mixture (IL + salt + water) at the biphasic region was gravimetrically prepared, vigorously stirred, and allowed to reach the equilibrium by the separation of the phases for at least 12 h at (298 ± 1) K. After the phases separation, both top and bottom phases were weighed. Finally, each individual TL was determined by application of the lever-arm rule to the relationship between the top phase weight and the overall system composition. The experimental binodal curves were fitted using Eq. (1) [29],

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

where [IL] and [salt] are, respectively, the IL and salt weight percentages and A, B, and C are constants obtained by the regression of the experimental binodal data.

For the determination of the TLs it was solved the following system of four equations (Eqs. (2)–(5)) and four unknown values ($[\mathrm{IL}]_{\mathrm{IL}}$, $[\mathrm{IL}]_{\mathrm{salt}}$, $[\mathrm{salt}]_{\mathrm{IL}}$ and $[\mathrm{salt}]_{\mathrm{salt}}$):

$$[\mathrm{IL}]_{\mathrm{IL}} = A\exp[(B \times [\mathrm{salt}]_{\mathrm{IL}}^{0.5}) - (C \times [\mathrm{salt}]_{\mathrm{IL}}^3)] \quad (2)$$

$$[\mathrm{IL}]_{\mathrm{salt}} = A\exp[(B \times [\mathrm{salt}]_{\mathrm{salt}}^{0.5}) - (C \times [\mathrm{salt}]_{\mathrm{salt}}^3)] \quad (3)$$

$$[\mathrm{IL}]_{\mathrm{IL}} = \frac{[\mathrm{IL}]_{\mathrm{M}}}{\alpha} - \frac{1-\alpha}{\alpha} \times [\mathrm{IL}]_{\mathrm{salt}} \quad (4)$$

$$[\mathrm{salt}]_{\mathrm{IL}} = \frac{[\mathrm{salt}]_{\mathrm{M}}}{\alpha} - \frac{1-\alpha}{\alpha} \times [\mathrm{salt}]_{\mathrm{salt}} \quad (5)$$

The subscripts IL, salt and M represent the IL, salt and mixture phases, respectively. The parameter α is the ratio between the top and the total weight of the mixture. The solution of the referred

system gives the concentration of the IL and salt in the top and bottom phases. For the calculation of the tie-line length (TLL) it was applied Eq. (6).

$$TLL = \sqrt{([\text{salt}]_{IL} - [\text{salt}]_{salt})^2 + ([IL]_{IL} - [IL]_{salt})^2} \quad (6)$$

The pH values of both the IL-rich and organic-salt-rich aqueous phases were measured at (298 ± 1) K using a METTLER TOLEDO SevenMulti pH meter with an uncertainty of ±0.02.

2.3. Partitioning of alkaloids

Aqueous solutions of each alkaloid were prepared with the following concentrations: 0.76 g dm^{-3} (4.7×10^{-3} mol dm^{-3}) for nicotine, 0.91 g dm^{-3} (4.7×10^{-3} mol dm^{-3}) for caffeine, 0.85 g dm^{-3} (4.7×10^{-3} mol dm^{-3}) for theophylline, 0.20 g dm^{-3} (1.1×10^{-3} mol dm^{-3}) for theobromine. At these concentrations all alkaloids can be considered completely solvated in aqueous media preventing thus solute–solute interactions.

The ternary mixture compositions were chosen based on the phase diagrams determined for each IL-$C_6H_5K_3O_7/C_6H_8O_7$ and IL-$C_6H_5K_3O_7$ systems. Moreover, to avoid discrepancies in the results which could arise from the different compositions of the phases, all the partitioning studies were performed at a constant TLL. The mixture compositions which correspond to a TLL of 40 are as follows: 27.0 wt% of [C_4mim]Cl + 30.0 wt% of $C_6H_5K_3O_7/C_6H_8O_7$, 26.0 wt% of [C_6mim]Cl + 30.0 wt% of $C_6H_5K_3O_7/C_6H_8O_7$, 27.0 wt% of [C_7mim]Cl + 30.0 wt% of $C_6H_5K_3O_7/C_6H_8O_7$, 27.5 wt% of [C_8mim]Cl + 30.0 wt% of $C_6H_5K_3O_7/C_6H_8O_7$, 28.2 wt% of [C_{10}mim]Cl + 30.0 wt% of $C_6H_5K_3O_7/C_6H_8O_7$, 26.8 wt% of [C_4mim]Cl + 30.0 wt% of $C_6H_5K_3O_7$, 26.0 wt% of [C_6mim]Cl + 30.0 wt% of $C_6H_5K_3O_7$, 27.0 wt% of [C_8mim]Cl + 30.0 wt% of $C_6H_5K_3O_7$, and 27.8 wt% of [C_{10}mim]Cl + 30.0 wt% of $C_6H_5K_3O_7$.

