



Characterization of aqueous biphasic systems composed of ionic liquids and a citrate-based biodegradable salt

Helena Passos, Ana R. Ferreira, Ana Filipa M. Cláudio, João A.P. Coutinho, Mara G. Freire*

Departamento de Química, CICECO, Universidade de Aveiro, Campus Universitário de Santiago, 3810-193 Aveiro, Portugal

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ABSTRACT

Albeit ionic-liquid-based aqueous biphasic systems (ABS) have been largely explored as liquid-liquid extractive approaches for a large array of (bio)molecules, the application of biodegradable and nontoxic salts as phase constituents of these systems has been seldom investigated. In this work 15 ionic liquids were evaluated toward their ability to form ABS in the presence of a common biodegradable organic salt: potassium citrate. The ternary phase diagrams, tie-lines, and respective tie-line lengths, were determined at 25 °C. The gathered data allowed the evaluation of the effects of the ionic liquid cation core, of the cation side alkyl chain length, and of the anion nature, to form two-phase systems. It is shown that the ionic liquids aptitude to undergo liquid-liquid demixing is mainly controlled by their hydrophobicity. The large differences observed between the phase diagrams behavior suggest the possibility of tailoring the aqueous phases' polarities for a specific extraction. Therefore, the partitioning of a hydrophobic amino acid produced by bacteria fermentation, L-tryptophan, was also addressed aiming at exploring the applicability of the proposed systems in the biotechnology field. Single-step extraction efficiencies of L-tryptophan for the ionic-liquid-rich phase range between 72% and 99%.

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1. Introduction

The recovery and purification of biomolecules from bioreaction media are gaining crucial importance due to a growing demand of fine chemicals which are biotechnologically manufactured. Liquid-liquid extraction approaches have often been a favored choice for bioseparations. They allow a high effectiveness, high yield, improved purity degree, a proper selectivity, technological simplicity, and low cost [1]. Considering that most often the biomolecules of interest are produced in aqueous media, organic solvents have been widely applied due to their immiscibility with water. Nevertheless, these solvents can promote the denaturation of enzymes and other biomolecules [2]. Organic solvents further present high volatility and toxicity, being therefore hazardous materials for the environment and health safety. In this context, the development and design of new and "greener" separation technologies for the improvement of clean manufacturing processes has been a hot topic of research in the past decades. Meanwhile, liquid-liquid extraction making use of aqueous biphasic systems (ABS) has found its platform in the biotechnological field [3].

ABS consist of two aqueous-rich phases that are formed by the addition of two aqueous solutions of structurally different polymers, a polymer and a salt, or two surfactants [3,4]. ABS are more

environmentally friendly techniques since volatile organic solvents are not employed in the whole process. Furthermore, since the two phases present distinctive chemical and physical properties, the partition of biomolecules between the two phases is a favored fate. Consequently, since the 1960s, and after the pioneer work of Albertsson [3], ABS have been largely explored in the separation, recovery, and purification of the most distinct biomaterials, such as proteins [5,6], enzymes [7,8], nucleic acids [9], antibodies [10], and antibiotics [11,12].

Apart from these conventional and mostly polymer-based systems, ionic liquids (ILs) have shown to be a possible alternative to be used in the preparation of ABS and partitioning studies [13–28]. The main interest on ionic liquids relays on their remarkable properties: negligible vapor pressure, non-flammability, high thermal and chemical stabilities, and a large liquid temperature range. Although most of these properties are also shared by polymers, one of the main advantages of ABS composed of ionic liquids relays on the possibility of controlling their phases' polarities by an appropriate choice of the ions that combined form a given ionic fluid [22]. On the other hand, polymers are more limited in what concerns their hydrophilic/lipophilic balance, and the functionalization of the polymer is a recurrent approach [23].

While a large set of literature data has been focused on ionic-liquid-based ABS, most of the salts used are based on phosphates, sulfates, and carbonate anions [13–20]. However, common inorganic salts lead to environmental concerns when discharged in high concentrations into the effluent streams. Recent works have

* Corresponding author. Tel.: +351 234401507; fax: +351 230370084.

E-mail address: maragfreire@ua.pt (M.G. Freire).

introduced biodegradable and nontoxic organic salts, such as the citrate-, tartrate- or acetate-based [29–36], or non-charged, biodegradable, nontoxic, and renewable feedstock organic compounds, such as carbohydrates [21] or amino acids [20], in the formulation of ionic-liquid-based ABS. Nevertheless, the works dealing with organic salts [29–39] addressed ionic liquids based essentially on the imidazolium cation with the bromide, chloride, and tetrafluoroborate counterions. Besides the water-stable chloride and bromide-based ionic liquids, [BF₄]-based fluids tend to be the most largely explored [29–39], and it should be stressed that [BF₄]-based ionic liquids are not water stable and form hydrofluoric acid in aqueous media [40].

Since citrate-based salts are biodegradable and nontoxic, we studied here a large array of ionic-liquid-based ABS, making use of potassium citrate, as “greener” alternatives to the previously studied systems [13–20]. Moreover, aiming at achieving a proof of principle on the applicability of the studied systems, the partitioning of L-tryptophan was also addressed. Aromatic amino acids, such as L-tryptophan, L-phenylalanine, and L-tyrosine, can be produced by bacterial fermentation [41]. Two representative producer organisms are *Corynebacterium glutamicum* and *Escherichia coli* [41]. In particular, L-tryptophan is one of the amino acids difficult to obtain in a high level of production yield [41]. Hence, several attempts at strain improvement have been explored, namely the recombinant DNA technology [42]. It has been shown that to guarantee the cell viability and increase the rate of tryptophan production, 4 main parameters should be optimized, namely the efflux of tryptophan, the growth rate, the inhibition constant, and the tryptophan repressor level [43]. In this context, the application of ionic-liquid-based ABS could be a valuable alternative to guarantee a continuous tryptophan extraction step during fermentation.

