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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

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HIGHLIGHTS

- The ionic liquids effect as adjuvants was studied in systems formed by water, polymer and salt.
- Ionic liquids may be as adjuvants to tailor the partition coefficients of biomolecules.
- There is a linear correlation between the biomolecules and the ionic liquids partition coefficients.
- The biomolecules partition is driven by the ionic liquids partition and by hydrophobicity differences.
- The effect of ionic liquids as adjuvants is more pronounced in systems composed of weak salting-out salts.

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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 ($(NH_4)_2SO_4$) 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.

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 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

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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 [7,11,15–19].

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 (Na_2SO_4) 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 Na_2SO_4 for the extraction of gallic, vanillic and syringic acids [20]. The addition of only 5 wt% of imidazolium-based ILs provides 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 [C_4mim][BF_4] (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 ([C_6mim]Cl) as adjuvant than in ternary ATPS (PEG + salt + water), the opposite behavior was observed in presence of the same ILs family with shorter ([C_2mim]Cl, [C_4mim]Cl) and longer ([C_8mim]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 ($(NH_4)_2SO_4$)) using ILs as adjuvants. The hypothesis to be evaluated in 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. 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 + $(NH_4)_2SO_4$ 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.

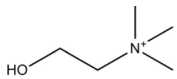
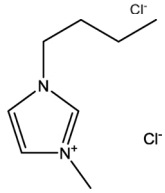
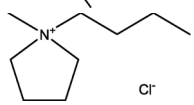
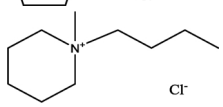
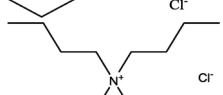
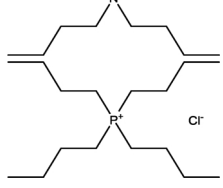
2. Experimental section

2.1. Materials

The ATPS studied in this work are constituted by water, ammonium sulfate ($(NH_4)_2SO_4$), polyethylene glycol with a molecular weight of 400 g·mol^{-1} (PEG 400) and several ILs at 5 wt%. PEG 400 was supplied by Sigma-Aldrich, while $(NH_4)_2SO_4$ (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, [C_4mim]Cl (> 99% pure); 1-butyl-1-methylpyrrolidinium chloride, [C_4mpyr]Cl (> 99% pure); 1-butyl-3-methylpiperidinium chloride, [C_4mpip]Cl (> 99% pure); tetrabutylammonium chloride, [N_{4444}]Cl (> 97 wt% pure); tetrabutylphosphonium chloride, [P_{4444}]Cl (> 96% pure); and cholinium chloride, [Ch]Cl (> 98 wt% pure). The ILs [C_4mim]Cl, [C_4mpyr]Cl and [C_4mpip]Cl were purchased from Iolitec, while [N_{4444}]Cl and [Ch]Cl were acquired from Sigma-Aldrich and Acros Organics, respectively. [P_{4444}]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.

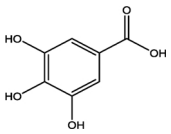
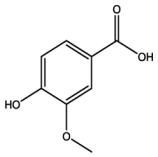
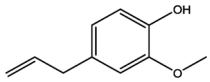
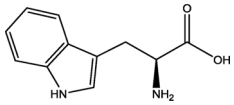
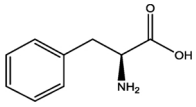
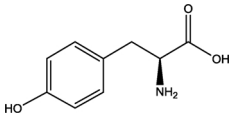
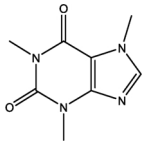
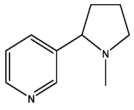
The biomolecules Vanillic Acid (VanAc, 97 wt% pure), Eugenol (Eug, 99 wt% pure), L-Tryptophan (Trp, 99 wt% pure) and L-Phenylalanine (Phen, 99 wt% pure) were acquired from Sigma-Aldrich.

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

IL	Chemical structure	α	V_m / (cm^3·mol^{-1})[a]
[Ch]Cl		1.555	123.06
[C_4mim]Cl		0.986	154.40
[C_4mpyr]Cl		0.537	169.49
[C_4mpip]Cl		0.527	173.84
[N_{4444}]Cl		0.423	304.33
[P_{4444}]Cl		0.428	307.55

[a] Molar Volumes (V_m) calculated with the COSMO-RS predictive model.

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

Biomolecule	Chemical structure	logK_{ow}	M_w /(g·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
Nicotine		1.17	162.23

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 (logK_{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 ± 10^{-4} g.

