Published in final edited form as:

Biochem Eng J. 2019 January 15; 141: 239–246. doi:10.1016/j.bej.2018.10.022.

Understanding the Effect of Ionic Liquids as Adjuvants in the Partition of Biomolecules in Aqueous Two-Phase Systems Formed by Polymers and Weak Salting-Out Agents

Catarina M. S. S. Neves^a, Rita de Cássia S. Sousa^{a,b}, Matheus M. Pereira^a, Mara G. Freire^a, and João A. P. Coutinho^{a,*}

^aCICECO - Aveiro Institute of Materials, Department of Chemistry, University of Aveiro, 3810-193 Aveiro, Portugal

^bDepartment of Chemistry, Federal University of Viçosa, 36570-000, Viçosa – MG, Brazil

Abstract

Ionic liquids (ILs) as adjuvants in polymer-salt aqueous two-phase systems (ATPS) have been used to improve the extraction of biomolecules. However, the impact of ILs as adjuvants on the partition of biomolecules is still poorly understood. Previous works mostly focused on ATPS based on strong salting-out agents, which may mask the IL effect. In this work, ATPS formed by polyethylene glycol (PEG 400) and a weak salting-out salt ((NH₄)₂SO₄) with a wide number of ILs as adjuvants (chloride-based combined with cholinium, imidazolium, pyrrolidinium, piperidinium, tetralkylammonium and tetralkylphosphonium cations) were investigated. The respective phase diagrams were determined, and the systems extraction performance for a wide range of biomolecules (phenolic compounds, alkaloids and amino acids) was investigated. The results obtained show that ILs as adjuvants in polymer-salt ATPS modulate the partition of biomolecules. In particular, more hydrophobic ILs significantly enhance the partition of more hydrophobic biomolecules to the PEG-rich phase (where the IL is enriched). Furthermore, the intensity of the IL effect is more pronounced when using weak salting-out agents. A linear correlation between the biomolecules and the ILs partition coefficients, and with the biomolecules octanol-water partition coefficients, was found. In most ATPS formed by polymers and salts using ILs as adjuvants, the biomolecules partition is driven by the ILs partition and by the difference in hydrophobicity between the coexisting phases.

Keywords

aqueous biphasic systems; partition; extraction; ionic liquids; adjuvants; biomolecules

1 Introduction

Aqueous two-phase systems (ATPS) are formed by the dissolution of two polymers, a polymer and a salt, or two salts in water [1–8]. Conventional polymer-based ATPS exhibit however a small range of polarities differences between the two phases, narrowing thus their

*Corresponding author. Tel.: +351 234401507. jcoutinho@ua.pt.

application in separation processes [7,9–11]. Alternative ATPS constituted by ionic liquids (ILs) and salts have been proposed to complement ATPS based on polymers [12]. As a result of the ILs diversity in terms of chemical structures, IL-ATPS provide a wider range of polarities between their phases [13,14]. This translates into more efficient, and often more selective, extraction and separation processes. However, due to the high solubility of most ILs in water at room temperature, usually large concentrations of salts are required to promote the ATPS formation, turning these processes more expensive and less sustainable. The use of small quantities of ILs as adjuvants in polymer-based ATPS appears as an alternative to overcome these difficulties [7,11,16–19].

Pereira et al. [7] studied the L-tryptophan partition in ATPS composed of polyethylene glycol with a molecular weight of 600 g·mol⁻¹ (PEG 600) and sodium sulfate (Na₂SO₄) with the addition of 5 wt% of imidazolium-based ILs as adjuvants. The authors showed that the partition coefficient (*K*) of L-tryptophan is mainly dependent on the IL nature and on the IL partition to the polymer-rich phase. ILs were also used in concentrations ranging between (5–10) wt% as adjuvants in ATPS constituted by PEG 300 and Na₂SO₄ for the extraction of gallic, vanillic and syringic acids [20]. The addition of only 5 wt% of imidazolium-based ILs provide enhanced extraction efficiencies (*EE*%), varying between 80% and 99%, when compared with the ternary ATPS with no IL added. Ferreira et al. [17] studied ATPS composed of PEG 400 and potassium citrate buffer at pH 7.0 with ILs as adjuvants to extract Immunoglobulin G (IgG) from rabbit serum. An increase in the *EE*% of IgG was observed using 5 wt% of ILs. All these results indicate that the chemical nature of the IL and its partition between the coexisting phases controls the preferential partition of biomolecules to the phase in which the IL is enriched. In addition to the previously described works and contrarily to those findings [7, 17, 20], in other works the use of ILs as adjuvants does not lead to the preferential migration of the target biomolecule to the polymer-rich phase or improvements on the partition coefficients. Aziz et al. [19] used ATPS formed by PEGs of different molecular weights and potassium phosphate, potassium citrate and sodium acetate to extract β-mannanase. The authors showed that 3 wt% of [C₄mim][BF₄] (1-butyl-3-methylimidazolium tetrafluoroborate) in the ATPS formulation enhances the β-mannanase partition to the salt-rich phase. Souza et al. [11] studied the partition of two dyes in ATPS composed of PEG 1500 and 8000 and a phosphate buffer at pH 7.0 with 5 wt% of ILs as adjuvants. It was shown that the ILs have a minor effect upon the ATPS formation, and that the partition coefficients values obtained in the systems with ILs are lower than in systems without them. The same authors [16] studied the extraction of lipase produced by submerged fermentation by *Bacillus* sp. ITP-001 using imidazolium-based ILs as adjuvants (5 wt%) in ATPS composed of PEG (1500, 4000, 6000 and 8000) and potassium phosphate buffer at pH 7. Although the authors obtained higher purification factors for the enzyme in quaternary systems containing 1-hexyl-3-methylimidazolium chloride ([C₆mim]Cl) as adjuvant than in ternary ATPS (PEG + salt + water), the opposite behavior was observed in presence of the same ILs family with shorter ([C₂mim]Cl, [C₄mim]Cl) and longer ([C₈mim]Cl) alkyl side chains length. Chloride-based ILs were also used as adjuvants in ATPS composed of PEG 400 and potassium citrate buffer pH 7.0 to enhance the extraction of phenolic antioxidants, alkaloids and amino acids [18]. The use of those ILs showed a favorable, yet small, effect on