Each mixture was vigorously stirred and left to equilibrate for at least 12 h (a time period established in previous optimizing experiments), to achieve the complete partitioning of each alkaloid between the two phases. For all the ternary mixtures evaluated, and at the compositions used, the IL-rich aqueous phase is the top phase while the salt-rich aqueous phase corresponds to the bottom phase. After a careful separation of both phases, the alkaloids quantification in each phase was carried out by UV-spectroscopy, using a SHIMADZU UV-1700, Pharma-Spec Spectrometer, at a wavelength of 260 nm for nicotine, 272 nm for theophylline and 273 nm for caffeine and theobromine, and using calibration curves previously established. At least three individual samples of each phase were quantified in order to determine the average in the alkaloids partition coefficients and the respective standard deviations. Possible interferences of the organic salt and ILs with the analytical method were investigated. Blank control samples were always used. The stability of the alkaloids in the coexisting phases of all systems was also confirmed and it is safe to admit that all the biomolecules are stable up to at least 72 h.

The partition coefficients of the studied biomolecules, K_{Nic} for nicotine, K_{Caf} for caffeine, K_{Tph} for theophylline and K_{Tbr} for theobromine are defined as the ratio of the concentration of the each biomolecule in the IL to that in the salt aqueous phases according to the following equation:

$$K_{Alk} = \frac{[Alk]_{IL}}{[Alk]_{salt}} \quad (7)$$

where $[Alk]_{IL}$ and $[Alk]_{salt}$ are the concentration of each alkaloid in the IL- and in the salt-rich aqueous phases, respectively.

2.4. Micelles characterization

The formation of micelles in the studied IL-based ATPS was evaluated by Transmission Electron Microscopy (TEM) using a TEM-MSC-JEOL 2100 microscope at an accelerating voltage of 200 kV. Samples for TEM observation were prepared by dropping onto a holey carbon-coated copper grid and allowing the solvent to evaporate at 150 °C. Both aqueous phases of each system, with the same composition of the mixtures used in the extraction studies, were analyzed.

3. Results and discussion

3.1. Phase diagrams and tie-lines

The experimental phase diagrams determined at 298 K and at atmospheric pressure for each IL + water + potassium citrate system are illustrated in Figs. 1 and 2. The solubility curves are presented in molality units for a better understanding of the impact of the ILs structure on the phase diagrams behavior, avoiding differences that could result from the different molecular weights of the ILs. The experimental weight fraction data of each phase diagram are reported in Supporting information.

Figs. 1 and 2 depict the effect of the imidazolium alkyl side chain length in the formation of ATPS, at pH 7 and 9, respectively. Aqueous solutions of $C_6H_5K_3O_7$ confer a pH *ca.* to 9 to all the systems, whereas the citrate buffer ($C_6H_5K_3O_7/C_6H_8O_7$) was prepared to fix the pH of the systems around 7. It should be remarked that the phase diagrams obtained for [C_4mim]Cl- and [C_6mim]Cl-$C_6H_5K_3O_7$ systems are in good agreement with those previously reported [17].