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

$$[\mathrm{PEG}] = Aexp[(B[\mathrm{Salt}]^{0.5}) - (C[\mathrm{Salt}]^3)] \quad (1)$$

where [PEG] and [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% $(NH_4)_2SO_4$ + 52 wt% H_2O + 5 wt% IL and 22 wt% PEG 400 + 18 wt% $(NH_4)_2SO_4$ + 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.

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

$$[\mathrm{PEG}]_B = Aexp[(B[\mathrm{Salt}]_B^{0.5}) - (C[\mathrm{Salt}]_B^3)] \quad (3)$$

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

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

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:

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

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% $(NH_4)_2SO_4$ + 52 wt% aqueous solution containing the target biomolecule + 0/5 wt% IL and 22 wt% PEG 400 + 18 wt% $(NH_4)_2SO_4$ + 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 g L^{-1} for Gallic and Vanillic Acids; 0.1 g L^{-1} for Eugenol and L-Tyrosine; 1.0 g L^{-1} for Caffeine, Nicotine and L-Tryptophan; and 3.0 g 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),

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

where $[Biom]_T$ and $[Biom]_B$ are the biomolecule concentrations ($g{\cdot}L^{-1}$) 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.

2.4. 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 $mol{\cdot}L^{-1}$) was prepared and diluted at appropriate concentrations (between 10^{-4} and 10^{-1} $mol{\cdot}L^{-1}$) to establish the calibration curve. A total ionic strength adjustment buffer (TISAB) was prepared by mixing aqueous solutions of potassium nitrate (KNO_3), acetic acid ($C_2H_4O_2$) and sodium acetate ($C_2H_3NaO_2$) at 0.1 $mol{\cdot}L^{-1}$. 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 + $(NH_4)_2SO_4$ + H_2O) and quaternary systems (PEG 400 + $(NH_4)_2SO_4$ + H_2O + 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: $[P_{4444}]Cl \approx [N_{4444}]Cl > [C_4mpip]Cl \approx [C_4mpyr]Cl \approx [C_4mim]Cl > [Ch]Cl$. 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 < [
C_4mim]Cl < $[C_4mpyr]Cl < [C_4mpip]Cl < [P_{4444}]Cl < [N_{4444}]Cl$.
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 more

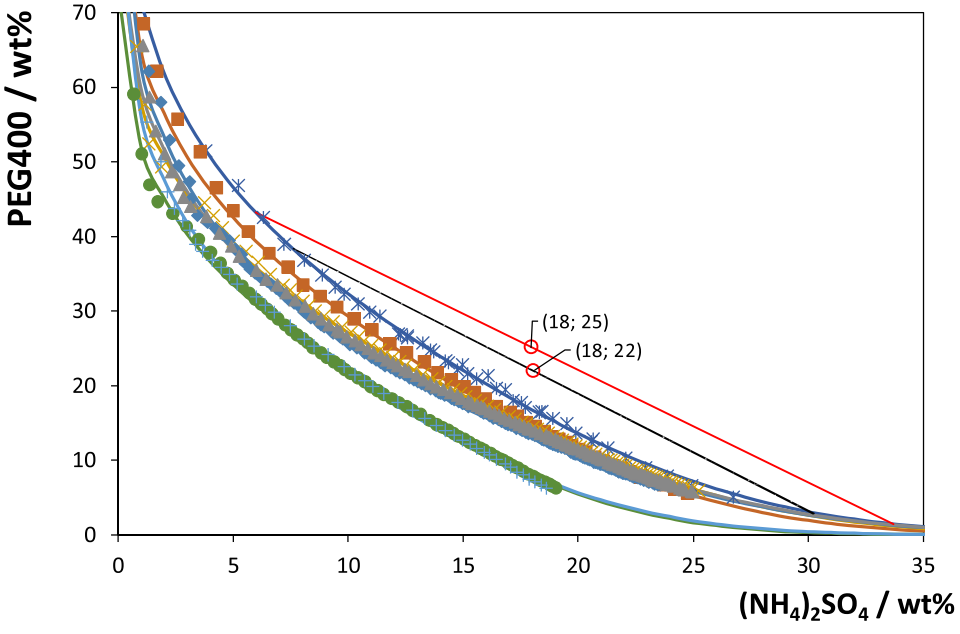


Fig. 1. Phase diagrams at (298 ± 1) K for the ternary system composed of PEG 400 + ammonium sulfate + water, ✳; and quaternary systems composed of PEG 400 + $(NH_4)_2SO_4$ + water + 5 wt% IL: ■, [Ch]Cl; ✕, [C_4mim]Cl; ◆, [C_4mpip]Cl; ▲, [C_4mpyr]Cl; ●, [N_{4444}]Cl; ┼, [P_{4444}]Cl. The lines were obtained by fitting the experimental data with Eq. (1). The symbol ○ 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) (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article).