2. Materials and methods

2.1. Materials

The determination of the liquid–liquid ternary phase diagrams was performed using aqueous solutions of potassium citrate tribasic monohydrate, C₆H₅K₃O₇·H₂O (≥99 wt% pure from Sigma–Aldrich), and individual aqueous solutions of the following ionic liquids: 1-butyl-3-methylimidazolium chloride, [C₄mim]Cl (99 wt%); 1-hexyl-3-methylimidazolium chloride, [C₆mim]Cl (>98 wt%); 1-butyl-3-methylpyridinium chloride, [C₄mpy]Cl (>98 wt%); 1-butyl-1-methylpiperidinium chloride, [C₄mpip]Cl (99 wt%); 1-butyl-1-methylpyrrolidinium chloride, [C₄mpyr]Cl (99 wt%); tetrabutylammonium chloride, [N₄₄₄₄]Cl (≥97 wt%); tetrabutylphosphonium chloride, [P₄₄₄₄]Cl (98 wt%); 1-butyl-3-methylimidazolium bromide, [C₄mim]Br (99 wt%); 1-butyl-3-methylimidazolium acetate, [C₄mim][CH₃CO₂] (>98 wt%); 1-butyl-3-methylimidazolium methanesulfonate, [C₄mim][CH₃SO₃] (99 wt%); 1-butyl-3-methylimidazolium trifluoroacetate, [C₄mim][CF₃CO₂] (>97 wt%); 1-butyl-3-methylimidazolium trifluoromethanesulfonate, [C₄mim][CF₃SO₃] (99 wt%); 1-butyl-3-methylimidazolium dicyanamide, [C₄mim][N(CN)₂] (>98 wt%); 1-butyl-3-methylimidazolium thiocyanate, [C₄mim][SCN] (>98 wt%); and 1-butyl-3-methylimidazolium dimethylphosphate, [C₄mim][PO₄(CH₃)₂] (>98 wt%).

All imidazolium-, pyridinium-, and pyrrolidinium-based ionic liquids were purchased from Iolitec. The tetrabutylphosphonium chloride was kindly supplied by Cytec Industries Inc. The tetrabutylammonium chloride was from Aldrich. To reduce the volatile impurities to negligible values, ionic liquid individual samples were purified under constant agitation, under vacuum, and at moderate temperature (60 °C), for a minimum of 24 h. After this procedure,

the purity of each ionic liquid was further checked by ¹H and ¹³C NMR spectra and found to be in accordance with the stated purity level provided by the suppliers.

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

L-Tryptophan (purity >99.0 wt%) was from Sigma.

2.2. Experimental procedure

The solubility curves were determined through the cloud point titration method (previously described by us [16–21]), and at (25 ± 1) °C and atmospheric pressure. Aqueous solutions of potassium citrate at circa 50 wt% and aqueous solutions of the different hydrophilic ionic liquids (with concentrations ranging from 60 wt% to 90 wt%) were prepared and used for the determination of the binodal curves. All the calculations considering the mass fractions or molality of the citrate-based salt were carried out discounting the complexed water.

Repetitive drop wise addition of the aqueous organic salt solution to the aqueous solutions of each ionic liquid was carried out until the detection of a cloudy (biphasic) solution, followed by the drop wise addition of ultrapure water until the finding of a monophasic region (clear and limpid solution). To complete the phase diagrams, the addition of ionic liquid aqueous solutions to the salt solution was also carried out. All the additions were made under constant stirring. The ternary system compositions were determined by weight quantification within ±10^{−4} g.

The experimental binodal curves were fitted by the following equation [44]:

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

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

The tie-lines (TLs) were determined by a gravimetric method originally proposed by Merchuk et al. [44] for polymer-based ABS, and later on applied by Rogers and co-workers [13] to ionic-liquid-based ABS. A ternary mixture composed of ionic liquid + C₆H₅K₃O₇ + water at the biphasic region was gravimetrically prepared within ±10^{−4} g, vigorously agitated, and left to equilibrate for at least 12 h and at (25 ± 1) °C, aiming at a complete separation of the coexisting phases. After such time, both phases were carefully separated and individually weighed.

Each TL was determined by the lever–arm rule through the relationship between the top phase composition and the overall system composition, and for which the following system of four equations (Eqs. (2)–(5)) and four unknown values ([IL]_{IL}, [IL]_{salt}, [salt]_{IL} and [salt]_{salt}) was solved [44]:

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

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

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

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

where subscripts “IL”, “salt”, and “M” designate the ionic-liquid-rich phase, the salt-rich phase and the mixture, respectively; [salt] and [IL] represent, respectively, the weight fractions of C₆H₅K₃O₇ and ionic liquid; and α is the ratio between the mass of the top phase and the total mass of the mixture. The system solution results in the composition (wt%) of the ionic liquid and organic salt in the top and bottom phases.

For the calculation of each tie-line length (TLL) the following equation was used:

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

where subscripts “IL” and “salt” symbolize, respectively, the ionic-liquid-rich phase and the salt-rich phase, and [salt] and [IL] are the weight fraction percentages of organic salt and ionic liquid, as described before.

The pH values (± 0.02) of the ionic-liquid-rich and organic-salt-rich aqueous phases were measured at $(25 \pm 1)^\circ\text{C}$ using an HI 9321 Microprocessor pH meter (HANNA instruments). The compositions adopted at the biphasic region are the same as those applied for the TLs determination. All mixtures were gravimetrically prepared within $\pm 10^{-4}$ g. The mixtures were prepared, vigorously stirred, and kept in small ampoules for phase separation for at least 12 h at $(25 \pm 1)^\circ\text{C}$. After the careful separation of the phases, the pH of each aqueous phase was measured.