In general, an increase in the length of the aliphatic chain of the imidazolium cation facilitates the creation of ATPS. In fact, as the IL becomes more hydrophobic, due to the alkyl chain length increase, there is a reduction on the water-IL affinity [33,34] and, therefore, an improved phase separation occurs. However, the increase of the alkyl side chain length also promotes the IL self-aggregation and changes the trend observed for the systems composed of [C_7mim]Cl, [C_8mim]Cl and [C_{10}mim]Cl. To better visualize this trend-shift in the phase behavior, Fig. 3 depicts the representation of the molality of IL at which it equals the molality of salt in the binodal curve at pH 7, [IL] = [salt], as a function of the alkyl side chain length of the IL cation. This effect has been previously documented and deeply discussed by us using ATPS composed of [$C_n$mim]Cl + K_3PO_4 [35].

Fig 4 shows the phase diagrams of two ILs at the two pH values to evaluate the effect of the pH in the formation of IL-based ATPS [36,37]. At pH 9 potassium citrate ions are in the form of K^+ and $C_6H_5O_7^{3-}$. The decrease of the pH decreases the negative charge of

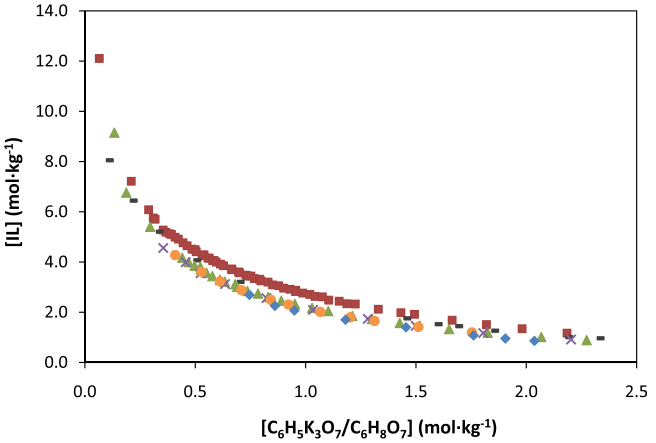


Fig. 1. Evaluation of the cation alkyl side chain length in the ternary phase diagrams composed of IL + H_2O + $C_6H_5K_3O_7/C_6H_8O_7$ (pH ≈ 7): [C_4mim]Cl (■); [C_5mim]Cl (–); [C_6mim]Cl (▲); [C_7mim]Cl (×); [C_8mim]Cl (●); [C_{10}mim]Cl (◆).

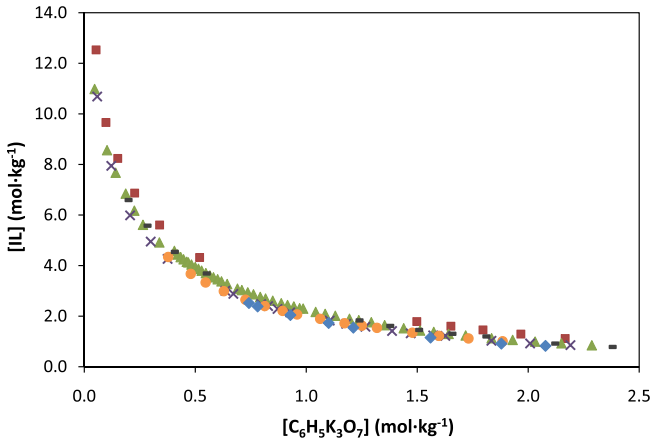


Fig. 2. Evaluation of the cation alkyl side chain length in the ternary phase diagrams composed of IL + H_2O + $C_6H_5K_3O_7$ (pH ≈ 9): [C_4mim]Cl (■); [C_5mim]Cl (–); [C_6mim]Cl (▲); [C_7mim]Cl (×); [C_8mim]Cl (●); [C_{10}mim]Cl (◆).