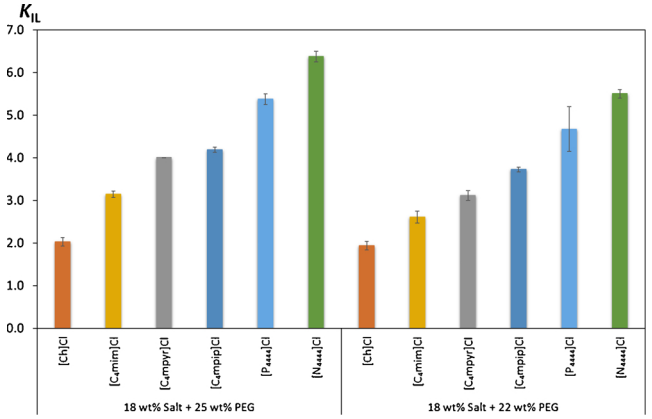


Fig. 2. Partition coefficients of ILs (K_{IL}) in ATPS composed of PEG 400 + $(NH_4)_2SO_4$ + water + 5 wt% IL at two mixture points.

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 ATPS 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 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 $(NH_4)_2SO_4$, 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 $C_6H_5O_7^{3-}$, 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, altough 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. 3–5. 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. 3–5, all biomolecules preferentially partition to the polymer-rich phase (more hydrophobic phase, $K_{Biom} > 1$), 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

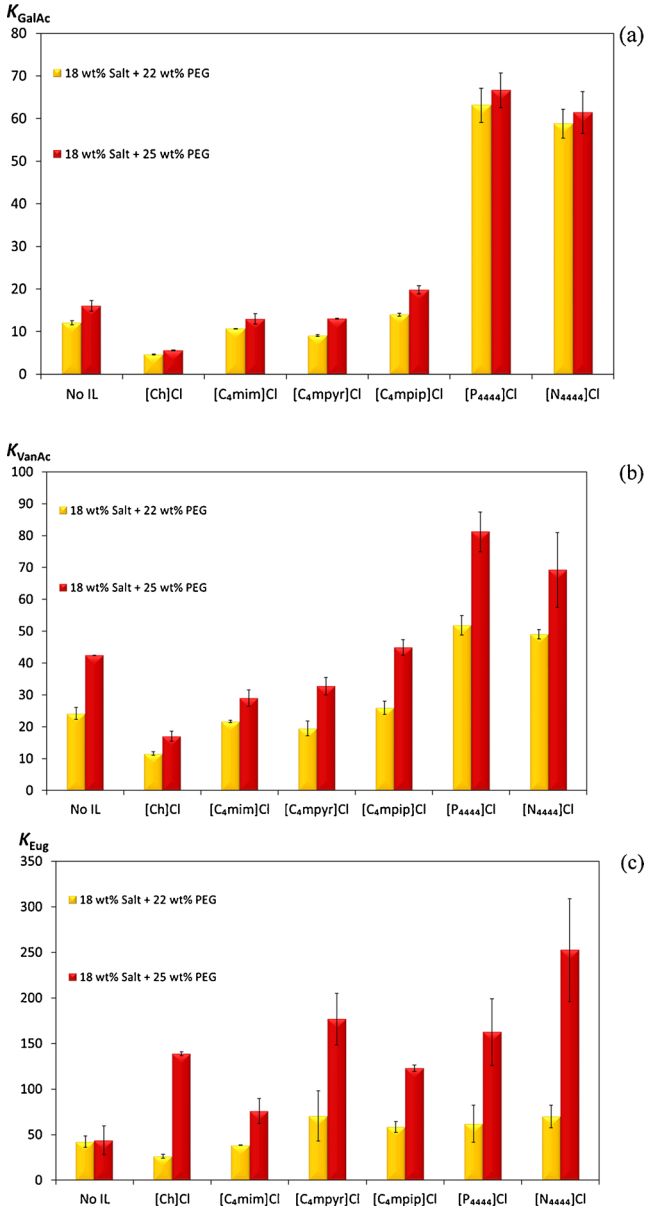


Fig. 3. Partition coefficients (K) of (a) Gallic Acid, (b) Vanillic Acid and (c) Eugenol in ATPS composed of PEG 400 + (NH4)2SO4 (no IL) and of PEG400 + (NH4)2SO4 + 5 wt% IL at two mixture points.

use of more hydrophobic ILs ([N_{4444}]Cl and [P_{4444}]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 [C_4mim]Cl, [C_4pyr]Cl and [C_4mpip]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 [P_{4444}]Cl.