The partitioning of L-tryptophan was evaluated in several systems composed of 20 wt% of $\text{C}_6\text{H}_5\text{K}_3\text{O}_7$, 40 wt% of each ionic liquid, and 40 wt% of an aqueous solution containing L-tryptophan at 0.77 g dm^{-3} ($3.8 \times 10^{-4} \text{ mol dm}^{-3}$). All mixtures were prepared by weight with an uncertainty of $\pm 10^{-4}$ g. The mixtures were prepared and vigorously stirred, and further allowed to equilibrate and phase separate for at least 12 h at $(25 \pm 1)^\circ\text{C}$. Preliminary optimization studies showed that the partitioning of L-tryptophan was completely attained after 12 h of equilibrium.

After the separation of the phases, the amount of L-tryptophan was quantified through UV-spectroscopy, using a SHIMADZU UV-1700, Pharma-Spec Spectrometer, at a wavelength of 279 nm, and using a calibration curve previously established. Slight interferences of the salt and some ionic liquids in the quantification of L-tryptophan were verified. Therefore, to minimize these interferences, ternary mixtures at the same weight fraction composition were prepared, using pure water instead of the L-tryptophan aqueous solution, and used as blank samples. At least three individual samples of each system were prepared for the quantification of L-tryptophan in both phases. The partition coefficient of L-tryptophan, K_{Trp} , was determined according to the following equation,

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

where $[\text{Trp}]_{\text{IL}}$ and $[\text{Trp}]_{\text{salt}}$ are the concentrations of L-tryptophan in the ionic-liquid-rich and in the citrate-rich phase, respectively.

The extraction efficiencies of L-tryptophan were determined according to,

$$\%EE_{\text{Trp}} = \frac{[\text{Trp}]_{\text{IL}} \times w_{\text{IL}}}{[\text{Trp}]_{\text{IL}} \times w_{\text{IL}} + [\text{Trp}]_{\text{salt}} \times w_{\text{salt}}} \times 100 \quad (8)$$

where w_{IL} and w_{salt} are the weight of the ionic-liquid-rich phase and of the citrate-rich phase, respectively, and $[\text{Trp}]_{\text{IL}}$ and $[\text{Trp}]_{\text{salt}}$ are the concentration of L-tryptophan in the ionic-liquid-rich phase and in the citrate-rich phase, respectively.

3. Results and discussion

Novel ternary phase diagrams were determined for several ionic liquids + water + potassium citrate, at 25°C and at atmospheric pressure. The ionic structures of the ionic liquids that were able to create liquid-liquid systems with this salt are depicted in Fig. 1. The respective ternary phase diagrams are illustrated in Figs. 2–4. The experimental weight fraction data of each phase diagram are given in Supporting Information.

The systems composed of potassium citrate, water, and $[\text{C}_4\text{mim}]\text{Br}$ or $[\text{C}_4\text{mim}]\text{Cl}$ have already been reported by

Zafarani-Moattar and Hamzehzadeh [34,35]. It should be remarked that a good agreement was observed between our data and literature data [34,35]. The respective comparisons are provided in Supporting Information.

In the studied ABS, the top phase corresponds to the ionic-liquid-rich phase, while the bottom phase is mainly composed of the citrate-based salt. The only exception was observed with the $[\text{C}_4\text{mim}][\text{CF}_3\text{SO}_3]$ -based system. This feature was observed before with other salts [16–18] and is a consequence of the high density of the fluorinated ionic liquid.

In Figs. 2–4, the solubility curves are presented in molality units for a better understanding of the impact of the ionic liquids structure on the phase diagrams behavior, avoiding differences that would result from different molecular weights. In all phase diagrams, the biphasic region is localized above the solubility curve. The larger this region, the higher the ability of the ionic liquid to undergo liquid-liquid demixing, *i.e.*, the easier the ionic liquid is salted-out by the citrate-based salt. The experimental phase diagrams are divided in different figures to allow the individual evaluation of the cation side alkyl chain length, the cation core, and the anion nature of the diverse ionic liquids on the formation of ABS.

Fig. 2 depicts the effect of the imidazolium side alkyl chain length in the formation of ABS. In general, an increase in the aliphatic chain facilitates the creation of ABS. Longer aliphatic chains at the cation contribute to an enhanced hydrophobicity displayed by the ionic liquid. Indeed, as the ionic liquid becomes more hydrophobic there is a reduction on the water-ionic-liquid affinity and, therefore, an improved phase separation occurs. Binary liquid-liquid equilibrium data further supports this idea: an increase in the cation side alkyl chain length decreases the solubility of the ionic liquid in water [45]. Moreover, this pattern is in close agreement with previous results on ionic-liquid-based ABS composed of distinct salts, such as Na_2SO_4 and K_3PO_4 [16,18].

It should be stressed that 3 additional ionic liquids were tested according to their ability to form liquid-liquid aqueous phases, namely 1-ethyl-3-methylimidazolium chloride, 1-allyl-3-methylimidazolium chloride, and 1-hydroxyethyl-3-methylimidazolium chloride. However, we could not detect the coexisting liquid aqueous phases with these ionic liquids. Only solid-liquid equilibria were observed. The solid phases were further identified by ^1H and ^{13}C NMR spectra and correspond merely to the ionic liquid. Therefore, it is safe to admit that there is no ion exchange among the studied ionic liquids and the citrate-based salt (at least not above the detection limit of the equipment). Since potassium citrate has a higher affinity for water compared to the ionic liquids, there is the preferential exclusion of the ionic liquid from the aqueous solution. The reason behind the formation of solid-liquid equilibrium instead of liquid-liquid equilibrium with these ionic liquids is a consequence of their higher melting temperature (85°C for 1-ethyl-3-methylimidazolium chloride [46], 52°C for 1-allyl-3-methylimidazolium chloride [47], and 86°C for 1-hydroxyethyl-3-methylimidazolium chloride [48]) compared with the remaining ionic liquids. Indeed, these ionic liquids are more structurally similar to conventional salts and thus present higher melting temperatures.