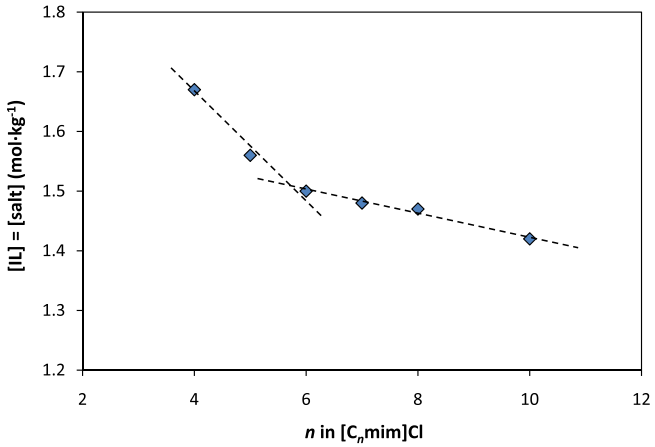


Fig. 3. Relationship between the saturation solubility, [IL] = [salt] in molality units in the binodal curve, and the alkyl chain length, *n*, in [$C_n$mim]Cl ILs, at pH 7 and 298 K.

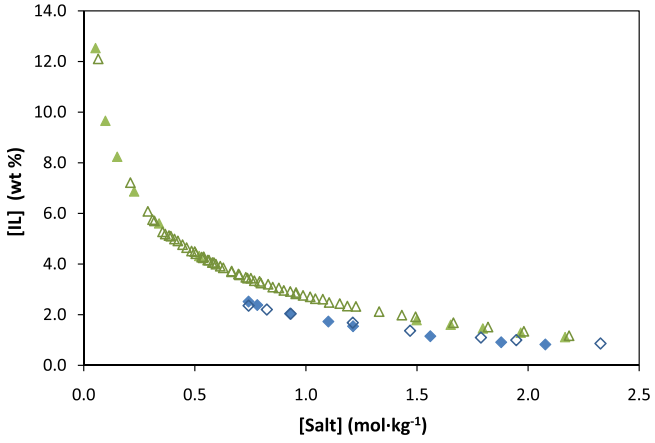


Fig. 4. Evaluation of the pH effect in the ternary phase diagrams composed of [C_4mim]Cl + H_2O + $C_6H_5K_3O_7$ (▲); [C_4mim]Cl + H_2O + $C_6H_5K_3O_7$/$C_6H_8O_7$ (△); [C_{10}mim]Cl + H_2O + $C_6H_5K_3O_7$ (◆); [C_{10}mim]Cl + H_2O + $C_6H_5K_3O_7$/$C_6H_8O_7$ (◇). Full symbols correspond to systems with a pH ≈ 9 while the empty symbols represent the phase diagrams at pH ≈ 7.

the anion from $C_6H_5O_7^{3-}$ to $C_6H_4O_7^{2-}$ and $C_6H_3O_7^{-}$ and thus reduce the ability for ATPS formation. Indeed, this trend is in accordance with the Hofmeister series [38,39]. There are previous studies

Table 2
Correlation parameters used to describe the experimental binodal data by Eq. (1).

IL	$A \pm \sigma$	$B \pm \sigma$	$10^5(C \pm \sigma)$	R^2
IL + $C_6H_5K_3O_7$/$C_6H_8O_7$ ATPS				
[C_4mim]Cl	88.1 ± 0.3	−0.190 ± 0.001	0.71 ± 0.01	0.9995
[C_5mim]Cl	81.8 ± 1.7	−0.172 ± 0.008	0.90 ± 0.07	0.9987
[C_6mim]Cl	99.5 ± 1.0	−0.224 ± 0.003	0.69 ± 0.03	0.9990
[C_7mim]Cl	98.2 ± 2.0	−0.216 ± 0.006	0.68 ± 0.04	0.9996
[C_8mim]Cl	104.7 ± 1.3	−0.222 ± 0.003	0.68 ± 0.02	0.9999
[C_{10}mim]Cl	115.7 ± 23.0	−0.236 ± 0.047	0.70 ± 0.20	0.9958
IL + $C_6H_5K_3O_7$ ATPS				
[C_4mim]Cl	86.0 ± 0.5	−0.180 ± 0.003	0.84 ± 0.03	0.9998
[C_5mim]Cl	89.7 ± 1.9	−0.202 ± 0.007	0.89 ± 0.06	0.9994
[C_6mim]Cl	87.9 ± 0.6	−0.183 ± 0.002	1.00 ± 0.03	0.9984
[C_7mim]Cl	93.0 ± 0.9	−0.206 ± 0.004	0.82 ± 0.04	0.9994
[C_8mim]Cl	108.8 ± 1.0	−0.238 ± 0.003	0.64 ± 0.02	0.9999
[C_{10}mim]Cl	131.9 ± 9.7	−0.272 ± 0.018	0.57 ± 0.07	0.9997