the partition coefficients of most of the biomolecules studied when compared with the ternary systems composed of PEG + salt + water and IL + salt + water.

The results previously reported in the literature show that, although it is possible to induce an enhanced performance of polymer-based ATPS by using ILs as adjuvants, this is not a universal trend, and that the outcome is influenced by a diversity of effects. The effect of ILs seems to be dependent not only on the nature of the biomolecule and of the IL, as highlighted in our previous work [18], but also on the salt used to form ATPS. In summary, contradictory results have been found, and in all works reported up to date the intensity of the IL impact seems to be masked by the salts used, which were most of the times strong salting-out agents. Therefore, this work focuses on the study of several quaternary ATPS constituted by PEG 400 and a weak salting-out inducing agent (ammonium sulfate $(\text{NH}_4)_2\text{SO}_4$) using ILs as adjuvants. The hypothesis to be evaluated on this work is if the salt impact is minimized on the biomolecules partition, it would be possible to better address and understand the IL impact on the biomolecules partition. For this purpose, a series of chloride-based ILs with different cations, namely cholinium, 1-butyl-3-methylimidazolium, 1-butyl-1-methylpyrrolidinium, 1-butyl-1-methylpiperidinium, tetrabutylammonium and tetrabutylphosphonium were used as adjuvants at 5 wt% in PEG 400 + $(\text{NH}_4)_2\text{SO}_4$ ATPS. The respective pseudo-ternary phase diagrams were determined at 298 K and the IL effect on the partition of phenolic compounds (vanillic acid, gallic acid and eugenol), alkaloids (nicotine and caffeine) and amino acids (L-tryptophan, L-phenylalanine and L-tyrosine) evaluated.

2 Experimental section

2.1 Materials

The ATPS studied in this work are constituted by water, ammonium sulfate $(\text{NH}_4)_2\text{SO}_4$, polyethylene glycol with a molecular weight of 400 $\text{g}\cdot\text{mol}^{-1}$ (PEG 400) and several ILs at 5 wt%. PEG 400 was supplied by Sigma-Aldrich, while $(\text{NH}_4)_2\text{SO}_4$ (99 wt% pure) was purchased from Merck. The water employed was double distilled, passed across a reverse osmosis system and finally treated with a Milli-Q plus 185 water purification apparatus. The ILs studied were: 1-butyl-3-methylimidazolium chloride, $[\text{C}_4\text{mim}]\text{Cl}$ (> 99% pure); 1-butyl-1-methylpyrrolidinium chloride, $[\text{C}_4\text{mpyr}]\text{Cl}$ (> 99% pure); 1-butyl-1-methylpiperidinium chloride $[\text{C}_4\text{mpip}]\text{Cl}$ (> 99% pure); tetrabutylammonium chloride, $[\text{N}_{4444}]\text{Cl}$ (> 97 wt% pure); tetrabutylphosphonium chloride $[\text{P}_{4444}]\text{Cl}$ (> 96% pure); and cholinium chloride, $[\text{Ch}]\text{Cl}$ (> 98 wt% pure). The $[\text{C}_4\text{mim}]\text{Cl}$, $[\text{C}_4\text{mpyr}]\text{Cl}$ and $[\text{C}_4\text{mpip}]\text{Cl}$ were purchased from Iolitec, while $[\text{N}_{4444}]\text{Cl}$ and $[\text{Ch}]\text{Cl}$ were acquired from Sigma-Aldrich and Acros Organics, respectively. $[\text{P}_{4444}]\text{Cl}$ was kindly supplied by Cytec Ind. The chemical structures, predictive hydrogen-bond acidity (α) [21] and molar volume (V_m) of the studied ILs are presented in Table 1.

The biomolecules Vanillic Acid (VanAc, 97 wt% pure), Eugenol (Eug, 99 wt% pure), L-Tryptophan (Trp, 99 wt% pure) and L-Phenylalanine (Phen, 99 wt% pure) were acquired from Sigma-Aldrich. Nicotine (Nic, 99 wt% pure), Caffeine (Caf, 99 wt% pure) and L-Tyrosine (Tyr, 99 wt% pure) were supplied by Fluka. Gallic Acid (GalAc, 99.5 wt% pure) was purchased from Merck. The molecular structures of the biomolecules investigated and

the logarithm of their octanol-water partition coefficient ($\log K_{ow}$) [22] and molecular weight (M_w) are given in Table 2.