According to Fig. 3, all the studied phenolic compounds extensively partition to the PEG-rich phase, with K_{Biom} values > > 1, following the trend: Eugenol (K_{Eug} = 26–253) > Vanillic Acid (K_{VanAc} = 12–81) > Gallic Acid (K_{GalAc} = 5–67). This trend closely follows the biomolecules logK_{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

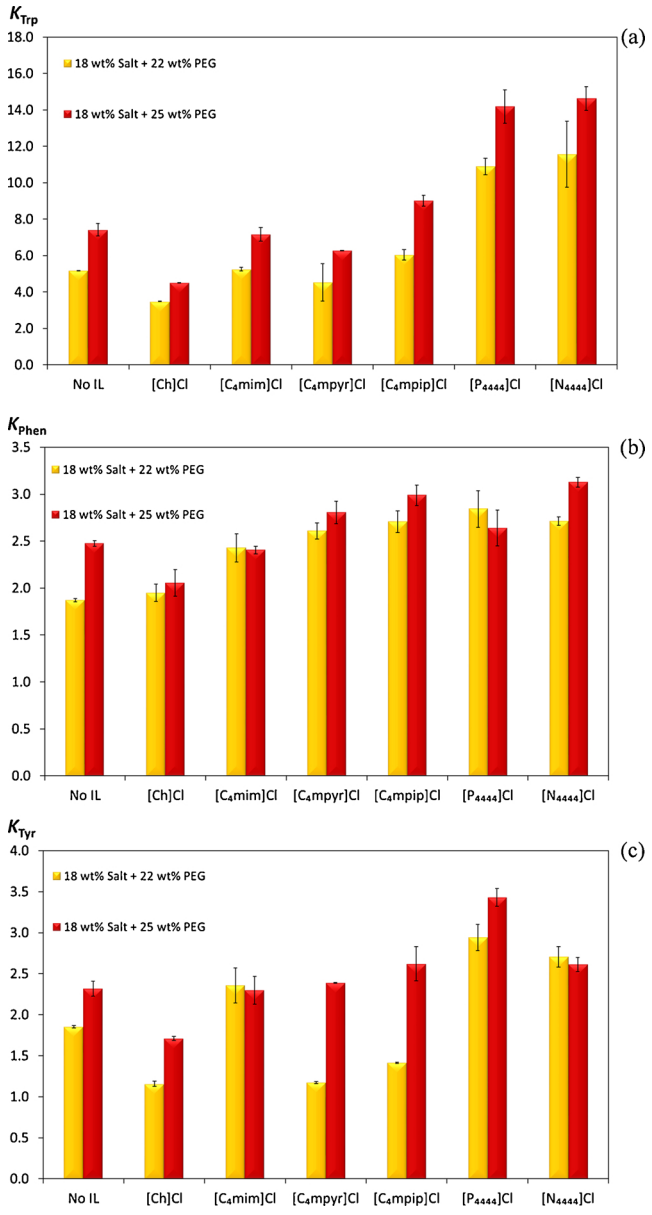


Fig. 4. Partition coefficients (K) of (a) L-Tryptophan, (b) L-Phenylalanine and (c) L-Tyrosine in ATPS composed of PEG 400 + $(NH_4)_2SO_4$ (no IL) and of PEG400 + $(NH_4)_2SO_4$ + 5 wt% IL at two mixture points.

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 logK_{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_{Trp} = 3–15) > Phenylalanine (K_{Phen} = 2–3) ≈ Tyrosine (K_{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 (logK_{ow} = 1.17 [22]) is more hydrophobic than caffeine (logK_{ow} = -0.07 [22]), the former more significantly partitions to the PEG-rich phase, indicating that a more complex