Despite the immense versatility inherent to the cation-anion permutations in ionic liquids, all authors who explored the use of organic salts in the creation of ionic-liquid-based ABS used imidazolium-based compounds [29–39]. The first investigation on the effect of the cation core regarding the formation of ABS was conducted by Bridges et al. [14] with imidazolium-, pyridinium-, ammonium-, and phosphonium-based chloride; yet, salted-out by the salts K_3PO_4 , K_2HPO_4 , K_2CO_3 , KOH and $(\text{NH}_4)_2\text{SO}_4$. Later on, Ventura et al. [49] compared the influence of imidazolium-, pyridinium-, pyrrolidinium-, and piperidinium-based fluids in the

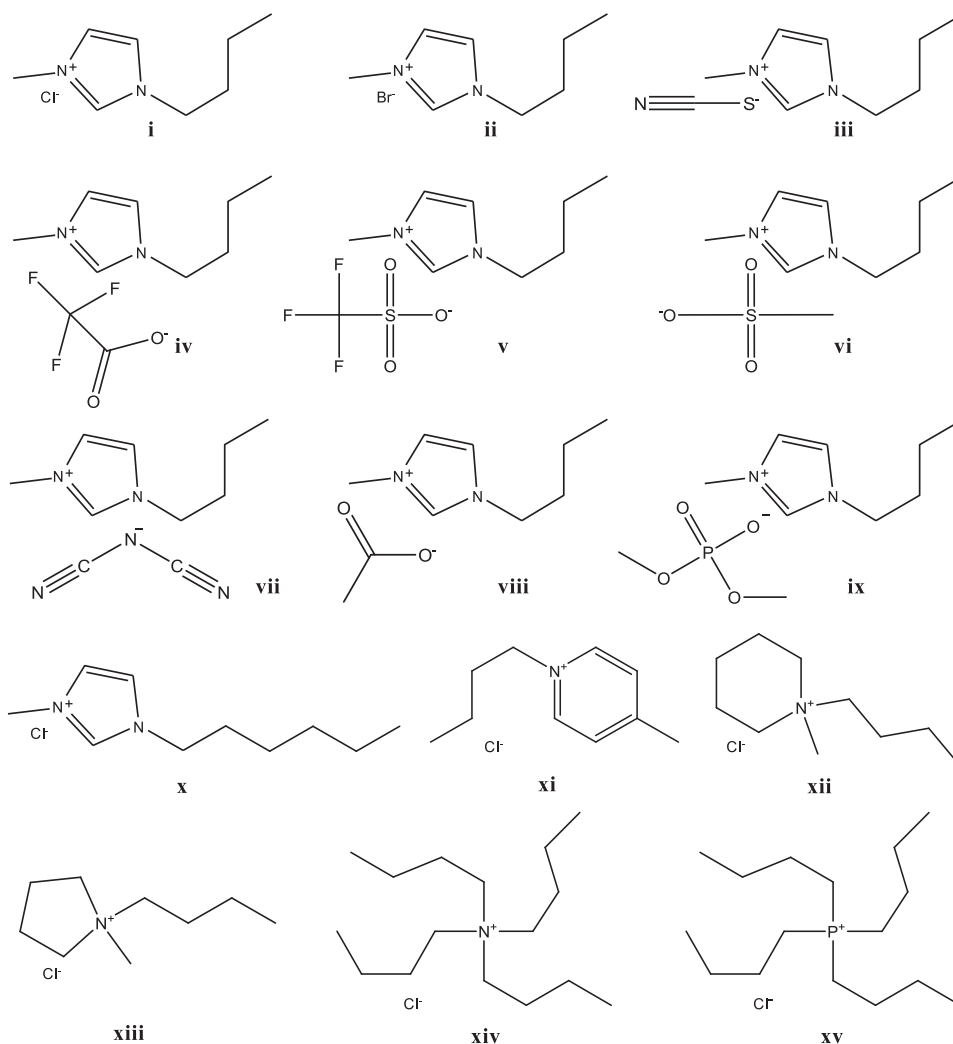


Fig. 1. Chemical structures of the ionic liquids used to form ABS: [C₄mim]Cl (i), [C₄mim]Br (ii), [C₄mim][SCN] (iii), [C₄mim][CF₃CO₂] (iv), [C₄mim][CF₃SO₃] (v), [C₄mim][CH₃SO₃] (vi), [C₄mim][N(CN)₂] (vii), [C₄mim][CH₃CO₂] (viii), [C₄mim][PO₄(CH₃)₂] (ix), [C₆mim]Cl (x), [C₄mpy]Cl (xi), [C₄mpip]Cl (xii), [C₄mpyr]Cl (xiii), [N₄₄₄₄]Cl (xiv), [P₄₄₄₄]Cl (xv).

formation of ABS with a phosphate buffer aqueous solution. Therefore, this study constitutes the most complete evaluation of the ionic liquid cation effect on ABS formation for a common organic salt.

The effect of the cation core is displayed in Fig. 3. The ionic liquid cation ability to form ABS, for instance at 0.5 mol kg⁻¹ of potassium citrate, follows the order: [P₄₄₄₄]⁺ > [N₄₄₄₄]⁺ ≫ [C₄mpy]⁺ ≈ [C₄mpip]⁺ > [C₄mpyr]⁺ > [C₄mim]⁺.

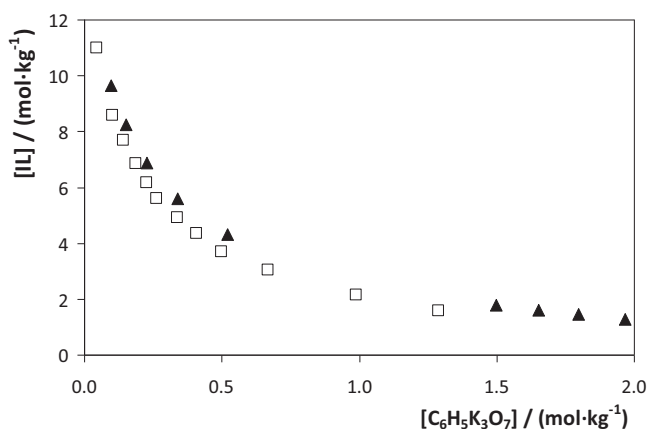


Fig. 2. Evaluation of the cation alkyl side chain length in the ternary phase diagrams composed of ionic liquid + water + C₆H₅K₃O₇: [C₄mim]Cl (▲), [C₆mim]Cl (□).