2.2 Phase diagrams

The binodal curves were obtained by the cloud point titration method at (298 ± 1) K and at atmospheric pressure [23]. Aqueous solutions of ammonium sulfate at 40 wt% and PEG 400 at 80 wt% were prepared for the determination of PEG-salt-water phase diagrams. For the determination of the liquid-liquid phase diagrams with ILs as adjuvants, water and the aqueous solutions of salt and PEG 400 contained 5 wt% of the respective IL, allowing to keep the IL concentration constant along all the phase diagram regions. Repetitive drop-wise addition of the PEG solution to the saline solution was carried out until the detection of a cloudy solution, followed by the drop-wise addition of water until the detection of a clear solution. This procedure was carried out under constant stirring and temperature. The systems compositions were determined by the weight quantification of all components added with an uncertainty of $\pm 10^{-4}$ g.

The experimental binodal curves were fitted using Eq. (1) [24]:

$$[\text{PEG}] = A \exp[(B[\text{Salt}]^{0.5}) - (C[\text{Salt}]^3)] \quad (1)$$

where $[\text{PEG}]$ and $[\text{Salt}]$ are the PEG 400 and salt weight percentages (wt%), and A , B and C are fitting parameters obtained by regression of the experimental data.

The tie-lines (TLs), which give the composition of each phase for a given mixture composition, were determined by a gravimetric method described by Merchuk *et al.* [24]. The TLs were determined for two mixture points at the biphasic region: 25 wt% PEG 400 + 18 wt% $(\text{NH}_4)_2\text{SO}_4$ + 52 wt% H_2O + 5 wt% IL and 22 wt% PEG 400 + 18 wt% $(\text{NH}_4)_2\text{SO}_4$ + 55 wt% H_2O + 5 wt% IL. These mixtures correspond to the ones used in the extraction experiments. These mixtures were vigorously stirred and allowed to equilibrate at (298 ± 1) K and atmospheric pressure for at least 12 h. The top and bottom phases were carefully separated and weighted. Finally, each TL was determined by the application of the lever-arm rule (Eqs. 2 to 5) to the relationship between the weight of the top and bottom phases and the overall system composition.

$$[\text{PEG}]_T = A \exp[(B[\text{Salt}]_T^{0.5}) - (C[\text{Salt}]_T^3)] \quad (2)$$

$$[\text{PEG}]_B = A \exp[(B[\text{Salt}]_B^{0.5}) - (C[\text{Salt}]_B^3)] \quad (3)$$

$$[\text{PEG}]_T = \frac{[\text{PEG}]_M}{\gamma} - \frac{1-\gamma}{\gamma} \times [\text{PEG}]_B \quad (4)$$

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

where the subscripts “T”, “B”, and “M” designate the top phase, the bottom phase and the mixture, respectively; and γ is the ratio between the mass of the top phase and the total mass of the mixture. In the studied systems, the top phase corresponds to the polymer-rich phase, whereas the bottom phase is enriched in salt and water.

For the calculation of the tie-line length (TLL), the following equation (Eq. 6) was used:

$$TLL = \sqrt{([\text{Salt}]_T - [\text{Salt}]_B)^2 + ([\text{PEG}]_T - [\text{PEG}]_B)^2} \quad (6)$$

2.3 Partition of biomolecules

To better understand the impact of the use of ILs as adjuvants in polymer-salt ATPS, partition studies of the selected biomolecules in ATPS with and without ILs were carried out. These studies were carried out at (298 ± 1) K, in two mixture points: 25 wt% PEG 400 + 18 wt% $(\text{NH}_4)_2\text{SO}_4$ + 52 wt% aqueous solution containing the target biomolecule + 0/5 wt% IL and 22 wt% PEG 400 + 18 wt% $(\text{NH}_4)_2\text{SO}_4$ + 55 wt% aqueous solution containing the target biomolecule + 0/5 wt% IL. These mixture points were chosen with the objective of addressing the phase composition effect in the biomolecules partition coefficients. Aqueous solutions of each biomolecule were prepared at the following concentrations: $0.5 \text{ g}\cdot\text{L}^{-1}$ for Gallic and Vanillic Acids; $0.1 \text{ g}\cdot\text{L}^{-1}$ for Eugenol and L-Tyrosine; $1.0 \text{ g}\cdot\text{L}^{-1}$ for Caffeine, Nicotine and L-Tryptophan; and $3.0 \text{ g}\cdot\text{L}^{-1}$ for L-Phenylalanine. All components were weighted and the ATPS vigorously stirred until all the components were dissolved, and then left to equilibrate at (298 ± 1) K for at least 12 h. The top and bottom phases were carefully separated and weighted. The biomolecule content in each phase was quantified through UV-spectroscopy, using a synergy/HT microplate reader (Biotek, USA), at a wavelength of 262 nm for Gallic Acid, 292 nm for Vanillic Acid, 279 nm for Eugenol and L-Tryptophan, 273 nm for Caffeine, 260 nm for Nicotine, 275 nm for L-Tyrosine and 258 nm for L-Phenylalanine, using established calibration curves. To avoid the interference of the ATPS components in the biomolecules quantification, blank control samples (without the presence of the biomolecule) were always used.

The partition coefficients of the biomolecules, K_{Biom} , were determined according to Eq. (7),

$$K_{\text{Biom}} = \frac{[\text{Biom}]_T}{[\text{Biom}]_B} \quad (7)$$

where $[\text{Biom}]_T$ and $[\text{Biom}]_B$ are the biomolecule concentrations ($\text{g}\cdot\text{L}^{-1}$) in the top and bottom phases, respectively. The partition coefficients obtained are an average of at least two individual measurements with the respective standard deviations.