reflecting the effect of the pH towards the phase diagrams behavior [27,36,37]. However, between the pH 9 and 7 evaluated here, the concentration of $C_6H_3O_7^{3-}$ decreases only slightly [40] which makes negligible the effect of pH on the formation of the ATPS studied. Thereby, there are almost no differences between the phase diagrams depicted in Fig. 4.

The experimental binodal data were further fitted using the empirical relationship described by Eq. (1). Table 2 presents the regression parameters estimated by the least-squares regression method, standard deviations (σ) and correlation coefficients (R^2). As can be seen by the good correlation coefficients obtained, Eq. (1) provides an accurate description of the experimental binodal curves.

The experimental TLs, along with their respective length (TLL), and the pH values of the two phases for each ATPS, and at the compositions for which the TLs were determined, are presented in Table 3. As expected the pH values of the systems composed of [$C_n$mim]Cl + $C_6H_5K_3O_7$/$C_6H_8O_7$ + H_2O are neutral (pH ≈ 7) and differences in the pH promoted by the ILs are not observed in the buffered systems. The pH values of the systems composed of [$C_n$mim]Cl + $C_6H_5K_3O_7$ + H_2O are alkaline and, although not being buffered solutions, the pH values are always close to 9. Only one exception was verified with the system constituted by [C_7mim]Cl + $C_6H_5K_3O_7$ + H_2O where the pH of both phases is approximately 7, probably due to the presence of some non-volatile impurities in the IL (although in a concentration low enough to be detected by NMR spectra).

3.2. Extraction of alkaloids

In Figs. 5 and 6 are depicted the results obtained for the partition coefficients of alkaloids in the several IL-based ATPS, at a common TTL ≈ 40, and at different pH values in the aqueous media. The partition coefficients of each alkaloid, and respective standard deviations, mixture compositions and respective TLs and TLLs, the ratio between the weight of the top phase and the total mixture, and pH values of the coexisting phases, are reported in Supporting information.

With the application of the citrate buffer ($C_6H_5K_3O_7$/$C_6H_8O_7$) all ATPS present a pH *ca.* to 7. At this pH value, caffeine, theophylline and theobromine are predominantly in a non-charged form and nicotine is predominantly present as a positively charged species – as can be seen in the dissociation curves shown in Supporting information. The partition coefficients obtained for the alkaloids at pH ≈ 7 are presented in Fig. 5. In all systems it is observed a preferential partitioning of the alkaloids for the IL-rich aqueous phase with partition coefficients of nicotine [4.28–8.43], caffeine [6.34–13.00], theophylline [5.16–12.33] and theobromine [3.12–

Table 3
Experimental data for TLs and TLLs of IL + $C_6H_5K_3O_7$/$C_6H_8O_7$ and IL + $C_6H_5K_3O_7$ ATPS and respective pH values of the coexisting phases.