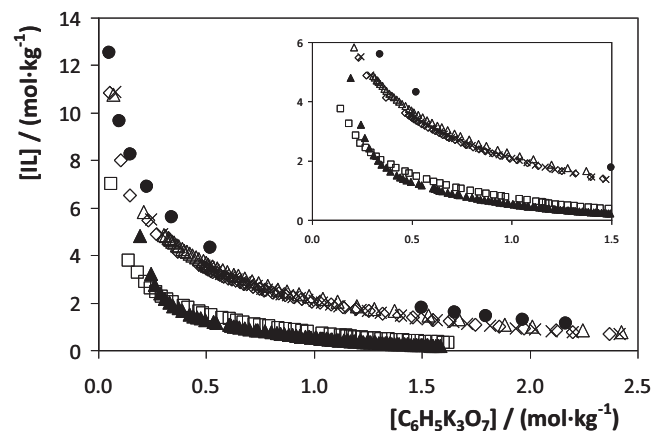


Fig. 3. Evaluation of the cation core in the ternary phase diagrams composed of ionic liquid + water + C₆H₅K₃O₇: [C₄mim]Cl (●), [C₄mpip]Cl (◇), [C₄mpyr]Cl (△), [C₄mpy]Cl (×), [P₄₄₄₄]Cl (▲), [N₄₄₄₄]Cl (□).

This trend reflects the aptitude of the ionic liquid cation to be solvated by water (since the chloride anion is the counterion common to all ionic liquids), and which is regulated by steric and entropic contributions [45,49]. This trend follows the hydrophobic sequence of the ionic liquid cations. Quaternary phosphonium- and ammonium-based cations are those that present the higher ability to form ABS since they present four butyl chains which are responsible for their higher hydrophobicity. Their water miscibility essentially results from the strong solvation of the chloride anion. From the comparison among the cyclic nitrogen-based ionic liquids, it is clear that the 6-sided ring cations, such as pyridinium and piperidinium are more able to induce ABS when compared with the smaller 5-sided rings of imidazolium and pyrrolidinium. Hence, the inherent hydrophobicity of the cation, which is also ruled by their carbon number, is the main factor behind the ability of these compounds to form ABS. The trends obtained here are in agreement with the results previously reported for additional ionic-liquid-based ABS composed of different salts [49] and also correlate with the solubility of these ionic liquids in water [50]. From the results obtained it is evident that a large number of combinations of ionic liquid cations can be employed in the formation of ionic-liquid-based ABS, and that these systems allow the tailoring of the phases' polarities aiming at performing specific extractions.

The effect of the anion nature on the ABS phase behavior was studied with several ionic liquids containing the common $[C_4mim]^+$ cation, while combined with the following anions: Cl^- , Br^- , $[CH_3SO_3]^-$, $[CH_3CO_2]^-$, $[CF_3SO_3]^-$, $[CF_3CO_2]^-$, $[SCN]^-$, $[N(CN)_2]^-$, and $[PO_4(CH_3)_2]^-$. The corresponding ternary phase diagrams are depicted in Fig. 4. Fig. 4 reveals that a large number of ionic liquids constituted by different anions can be combined with organic salts besides the chloride-, bromide-, and tetrafluoroborate-based fluids commonly investigated [29–39]. At 0.5 mol kg^{-1} of potassium citrate, the ionic liquid anion ability to form ABS is: $[CH_3CO_2]^- < [PO_4(CH_3)_2]^- < [CH_3SO_3]^- < Cl^- \ll Br^- < [CF_3CO_2]^- \ll [N(CN)_2]^- < [SCN]^- < [CF_3SO_3]^-$. This rank is in close agreement with previous works using inorganic salts such as K_3PO_4 [17] and Na_2SO_4 [18]. The good agreement on the anion trend observed among systems constituted by different salts suggests that the ionic liquid pattern for creating ABS is not affected by the salt nature or the pH of the aqueous salt solution employed.

The anions of ionic liquids compete with the salt ions for the formation of hydration complexes. Usually this competition is won by the ions with a higher charge density, *i.e.*, ions that are capable of stronger interactions with water. For a common cation, ionic liquids composed of anions with lower hydrogen bond basicity are more able to form ABS. Indeed, a close agreement exists between the

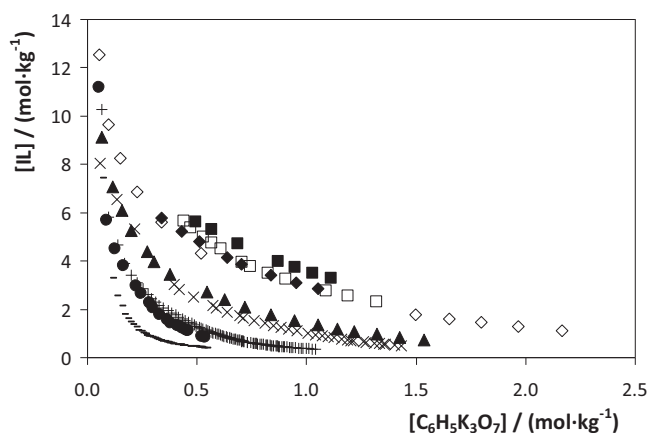


Fig. 4. Evaluation of the anion nature in the ternary phase diagrams composed of ionic liquid + water + $C_6H_5K_3O_7$: $[C_4mim][CF_3SO_3]$ (–), $[C_4mim][SCN]$ (●), $[C_4mim][N(CN)_2]$ (+), $[C_4mim][CF_3CO_2]$ (×), $[C_4mim]Br$ (▲), $[C_4mim]Cl$ (◇), $[C_4mim][CH_3SO_3]$ (◆), $[C_4mim][PO_4(CH_3)_2]$ (□), $[C_4mim][CH_3CO_2]$ (■).

tendency on the ionic liquids ability to form ABS and the hydrogen bond basicity values determined by solvatochromic probes [51], and as previously identified by us [17,18]. Moreover, the fluorination of the anions, which further implies a lower ability of the anion for hydrogen-bonding, leads to an enhanced capability of the ionic liquid to undergo liquid–liquid demixing. For instance, $[C_4mim][CF_3CO_2]$ and $[C_4mim][CF_3SO_3]$ are more easily salted-out than $[C_4mim][CH_3CO_2]$ and $[C_4mim][CH_3SO_3]$, respectively. Therefore, fluorinated ionic liquids require a less amount of salt to form two aqueous liquid phases.