2.3 Partition of ILs

The partition coefficients of each IL in ATPS were also determined to address their effect on the biomolecules partition between the two phases. The systems were prepared as described for the biomolecules partition. The IL concentration in each phase was quantified using a Metrohm 904 Titrando ion chloride electrode. A stock aqueous solution of potassium chloride (KCl, 1 mol·L⁻¹) was prepared and diluted at appropriate concentrations (between 10⁻⁴ and 10⁻¹ mol·L⁻¹) to establish the calibration curve. A total ionic strength adjustment buffer (TISAB) was prepared by mixing aqueous solutions of potassium nitrate (KNO₃), acetic acid (C₂H₄O₂) and sodium acetate (C₂H₃NaO₂) at 0.1 mol·L⁻¹. This solution was added to all standard solutions and samples to maintain the ionic strength during the measurements. The partition coefficient of each IL, K_{IL} , was determined according to Eq. (8).

$$K_{IL} = \frac{[IL]_T}{[IL]_B} \quad (8)$$

where $[IL]_T$ and $[IL]_B$ are the concentrations (g·L⁻¹) of the IL in the top and in the bottom phases, respectively. The top phase corresponds to the PEG-rich phase in all systems investigated. The partition coefficients obtained are an average of at least two individual measurements with the respective standard deviations.

3 Results and Discussion

3.1 Phase diagrams

The binodal curves for the ternary system (PEG 400 + (NH₄)₂SO₄ + H₂O) and quaternary systems (PEG 400 + (NH₄)₂SO₄ + H₂O + 5 wt% IL) were determined at (298 ± 1) K and atmospheric pressure. The respective phase diagrams in an orthogonal representation, where the amount of water is omitted, are depicted in Fig. 1. It should be remarked that the IL concentration was always kept at 5 wt% and was considered as part of the solvent in the phase diagrams representation. The detailed experimental data and the regression parameters obtained by Eq. (1) are reported in the Supporting Information (Tables S1 and S2).

All mixture compositions above each binodal curve shown in Fig. 1 result in two-phase systems formation, and the closer to the axes the curve is the lower the amount of the phase-forming components required to create ATPS. The obtained results show that the addition of any of the ILs here studied leads to an increase in the biphasic region, *i.e.* due to the presence of an IL, lower amounts of salt and polymer are necessary to promote phase separation, and as observed in previous works [20,25]. The ability of ILs as adjuvants to induce the formation of two-phase systems follows the trend: [P₄₄₄₄]Cl ≈ [N₄₄₄₄]Cl > [C₄mpip]Cl ≈ [C₄mpyr]Cl ≈ [C₄mim]Cl > [Ch]Cl. The most hydrophilic IL ([Ch]Cl) is the one with the lowest impact on phase formation, followed by the cyclic and aromatic ILs that display a similar behavior. On the other hand, the quaternary ammonium and phosphonium salts have the most significant effect upon the phase diagrams, leading to a more significant increase of the biphasic region. This trend correlates with the trend observed in ATPS

formed by ILs and salts [20,26], where more hydrophobic and higher volume ILs are more easily salted-out and more easily create two-phase systems [27] (the ILs hydrogen-bond acidity and molar volume data are given in Table 1). Overall, it seems that mixtures of IL–PEG are more hydrophobic than the respective ternary system with no IL added. All ILs preferentially migrate to the PEG-rich phase (as will be discussed below) increasing its hydrophobicity, being thus more easily salted-out by the salt in aqueous media.

3.2 Partition of ILs

All the investigated ILs display partition coefficients (K_{IL}) higher than 1.0, meaning that there is the preferential IL partition to the most hydrophobic phase (PEG-rich phase), as shown in Fig. 2. The K_{IL} values range between 1.94 and 6.38 (detailed data are given in the Supporting information, Table S4). The K_{IL} values increase in the following order: [Ch]Cl < [C4mim]Cl < [C4mpyr]Cl < [C4mpip]Cl < [P4444]Cl < [N4444]Cl. Higher partition coefficients of the IL to the PEG-rich phase are observed with the more hydrophobic ILs in both tie-lines investigated. This sequence closely follows the trend obtained for the ILs impact on the phase diagrams discussed above. According to characteristics of the ILs described in Table 1, ILs with higher molar volumes (V_m) are also more easily salted-out and more easily create ATPS [27]. ILs with a lower value of hydrogen-bond acidity (α) are also less able to establish hydrogen-bonds with water, and are therefore more easily salted-out to the PEG-rich phase [26,28].

3.3 Partition of biomolecules

In a previous work [18] we studied the effect of ILs as adjuvants in ATPS formed by PEG and potassium citrate. It was found that the partition of more hydrophilic biomolecules in IL + salt ABS seems to be ruled by specific interactions with the IL, whereas the partition of more hydrophobic biomolecules in PEG + salt and PEG + salt + IL is to be governed by the differences in the phases hydrophobicities. Although some novel insights have been unveiled, potassium citrate is a strong salting-out agent (according to the Hofmeister series [29]) that may mask the IL effect on the biomolecules partition. In fact, only small differences have been observed in the partition behavior of the several biomolecules [18]. In this work, a weaker salting-out agent according to the Hofmeister series [29], namely $(\text{NH}_4)_2\text{SO}_4$, was used aiming at better understanding the impact of ILs when used as adjuvants in polymer-salt ATPS. According to the Hofmeister series [29], the divalent SO_4^{2-} is a weaker salting-out anion than the trivalent anion $\text{C}_6\text{H}_5\text{O}_7^{3-}$, i.e. the former has a higher aptitude to be solvated by water and to create hydration complexes. The salting-out aptitude of each ion is closely correlated to their molar Gibbs energy of hydration and molar entropy of hydration, although the molar entropy of hydration of the salt ions is the driving force in aqueous two-phase system formation as previously demonstrated [30].