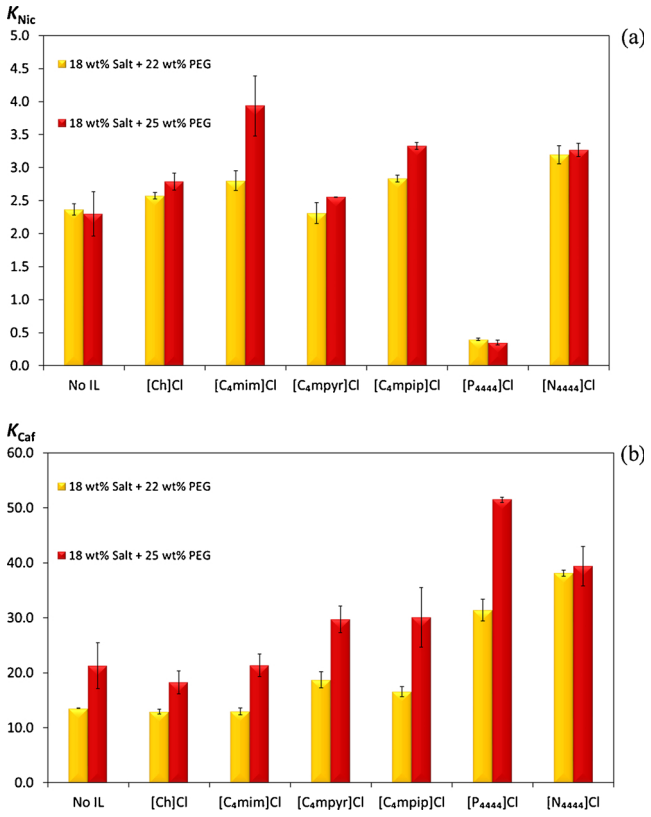


Fig. 5. Partition coefficients (K) of (a) Nicotine and (b) Caffeine in ATPS composed of PEG 400 + $(NH_4)_2SO_4$ (No IL) and of PEG400 + $(NH_4)_2SO_4$ + 5 wt% IL at two mixture points.

phenomenon takes place. In fact, the use of most hydrophobic ILs as adjuvants, such as $[N_{4444}]Cl$ and $[P_{4444}]Cl$, 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.

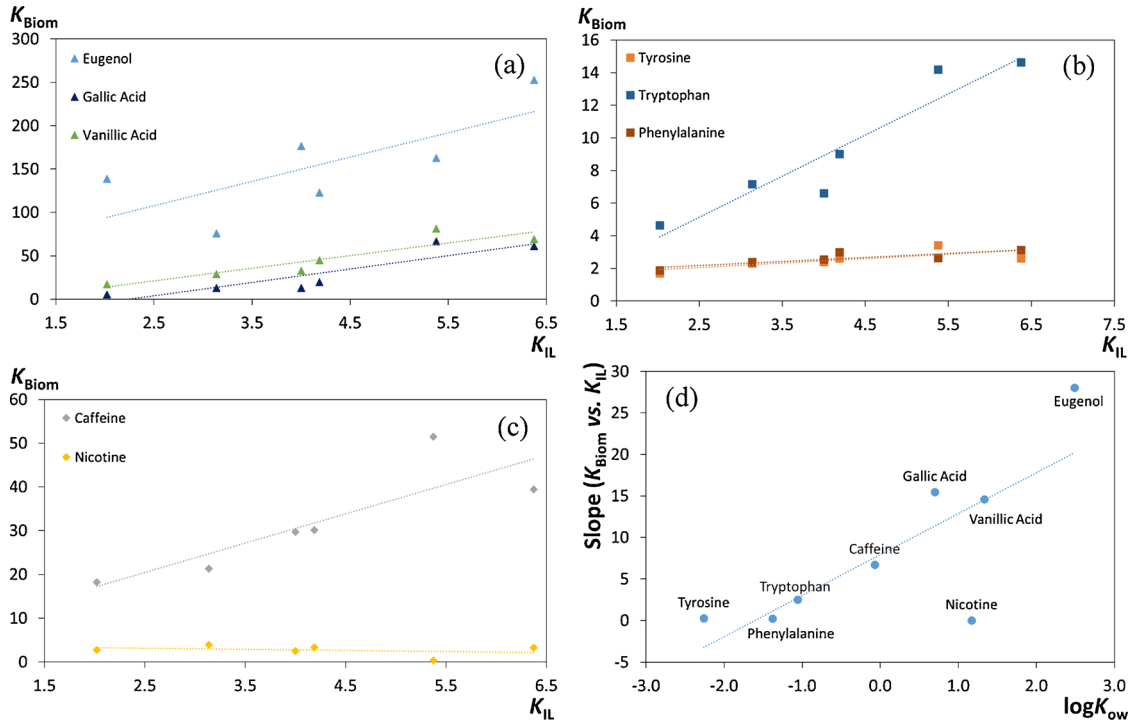


Fig. 6. Correlation between K_{Biom} and K_{IL} in the quaternary ATPS for the mixture point 25 wt% PEG 400 + 18 wt% $(NH_4)_2SO_4$ + 52 wt% H_2O + 5 wt% IL: (a) antioxidants; (b) amino acids and (c) alkaloids. (d) Slope of the curve of K_{Biom} *vs.* K_{IL} as a function of log K_{ow}. Dotted line corresponds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

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. 6. 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 logK_{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 ($(NH_4)_2SO_4$) 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.

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Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.bej.2018.10.022.

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