For the studied systems, the experimental binodal data were further fitted by the empirical relationship described by Eq. (1). The regression parameters were estimated by the least-squares regression, and their values and corresponding standard deviations (σ) are provided in Table 1. In general, good correlation coefficients were obtained for all systems indicating that these fittings can be used to predict data in a given region of the phase diagram where no experimental results are available.

The experimental tie-lines, along with their respective length, are reported in Table 2. An example of the tie-lines obtained for this type of systems is depicted in Fig. 5.

The pH values of both phases in each ABS, and for the compositions for which the TLs were determined, are given in Table 2. The pH values of these systems are in the alkaline region (pH 8–10) and indicate their possible applicability for the extraction of a given (bio)molecule. Since an aqueous solution at 50 wt% of potassium citrate presents a pH value of *circa* 9.2, the differences observed in

Table 1
A, B and C are constants obtained by the regression of the experimental binodal data through the application of Eq. (1) (and respective standard deviations, σ , and correlation coefficients, R^2) for the several systems at $25^\circ C$.

Ionic liquid	$A \pm \sigma$	$B \pm \sigma$	$10^5 (C \pm \sigma)$	R^2
$[C_4mim]Cl$	86.0 ± 0.5	-0.180 ± 0.003	0.84 ± 0.03	0.9998
$[C_4mim]Br$	92.4 ± 0.6	-0.228 ± 0.003	1.87 ± 0.06	0.9997
$[C_4mim][CF_3SO_3]$	208.8 ± 5.0	-0.829 ± 0.012	1.00 ± 1.44	0.9968
$[C_4mim][CF_3CO_2]$	93.7 ± 1.9	-0.222 ± 0.008	3.40 ± 0.15	0.9958
$[C_4mim][SCN]$	120.9 ± 4.3	-0.470 ± 0.019	13.34 ± 2.37	0.9951
$[C_4mim][N(CN)_2]$	121.8 ± 1.8	-0.444 ± 0.006	6.56 ± 0.24	0.9968
$[C_4mim][CH_3SO_3]$	85.0 ± 2.8	-0.123 ± 0.010	1.02 ± 0.15	0.9987
$[C_4mim][CH_3CO_2]$	82.6 ± 3.7	-0.117 ± 0.013	0.95 ± 0.13	0.9994
$[C_4mim][PO_4(CH_3)_2]$	112.2 ± 2.2	-0.179 ± 0.006	0.54 ± 0.05	0.9996
$[C_6mim]Cl$	85.9 ± 1.0	-0.173 ± 0.005	1.61 ± 0.14	0.9983
$[C_4mpip]Cl$	87.1 ± 0.3	-0.210 ± 0.001	0.85 ± 0.01	0.9997
$[C_4mpyr]Cl$	87.8 ± 0.5	-0.214 ± 0.002	0.74 ± 0.02	0.9993
$[C_4mpy]Cl$	94.3 ± 0.4	-0.232 ± 0.001	0.80 ± 0.01	0.9996
$[P_{4444}]Cl$	170.0 ± 5.7	-0.484 ± 0.011	1.64 ± 0.14	0.9944
$[N_{4444}]Cl$	99.3 ± 0.9	-0.318 ± 0.003	1.79 ± 0.06	0.9990

Table 2Weight fraction percentage (wt%) for the coexisting phases of ionic liquid ([IL]) + potassium citrate ([salt]) + H₂O, and respective values of TLL, and pH values of each phase.

Ionic liquid	Weight fraction percentage (wt%)								TLL
	[IL] _{IL}	[salt] _{IL}	pH _{IL}	[IL] _M	[salt] _M	[IL] _{salt}	[salt] _{salt}	pH _{salt}	
[C ₄ mim]Cl	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 ₄ mim]Br	60.81	3.36	9.19	29.92	24.91	5.14	42.20	9.23	67.87
	73.40	1.02	9.52	40.01	25.03	1.07	53.04	9.04	89.08
[C ₄ mim][CF ₃ SO ₃]	74.74	1.54	7.17	40.03	14.91	1.76	29.66	7.65	78.21
	82.48	1.26	7.20	39.82	20.41	0.73	37.96	7.67	89.61
[C ₄ mim][SCN]	56.77	2.57	7.79	14.93	15.05	9.61	16.64	7.84	49.21
	70.64	1.31	7.26	30.19	20.14	0.04	34.18	7.81	77.88
	74.25	1.08	7.06	39.94	20.02	0.00	42.07	7.78	84.81
[C ₄ mim][N(CN) ₂]	47.57	4.43	8.76	21.82	15.14	9.67	20.19	8.83	41.05
	52.21	3.62	8.80	24.70	15.13	6.98	22.55	8.68	49.03
	58.81	2.68	8.87	29.95	14.97	3.98	26.04	8.66	59.60
	86.94	0.58	8.75	39.92	19.98	0.37	36.30	8.63	93.65
[C ₄ mim][CH ₃ SO ₃]	61.89	6.49	9.36	36.88	29.91	2.95	61.68	8.93	80.74
[C ₆ mim]Cl	54.20	6.90	8.39	44.89	15.02	2.62	51.86	8.88	68.43
[C ₄ mpip]Cl	49.13	7.38	9.41	32.05	24.96	5.83	51.94	9.29	62.13
	54.23	5.04	9.52	40.00	20.06	3.98	58.11	9.32	73.08
	59.43	3.32	9.61	43.00	19.96	2.44	61.04	9.49	81.11
[C ₄ mpyr]Cl	54.34	4.99	9.31	39.93	19.92	4.54	56.62	10.09	71.73
	54.23	5.04	9.31	40.00	20.06	3.98	58.11	10.58	73.08
	55.52	4.56	9.30	41.98	18.59	4.07	57.86	10.04	74.08
[C ₄ mpy]Cl	45.12	9.90	8.31	30.03	25.05	8.70	46.47	8.23	51.61
	55.51	5.20	8.18	34.76	25.10	5.04	53.61	8.18	69.94
[P ₄₄₄₄]Cl	39.56	8.94	7.99	30.06	14.96	8.93	28.33	7.86	36.25
	50.66	6.23	8.32	34.80	15.21	7.14	30.88	8.22	50.01
	67.27	3.67	8.39	39.92	20.06	1.99	42.80	8.29	76.11
[N ₄₄₄₄]Cl	42.92	6.86	8.81	26.97	20.05	5.19	38.07	8.95	48.96
	52.43	4.02	9.12	31.81	20.18	3.08	42.70	9.03	62.70
	64.74	1.81	9.58	39.87	20.21	1.36	48.71	9.32	78.84