Partition studies of a wide range of biomolecules (phenolic compounds, amino acids and alkaloids) at two mixture compositions have been carried out. The composition of the phases and respective TLL at which the partition studies were carried out are given in Table S3 in the Supporting Information. The partition coefficients obtained at (298 ± 1) K are shown in Figs. 3 to 5. The respective detailed data are given in Table S4 in the Supporting Information. In general, it is observed a significant effect upon the partition coefficients of

all biomolecules by using ILs as adjuvants in PEG + salt ATPS when ammonium sulfate is used instead of potassium citrate, validating our hypothesis.

According to Figs. 3 to 5, all biomolecules preferentially partition to the polymer-rich phase (more hydrophobic phase, $K_{\text{Biom}} > 1$), suggesting a moderate salting-out effect of ammonium sulphate and preferential interactions between the biomolecules and the PEG-rich phase components. Moreover, based on the partition coefficient values, both phenomena can be modulated by the ILs presence and nature. Unlike observed in our previous work [18], significant differences between the various ILs investigated are now observed, showing that the nature of the IL cation has a relevant influence on the biomolecules partition. The use of more hydrophobic ILs ([N₄₄₄₄]Cl and [P₄₄₄₄]Cl) as adjuvants leads to a strong enhancement of the biomolecules partition to the PEG-rich phase, followed by a less relevant, yet positive, effect observed with [C₄mim]Cl, [C₄pyr]Cl and [C₄mpip]Cl (ILs with intermediate polarity as addressed by the hydrogen-bond acidity data shown in Table 1). On the other hand, the most hydrophilic IL ([Ch]Cl) has the opposite effect, leading to a decrease on the partition coefficients of almost all biomolecules investigated when compared with the system where no IL was added. Although these results seem to be driven by the difference in hydrophobicity between the two phases, in some cases specific interactions may be present, e.g. observed with nicotine in the ATPS containing [P₄₄₄₄]Cl.

According to Fig. 3, all the studied phenolic compounds extensively partition to the PEG-rich phase, with K_{Biom} values $\gg 1$, following the trend: Eugenol ($K_{\text{Eug}} = 26 - 253$) > Vanillic Acid ($K_{\text{VanAc}} = 12 - 81$) > Gallic Acid ($K_{\text{GalAc}} = 5 - 67$). This trend closely follows the biomolecules $\log K_{\text{ow}}$ values: Gallic Acid is 0.70; Vanillic Acid is 1.33; and Eugenol is 2.39 [22]. In summary, all phenolic compounds studied show a higher affinity to the more hydrophobic PEG-rich phase, which increases when using more hydrophobic ILs, such as quaternary ammonium and phosphonium salts.

For the amino acids partition assays, given in Fig. 4, the K_{Biom} values increase with the amino acids $\log K_{\text{ow}}$ values (Tryptophan is -1.06; Phenylalanine is -1.38; and Tyrosine is -2.26 [22]). As observed with the phenolic compounds, the higher the hydrophobicity of the biomolecule, the higher is the partition coefficient or preferential migration to the PEG-rich phase, following the order: Tryptophan ($K_{\text{Trp}} = 3 - 15$) > Phenylalanine ($K_{\text{Phen}} = 2 - 3$) $\approx$ Tyrosine ($K_{\text{Tyr}} = 1 - 3$). A lower impact was observed by the ILs used as adjuvants when addressing their effect on the amino acids partition compared to the phenolic compounds discussed before. According to these biomolecules K_{ow} values, the studied amino acids are less hydrophobic than Eugenol, Vanillic Acid, and Gallic Acid, and as such their partition coefficients are lower as well as the IL impact.

For the alkaloids – results given in Fig. 5 – an opposite trend was observed. Although nicotine ($\log K_{\text{ow}} = 1.17$ [22]) is more hydrophobic than caffeine ($\log K_{\text{ow}} = -0.07$ [22]), the former more significantly partitions to the PEG-rich phase, indicating that a more complex phenomenon takes place. In fact, the use of most hydrophobic ILs as adjuvants, such as [N₄₄₄₄]Cl and [P₄₄₄₄]Cl, is more relevant to improve the extraction of caffeine to the PEG-rich phase. On the other hand, nicotine is better extracted to the PEG-rich phase when using

imidazolium-based ILs. The partition coefficients of caffeine range between 13 and 52, whereas those of nicotine range from 0.4 to 4.

In general, for all biomolecules, the K values increase with the TLL. The respective TL and TLL data are given in Table S3 in the Supporting Information. With the increasing difference in the phase's compositions as the TLL increases, the PEG-rich phase becomes more hydrophobic, thus improving the partition of the investigated biomolecules to this phase.