IL	Weight fraction composition (wt%)								TLL
	$[IL]_{IL}$	$[salt]_{IL}$	pH_{IL}	$[IL]_M$	$[salt]_M$	$[IL]_{salt}$	$[salt]_{salt}$	pH_{salt}	
IL + $C_6H_5K_3O_7$/$C_6H_8O_7$ ATPS									
[C_4mim]Cl	41.44	14.80	7.13	27.04	29.97	13.11	44.65	7.11	41.16
	48.17	9.86	7.19	28.99	30.01	8.47	51.56	7.09	57.58
	54.85	6.17	7.34	40.67	20.87	5.39	57.45	7.41	71.25
[C_6mim]Cl	37.80	17.27	7.01	25.89	29.99	11.65	45.19	7.06	38.25
	45.15	12.03	7.06	31.71	25.78	9.52	48.46	7.08	50.96
	54.19	7.28	7.13	35.13	25.92	6.48	53.94	7.33	66.74
[C_7mim]Cl	42.99	14.00	6.85	27.03	29.97	13.43	43.57	6.93	41.81
	48.24	10.61	6.85	34.89	23.92	9.63	49.12	6.86	54.54
	55.24	7.05	7.05	35.31	26.24	7.24	53.26	7.11	66.63
[C_8mim]Cl	43.75	14.69	6.94	27.58	29.96	15.37	41.48	6.89	39.03
	52.11	9.70	7.00	34.99	26.01	8.49	51.26	7.06	60.24
	63.92	4.92	7.15	40.08	26.01	5.81	56.32	7.37	77.57
[C_{10}mim]Cl	42.56	16.79	6.84	33.49	24.96	15.99	40.71	6.72	35.75
	43.47	16.18	7.16	28.19	29.99	14.89	42.03	7.17	38.53
	43.73	16.01	6.84	33.30	25.44	14.74	42.21	6.73	39.08
IL + $C_6H_5K_3O_7$ ATPS									
[C_4mim]Cl	41.26	15.32	9.18	26.68	29.92	13.04	43.59	9.06	39.94
	52.68	7.33	9.00	39.74	20.35	5.83	54.47	8.98	66.46
	56.57	5.39	8.99	42.82	19.08	4.57	57.16	9.07	73.37
[C_6mim]Cl	40.03	16.44	9.01	25.96	29.97	10.51	44.83	9.13	40.95
	53.19	7.41	8.28	44.89	15.02	7.22	49.53	8.24	62.35
[C_7mim]Cl	51.43	8.15	7.43	34.97	24.00	7.62	50.33	7.64	60.82
	56.66	5.75	7.77	34.96	25.98	6.33	52.68	7.79	68.82
[C_8mim]Cl	39.49	17.02	9.15	27.03	29.87	11.97	45.40	9.20	39.53
	47.50	11.82	8.34	33.12	25.84	10.00	48.40	8.33	52.38
	58.37	6.80	8.44	36.91	25.91	8.16	51.51	8.50	67.22
[C_{10}mim]Cl	38.70	19.03	8.28	27.75	29.89	13.09	44.42	8.39	36.06

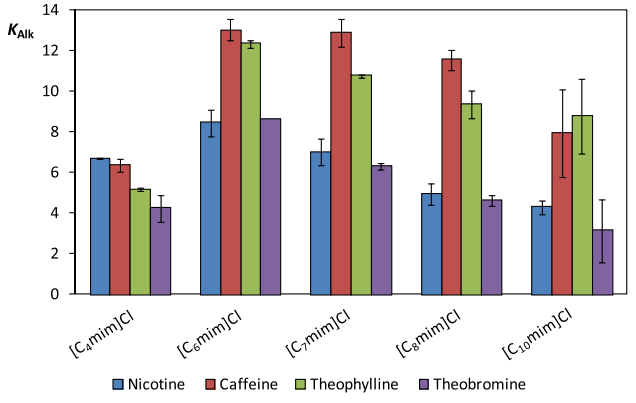


Fig. 5. Partition coefficients of alkaloids between the IL- and $C_6H_5K_3O_7$/$C_6H_8O_7$-rich aqueous phases at pH 7 and 298 K.

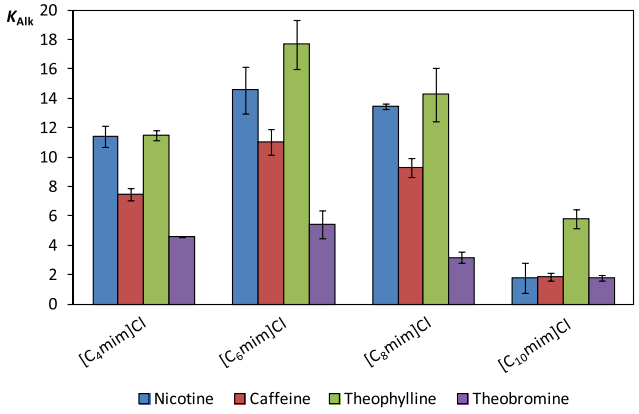


Fig. 6. Partition coefficients of alkaloids between the IL- and $C_6H_5K_3O_7$-rich aqueous phases at pH 9 and 298 K.