the pH values are due to the presence of the ionic liquids in the aqueous media.

Figs. 6 and 7 depict the results obtained for the partition coefficients and percentage extraction efficiencies of L-tryptophan in several ionic-liquid-based ABS. The composition of the coexisting phases corresponding to the common initial mixture (20 wt% of C₆H₅K₃O₇, 40 wt% of ionic liquid, and 40 wt% of an aqueous solution) is presented in Table 2.

The partition coefficients of L-tryptophan range between 3 and 67, indicating thus a preferential partitioning of the amino acid

for the ionic-liquid-rich phase in all the investigated systems. The single-step extraction efficiencies range between 72% and 99%. Although small deviations are verified in this work, it should be stressed that an increase in the partition coefficient does not necessarily involve the increase in the extraction efficiency or *vice versa*. The percentage extraction efficiencies are a result of the concentration of L-tryptophan in each phase combined with their total weight.

For the common mixture composition the partition coefficient of L-tryptophan increases in the order:

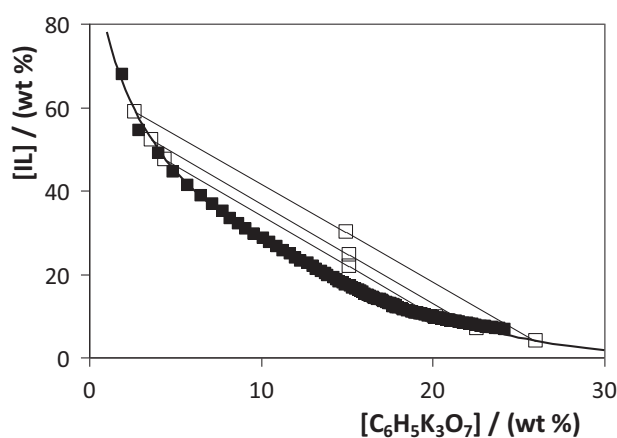


Fig. 5. Phase diagram for the ternary system composed of [C₄mim][N(CN)₂] + C₆H₅K₃O₇ + H₂O, at 298 K and atmospheric pressure: binodal curve data (■), TL data (□), adjusted binodal data through Eq. (1) (—).

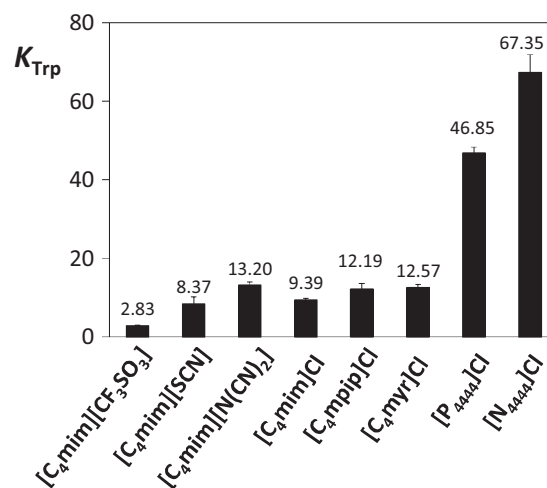


Fig. 6. Partition coefficients of L-tryptophan (K_{Trp}) in ABS composed of ionic liquids and C₆H₅K₃O₇ at 25 °C.

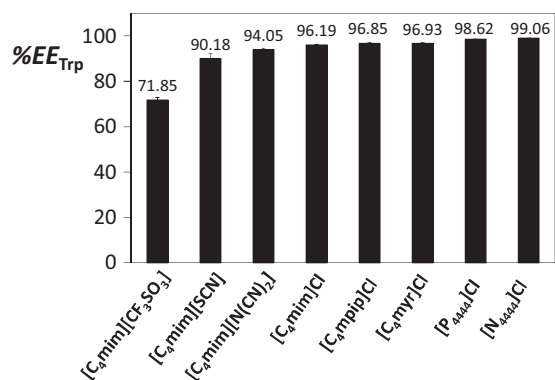


Fig. 7. Extraction efficiencies of L-tryptophan (%EE_{Trp}) in ABS composed of ionic liquids and C₆H₅K₃O₇ at 25 °C.

[C₄mim][CF₃SO₃] < [C₄mim][SCN] < [C₄mim]Cl < [C₄mpip]Cl ≈ [C₄mpyr]Cl < [C₄mim][N(CN)₂] < [P₄₄₄₄]Cl < [N₄₄₄₄]Cl.