The results reported in the literature [11,18,20,31–32] and those obtained in this work confirm that the chemical nature of the salt and of the IL are key features to manipulate the preferential partition of biomolecules to a given phase. The results obtained support the idea that ILs, when used as adjuvants in polymer-based ATPS, can modulate the partition behavior of biomolecules according to their hydrophobicity. Yet, a comparison with results from previous works [11,18,20] shows that the intensity of this effect is strongly dependent of the nature of the salt used. Strong salting-out inducing salts induce the partition of the biomolecules to the most hydrophobic phase, minimizing and masking the effect of the IL as additive. Weaker salting-out agents allow the enhancement of the IL effect as additive, not only in terms of tuning the hydrophobicity of the phases, but also by promoting the occurrence of specific interactions between the ILs and the biomolecules. In summary, the use of ILs as adjuvants in conventional polymer-salt systems can provide improved extraction efficiencies, and these can be maximized by a correct selection of the salt and IL employed.

3.4 Correlation between the biomolecules and ILs partition

The relationship between the partition coefficients of all biomolecules as function of the IL partition coefficient is shown in Fig. 9. A similar approach was previously reported by Sousa et al. [18]. In that work, the partition coefficients of the biomolecules increased with the IL content at the PEG-rich phase, and this increase was proportional to the $\log K_{ow}$ of the biomolecules. The same behavior is here observed. There is a linear relationship between the partition coefficients of the biomolecules to the PEG-rich phase and the IL partition coefficient, and this dependence is further proportional to the octanol-water partition coefficients of the studied biomolecules. However, the effect of ILs as adjuvants is more pronounced in this work, as assessed by the axes scales and linear correlations slopes, by using a weaker salting-out salt such as ammonium sulfate. By using a weaker salting-out inducing agent the effect of ILs as additives is not masked by the effect of the salt upon the biomolecules partition. The presence of IL to enhance the extraction of biomolecules to the PEG-rich phase is significantly more relevant when dealing with more hydrophobic biomolecules (e.g. Eugenol), while the opposite is observed with more hydrophilic biomolecules (e.g. L-Tyrosine, the most hydrophilic compound here investigated). Nicotine was identified as an outsider to this trend, and for this particular case specific interactions may govern its partition between the coexisting phases. Nicotine was also identified as an outsider in our previous work [18]. In summary, the favorable partition of more hydrophobic biomolecules in PEG + salt + IL ATPS seems to be governed by the ILs partition and by the

differences in the phases hydrophobicities, in which the addition of more hydrophobic ILs is beneficial to improve the extraction performance.

4 Conclusions

ILs have been proposed as adjuvants in ternary systems of PEG + salt + water to improve the extraction of biomolecules. However, these works showed inconsistent results, where the IL presence can increase or decrease the partition intensity of the target biomolecule to the polymer-rich phase, which may be a reflection of the low IL impact that is masked by the strong salting-out salts used. In this work, a weak salting-out inducing agent $((\text{NH}_4)_2\text{SO}_4)$ was used to prepare ATPS, aiming at minimizing the salting-out effect of the salt upon the partition of the biomolecules, thus allowing to better identify the IL adjuvant effect. A large number of chloride-based ILs and a wide number of biomolecules (including phenolic compounds, amino acids and alkaloids) were investigated. It is here shown that, unlike with other salts previously studied, the use of ILs as adjuvants, even at a low concentration of 5 wt%, has a significant effect upon the partition coefficients of most biomolecules studied when compared with the ATPS with no IL added. The effect of the IL cation nature was clearly identified, where more hydrophobic cations induce a more pronounced increase on the partition of more hydrophobic biomolecules to the PEG-rich phase. A linear correlation between the partition coefficients of the biomolecules and of the ILs to the PEG-rich phase, and among these and the octanol-water partition coefficients of the studied biomolecules, was found. In summary, in most ATPS formed by polymers and salts using ILs as adjuvants, the biomolecules partition is driven by the ILs partition and by the differences in hydrophobicities between the two phases, in which the addition of hydrophobic ILs is particularly relevant to improve the biomolecules partition to the polymer-IL-enriched phase.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgements

This work was developed in the scope of the project CICECO-Aveiro Institute of Materials (Ref. FCT UID /CTM / 50011/2013), financed by national funds through the FCT/MEC and co-financed by FEDER under the PT2020 Partnership Agreement, and the Multibiorefinery (POCI-01-0145-FEDER-016403) project. C. M. S. S. Neves acknowledges FCT for the postdoctoral grant SFRH/BPD/109057/2015. M. M. Pereira acknowledges the PhD grant (2740-13-3) and financial support from Coordenação de Aperfeiçoamento de Pessoal de Nível Superior – Capes. Rita de Cássia S. Sousa acknowledges the Post-doctoral grant (200833/2015-4/PDE) and financial support from Conselho Nacional de Desenvolvimento Científico e Tecnológico – CNPq. M. G. Freire acknowledges the European Research Council (ERC) for the Starting Grant ECR-2013-StG-337753.