8.62] larger than 1. Taking into account only the non-charged alkaloids, it is clear that caffeine partition coefficients are higher than those obtained for theophylline and theobromine, which is in agreement with the octanol-water partition coefficients (K_{OW}) of these compounds (*cf.* Table 1). Furthermore, unlike theobromine, the theophylline molecules have a free imidazolium ring to interact with the IL cation by $\pi\cdots\pi$ interactions, and what can explains the higher affinity of theophylline for the IL-rich phase. At a pH close to 7, nicotine presents the lower partition coefficient values and which are probably related with the charged nature of the molecule that favors the alkaloid migration for the most hydrophilic and charged citrate-based rich phase.

Through an overall analysis of the results it is possible to distinguish a well-defined trendshift in the partition coefficients of the alkaloids: for ILs up to [C_6mim]Cl there is a substantial increase in the partition coefficients with the cation alkyl side chain length increase, i.e. with the IL hydrophobicity. For heavier ILs it is observed a progressive decrease in the partition coefficients with the increase of the size of the aliphatic moiety. This behavior is common for all the alkaloids studied, independently of their charge or hydrophobicity that have an impact upon their individual partition coefficients, but not on the trendshift observed with the cation alkyl side chain length.

Fig 6 depicts the partition coefficients of all the studied alkaloids at pH 9. The extractions with the [C_7mim]Cl-$C_6H_5K_3O_7$ system were not studied in this case because, as mentioned above, the pH of this system is approximately equal to 7. At pH 9, nicotine is

preferentially as a neutral molecule, theophylline is negatively charged, theobromine is preferentially non-charged and, finally caffeine is completely in a neutral form as for pH 7 (*cf.* Supporting information). All systems display again a preferential partitioning of the alkaloids for the IL-rich aqueous phase with partition coefficients of nicotine, caffeine, theophylline and theobromine ranging between [1.80–14.58], [1.88–11.04], [5.81–17.68] and [1.80–5.41], respectively. Cláudio et al. [16] studied recently the effect of the pH in the partitioning of gallic acid in IL-salt-based ATPS, and concluded that non-charged molecules have preferential affinity for the IL-rich phase (more hydrophobic phase). Considering nicotine at both pH values, it is clear that the partition of this alkaloid follows the same behavior previously reported [16], with higher partition coefficients obtained at pH 9, e.g. when the molecule is in a neutral form. Considering only the neutral molecules, the partition coefficients of nicotine, caffeine and theobromine follow, as observed at pH 7, the trend of K_{OW}.

Although the pH of the medium affects the partition coefficients of the various alkaloids, the general behavior observed for the partition coefficients at pH 9 is the same as that obtained with pH 7. There is an increase in the partition coefficients with the cation alkyl side chain length up to [C_6mim]Cl followed by an inversion on the behavior for heavier ILs. In summary, the trendshift observed seems to be independent of the pH of the aqueous medium, and hydrophobicity or charge of the biomolecules. For all the alkaloids studied, at both pH values, at neutral, positively or negatively charged states, this peculiar behavior is always observed.

It seems clearly to establish that a change in the behavior of the systems is observed once the cation alkyl side chain length becomes larger than hexyl that has a deleterious effect upon the partition coefficients. Recently, Santos and co-workers [41–43] have reported trendshifts on the thermophysical properties of imidazolium-based ILs for cations larger than $[C_6mim]^+$. In aqueous solutions this change in behavior translates into the formation of micelles.

ILs based on the $[C_nmim]^+$ cation are structurally similar to cationic surfactants. Due to their amphiphilic nature [44,45], that results from their chemical structure which consists of a charged hydrophilic head group (imidazolium cation) and one hydrophobic tail (alkyl side chain), they present surface activity in solution and, if the cation alkyl chain is long enough, they inherently tend to self-aggregate [46–54]. In the case of [$C_n$mim]Cl ILs the micelle formation is observed in solutions containing ILs with alkyl chains larger than hexyl [55]. However, to the best of our knowledge, the formation of micelles in [C_7mim]Cl has not been previously studied being reported only for systems with [C_8mim]Cl or longer alkyl chains [55].