References

- [1]. Albertsoon, PA. Partition of cell particles and macromolecules. 1986.
- [2]. Z, BY. Aqueous Two-Phase Partitioning, Physical Chemistry and Bioanalytical Applications. Marcel Dekker; New York: 1995.
- [3]. F, D, Walter, H, Brooks, DE. Partitioning in Aqueous Two-Phase Systems. Academic Press; New York: 1985.
- [4]. Hatti-Kaul R. Aqueous Two-Phase Systems: A General Overview. Mol Biotechnol. 2001; 19:269–278. DOI: 10.1385/MB:193:269 [PubMed: 11721623]

- [5]. Hatti-Kaul R. Aqueous Two-Phase Systems. *J Chem Inf Model*. 2000; 11:1689–1699. DOI: 10.1385/1592590284
- [6]. 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. DOI: 10.1002/chem.201101780 [PubMed: 22223510]
- [7]. Pereira JFB, Lima AS, Freire MG, Coutinho JAP. Ionic liquids as adjuvants for the tailored extraction of biomolecules in aqueous biphasic systems. *Green Chem*. 2010; 12:1661–1669. DOI: 10.1039/C003578E
- [8]. Lin X, Wang Y, Zeng Q, Ding X, Chen J. Extraction and separation of proteins by ionic liquid aqueous two-phase system. *Analyst*. 2013; 138:6445–53. DOI: 10.1039/c3an01301d [PubMed: 24013164]
- [9]. Hamzehzadeh S, Vasiresheh M. Ionic liquid 1-butyl-3-methylimidazolium bromide as a promoter for the formation and extraction capability of poly(ethylene glycol)-potassium citrate aqueous biphasic system at T=298.15K. *Fluid Phase Equilib*. 2014; 382:80–88. DOI: 10.1016/j.fluid.2014.08.029
- [10]. Pereira JFB, Rebelo LPN, Rogers RD, Coutinho JAP, Freire MG. Combining ionic liquids and polyethylene glycols to boost the hydrophobic–hydrophilic range of aqueous biphasic systems. *Phys Chem Chem Phys*. 2013; 15:19580.doi: 10.1039/c3cp53701c [PubMed: 24145792]
- [11]. De Souza RL, Campos VC, Ventura SPM, Soares CMF, Coutinho JAP, Lima AS. Fluid Phase Equilibria Effect of ionic liquids as adjuvants on PEG-based ABS formation and the extraction of two probe dyes. *Fluid Phase Equilib*. 2014; 375:30–36. DOI: 10.1016/j.fluid.2014.04.011
- [12]. Gutowski KE, Broker GA, Willauer HD, Huddleston JG, Swatoski 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. DOI: 10.1021/ja0351802 [PubMed: 12769563]
- [13]. 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. DOI: 10.1039/c2cs35151j [PubMed: 22692625]
- [14]. Luís A, Dinis TBV, Passos H, Taha M, Freire MG. Good's buffers as novel phase-forming components of ionic-liquid-based aqueous biphasic systems. *Biochem Eng J*. 2015; 101:142–149. DOI: 10.1016/j.bej.2015.05.008 [PubMed: 26435687]
- [15]. Rogers RD, Seddon KR. Chemistry. Ionic liquids – solvents of the future? *Science*. 2003; 302:792–3. DOI: 10.1126/science.1090313 [PubMed: 14593156]
- [16]. Souza RL, Ventura SPM, Soares CMF, Coutinho JAP, Lima AS. Lipase purification using ionic liquids as adjuvants in aqueous two-phase systems. *Green Chem*. 2015; 17:3026–3034. DOI: 10.1039/C5GC00262A
- [17]. Ferreira AM, Faustino VFM, Mondal D, Coutinho JAP, Freire MG. Improving the extraction and purification of immunoglobulin G by the use of ionic liquids as adjuvants in aqueous biphasic systems. *J Biotechnol*. 2016; 236:166–175. DOI: 10.1016/j.jbiotec.2016.08.015 [PubMed: 27568168]
- [18]. de CS Sousa R, Pereira MM, Freire MG, Coutinho JAP. Evaluation of the effect of ionic liquids as adjuvants in polymer-based aqueous biphasic systems using biomolecules as molecular probes. *Sep Purif Technol*. 2017; 196:244–253. DOI: 10.1016/j.seppur.2017.07.018 [PubMed: 30271267]
- [19]. Aziz NFHA, Abbasiliasi S, Ng HS, Phapugrangkul P, Bakar MHA, Tam YJ, Tan JS. Purification of β -mannanase derived from *Bacillus subtilis* ATCC 11774 using ionic liquid as adjuvant in aqueous two-phase system. *J Chromatogr B*. 2017; 1055–1056:104–112. DOI: 10.1016/j.jchromb.2017.04.029
- [20]. Almeida MR, Passos H, Pereira MM, Á.Lima S, Coutinho JAP, Freire MG. Ionic liquids as additives to enhance the extraction of antioxidants in aqueous two-phase systems. *Sep Purif Technol*. 2014; 128:1–10. DOI: 10.1016/j.seppur.2014.03.004

- [21]. Kurnia KA, Lima F, Cláudio AFM, Coutinho JAP, Freire MG. Hydrogen-bond acidity of ionic liquids: an extended scale. *Phys Chem Chem Phys*. 2015; 17:18980–18990. DOI: 10.1039/C5CP03094C [PubMed: 26129926]
- [22]. PubChem. Open Chemistry database. PubChem Substance and Compound database. 2016. <https://pubchem.ncbi.nlm.nih.gov/>
- [23]. 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. DOI: 10.1021/jp900293v [PubMed: 19309125]
- [24]. Merchuk JC, Andrews BA, Asenjo JA. Aqueous two-phase systems for protein separation. Studies on phase inversion. *J Chromatogr B*. 1998; 711:285–293. DOI: 10.1016/S0378-4347(97)00594-x
- [25]. de CS Sousa R, Neves CMSS, Pereira MM, Freire MG, Coutinho JAP. Potential of Aqueous Two-Phase Systems for the Separation of Levodopa from Similar Biomolecules. *J Chem Technol Biotechnol*. 2017; 93:1940–1947. DOI: 10.1002/jctb.5553 [PubMed: 30275632]
- [26]. 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; 67:68–76. DOI: 10.1016/j.bej.2012.05.004
- [27]. Ventura SPM, Sousa SG, Serafim LS, Lima ÁS, 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. DOI: 10.1021/je200714h
- [28]. Ventura SPM, Sousa SG, Serafim LS, Lima ÁS, Freire MG, Coutinho JAP. Ionic-liquid-based aqueous biphasic systems with controlled pH: The ionic liquid anion Effect. *J Chem Eng Data*. 2012; 57:507–512. DOI: 10.1021/je2010787
- [29]. Hofmeister F. Zur lehre von der wirkung der salze [Title translation: About the science of the effect of salts]. *Arch Exp Pathol Pharmacol*. 1888; 24:247–260.
- [30]. Shahriari S, Neves CMSS, Freire MG, Coutinho JAP. Role of the Hofmeister Series in the Formation of Ionic-Liquid-Based Aqueous Biphasic Systems. *J Phys Chem B*. 2012; 116:7252–7258. DOI: 10.1021/jp300874u [PubMed: 22594382]
- [31]. Dreyer S, Salim P, Kragl U. Driving forces of protein partitioning in an ionic liquid-based aqueous two-phase system. *Biochem Eng J*. 2009; 46:176–185. DOI: 10.1016/j.bej.2009.05.005
- [32]. Li X, Liu Y, Li F. Effects of DC electric field on phase equilibrium and partitioning of ionic liquid-based aqueous two-phase systems. *Biochem Eng J*. 2017; 126:146–154. DOI: 10.1016/j.bej.2016.09.008

Fig. 1.

Phase diagrams at (298 ± 1) K for the ternary system composed of PEG 400 + ammonium sulfate + water, $*$; and quaternary systems composed of PEG 400 + $(\text{NH}_4)_2\text{SO}_4$ + water + 5 wt% IL: $\blacksquare$, [Ch]Cl; $\times$, [C₄mim]Cl; $\blacklozenge$, [C₄mpip]Cl; $\blacktriangle$, [C₄mpyr]Cl; $\bullet$, [N₄₄₄₄]Cl; $+$, [P₄₄₄₄]Cl. The lines were obtained by fitting the experimental data with Eq. (1). The symbol $\circ$ represents the mixture compositions used in the partition experiments and the red lines the TLs corresponding to the ternary system obtained by Eqs. (1 to 5).

Fig. 2. Partition coefficients of ILs (K_{IL}) in ATPS composed of PEG 400 + $(\text{NH}_4)_2\text{SO}_4$ + water + 5 wt% IL at two mixture points.

Fig. 3. Partition coefficients (K) of (a) Gallic Acid, (b) Vanillic Acid and (c) Eugenol in ATPS composed of PEG 400 + $(NH_4)_2SO_4$ (no IL) and of PEG400 + $(NH_4)_2SO_4$ + 5 wt% IL at two mixture points.

Fig. 4. Partition coefficients (K) of (a) L-Tryptophan, (b) L-Phenylalanine and (c) L-Tyrosine in ATPS composed of PEG 400 + $(\text{NH}_4)_2\text{SO}_4$ (no IL) and of PEG400 + $(\text{NH}_4)_2\text{SO}_4$ + 5 wt% IL at two mixture points.

Fig. 5. Partition coefficients (K) of (a) Nicotine and (b) Caffeine in ATPS composed of PEG 400 + $(\text{NH}_4)_2\text{SO}_4$ (No IL) and of PEG400 + $(\text{NH}_4)_2\text{SO}_4$ + 5 wt% IL at two mixture points.

Fig. 6. Correlation between K_{Biom} and K_{IL} in the quaternary ATPS for the mixture point 25 wt% PEG 400 + 18 wt% $(NH_4)_2SO_4$ + 52 wt% H_2O + 5 wt% IL: (a) antioxidants; (b) amino acids and (c) alkaloids. (d) Slope of the curve of K_{Biom} vs. K_{IL} as a function of $\log K_{ow}$. Dotted line corresponds to linear regressions.

Table 1
Chemical structures, predicted hydrogen-bond acidity (α) [21] and molar volume (V_m) of ILs.

IL	Chemical structure	α	$V_m / (\text{cm}^3 \cdot \text{mol}^{-1})^a$
[Ch]Cl		1.555	123.06
[C ₄ mim]Cl		0.986	154.40
[C ₄ mpyr]Cl		0.537	169.49
[C ₄ mpip]Cl		0.527	173.84
[N ₄₄₄₄]Cl		0.423	304.33
[P ₄₄₄₄]Cl		0.428	307.55

^aMolar Volumes (V_m) calculated with the COSMO-RS predictive model.

Table 2
Molecular structures and properties of the studied biomolecules [22].

Name	Chemical structure	$\log K_{ow}$	$M_w / (\text{g} \cdot \text{mol}^{-1})$
Gallic Acid		0.70	170.12
Vanillic Acid		1.33	168.14
Eugenol		2.49	164.20
L-Tryptophan		-1.06	204.23
L-Phenylalanine		-1.38	165.19
L-Tyrosine		-2.26	181.17
Caffeine		-0.07	194.19

Name	Chemical structure	$\log K_{ow}$	M_w /(g·mol ⁻¹)
Nicotine		1.17	162.23