To confirm the presence of micelles in the systems studied here, Fig. 7 presents TEM images of the IL-rich phase of ATPS composed of [C_8mim]Cl- and [C_{10}mim]Cl-$C_6H_5K_3O_7$. In Fig. 7 it is possible to distinguish the IL aggregates in aqueous solutions, which support the justifications given for the obtained results. Indeed, our results are similar to previously reported ones [56,57]. Furthermore, Fig. 7b and c shows that the aggregates formed in the [C_{10}mim]Cl-based ATPS are larger than those occurring in the [C_8mim]Cl systems. Moreover, it should be remarked that this type of aggregates were only visible in the IL-rich phase. Additional capturing of microscopy images confirmed the absence of aggregates in ATPS composed of [C_4mim]Cl and [C_6mim]Cl.

The partition coefficients reported show that the influence of the IL on the extraction of the alkaloids depends, among others, on the cation alkyl side chain length. Up to a certain alkyl side chain length the dispersive-type interactions between the alkaloids and the aliphatic moieties of the imidazolium-based ions seem to favor the extraction. With the micelle formation the cation alkyl chains become less available decreasing the dispersive-type interactions and induce the observed trendshift.

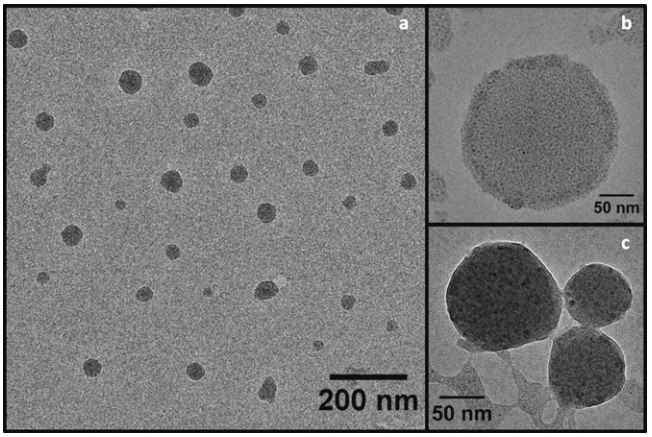


Fig. 7. TEM images of aggregates in the IL-rich phases of: (a and b) [C_{10}mim]Cl- and (c) [C_8mim]Cl-based ATPS.

4. Conclusions

The formation of ATPS composed of 1-alkyl-3-methylimidazolium chloride ILs, [$C_n$mim]Cl (n = 4, 6, 7, 8, and 10), and aqueous solutions of potassium citrate (at different pH values) was studied to infer on the effect of the cation alkyl side chain length, and thus on the possibility of their self-aggregation, upon the partition of biomolecules.

The ternary phase diagrams were initially determined, and these systems were further applied, at a fixed tie-line length, in the extraction of four alkaloids (nicotine, caffeine, theophylline, and theobromine) of different hydrophobicities. In all the partitioning investigations it was observed a maximum in the partition coefficient with [C_6mim]Cl, and that was independent of the pH of the medium and of the biomolecule charge or hydrophobicity. The results suggest that the observed trendshift on the partition coefficients is related with the ILs self-aggregation aptitude which is not favorable for the extraction of biomolecules. The formation of micelles in ATPS composed of ILs with long aliphatic chains was confirmed by TEM.

Acknowledgments

The authors are grateful for financial support from FCT - *Fundação para a Ciência e a Tecnologia* for the projects PTDC/QUI-QUI/121520/2010 and Pest-C/CTM/LA0011/2011 and post-doctoral and doctoral grants SFRH/BPD/41781/2007 and SFRH/BD/85248/2012 of M.G. Freire and H. Passos, respectively. L.P. Costa is grateful to CNPq, Capes and Fapitec (Brazilian Funding Agencies) for financial support. Contributions from LNNano-LME (Campinas-SP, Brazil) for TEM analysis are also gratefully acknowledged.

Appendix A. Supplementary material

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.seppur.2013.02.008.

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