It is well known that, depending on the pH of the solution, amino acids can be present in the form of different species due to the ionization/protonation of their characteristic functional groups: –COOH and –NH₂. Since for tryptophan, the protonation constants values are pK_{a1} = 2.38 and pK_{a2} = 9.39 [52], the amino acid is predominately on its cationic form (and therefore positively charged) in the systems with a pH below 2.38, it is mainly a zwitterion (with no net charge) if the pH is between 2.38 and 9.39, and it is negatively charged if the pH of the coexisting phases is higher than 9.39. The pH values of the ionic-liquid-rich phase in the mixtures used for the partitioning experiments are between pK_{a1} and pK_{a2}. However, most of the pH values of the phases are alkaline and near the value of pK_{a2} meaning that in some examples there are already significant amounts of charged L-tryptophan that act as excellent counter ions for the cationic more hydrophobic phase containing the ionic liquid. In fact, two systems present pH values ranging between 7.06 and 7.20, and those are the ones where the lower partitioning coefficients were observed ([C₄mim][CF₃SO₃] and [C₄mim][SCN]). For pH values above 8 the partition coefficients of L-tryptophan range between 13 and 67. Although [N₄₄₄₄]Cl presents the highest partition coefficient, which could be associated with high pH values, high partition coefficients in the [C₄mpyr]Cl-based system are not observed in spite of the similar high pH values at the coexisting phases. Moreover, no large differences in the partition coefficients of L-tryptophan are observed among the chloride and cyclic nitrogen-based ionic liquids, namely [C₄mim]Cl, [C₄mpyr]Cl and [C₄mpip]Cl. Indeed, the systems containing these ionic liquids present a similar TLL for the extraction point, reflecting thus the similarity in the composition of the coexisting phases among the 3 systems. Rogers and co-workers [13] already showed that the ionic-liquid-rich phase becomes increasingly hydrophobic as the divergence between the two phases increases, i.e. with an increase in the TLL, making use of partitioning experiments of a series of short chain alcohols. On the other hand, larger differences are observed between these and [P₄₄₄₄]Cl and [N₄₄₄₄]Cl. Systems formed by the two later ionic liquids show a better performance for extracting the amino acid, with extraction efficiencies in the order of 99%. These systems also show longer TLLs which further induce coexisting phases of progressive different polarities, albeit presenting different pH values. Finally, the effect of the ionic liquid anion seems to have a stronger effect on the partition coefficients of L-tryptophan. For the [C₄mim]-based ionic liquids the partitioning of the amino acid for the ionic-liquid-rich phase follows the order: [C₄mim][CF₃SO₃] < [C₄mim][SCN] < [C₄mim]Cl < [C₄mim][N(CN)₂]. In addition, this rank does not reflect the compositions of the

coexisting phases as shown by the TLL values. Therefore, the results obtained seem to indicate that the partition coefficient of L-tryptophan depends on a delicate balance between the amount of charged species and the composition of the phases.

In our previous works, the partition coefficients of L-tryptophan in ABS composed of diverse ionic liquids and K₃PO₄ have shown to be more dependent on the ionic liquid cation than on the ionic liquid anion [16,17]. This pattern is a direct consequence of the anionic nature of L-tryptophan in the systems formed by the highly alkaline K₃PO₄ (pH values > 12 in the coexisting phases). In the systems here studied, with lower pH values, the effect of the ionic liquid anion seems to be more relevant (especially if the extraction efficiencies are evaluated).

The partition coefficients obtained in this work are lower than those obtained before using K₃PO₄ and similar ionic liquids [16,17]. However, it should be remarked that high extraction efficiencies are still observed here while using a biodegradable organic salt such as potassium citrate. Moreover, the extraction efficiencies obtained with the citrate salt are higher than those previously observed by us with ABS formed by [C₄mim][CF₃SO₃] and a large variety of carbohydrates, where the extraction efficiencies were close to 50% [21].

Zafarani-Moattar and Hamzehzadeh [39] provided the partitioning coefficients of several amino acids in ABS composed of 1-butyl-3-methylimidazolium bromide and potassium citrate at 25 °C, and also investigated the effect of the pH of the aqueous media towards the partitioning behavior. The authors [39] concluded that hydrophobic interactions were the main driving force for the amino acids partitioning, although salting-out effects and electrostatic interactions also play a role. The partitioning coefficients of L-tryptophan increase with the TLL and with the pH increase (from pH 5.00 to 7.00) [39]. However, based on the range of the partitioning coefficient values obtained by the authors [39] and those obtained here it seems that the tailoring of the partitioning of amino acids is more easily attained by the manipulation of the ionic liquid structure (if compared with the phases composition and pH values of the coexisting phases).

Finally, it should be stressed that ionic-liquid-based ABS are enhanced techniques for extraction purposes when compared with conventional ABS systems. The partition coefficients of L-tryptophan obtained in this work are significantly higher than those observed with polymer-based ABS: K_{Trp} ≈ 1 in polymer-polysaccharide ABS [53,54] and K_{Trp} ≈ 1–7 in polymer-salt ABS [55].

4. Conclusions

In biotechnology, the extraction and purification of bio(molecules) by means of ABS is a promising tool. Ionic-liquid-based ABS have been studied as novel extractive systems for a large variety of compounds; yet, most of these works exploited high charge density inorganic salts. Aiming at developing more benign systems, we propose here the use of a biodegradable salt as a main constituent of ionic-liquid-based ABS. Novel ternary phase diagrams, tie-lines, and tie-line lengths were determined making use of a large range of ionic liquids and a common organic salt: potassium citrate. The large array of ionic liquids investigated and the distinct phase behaviors observed indicate that the tailoring of the phases' polarities can be achieved. In general, the higher the hydrophobicity of the ionic liquid, the better is the ionic liquid performance in inducing the liquid–liquid demixing.

The applicability of the studied ABS was evaluated through the determination of the partition coefficients and extraction efficiencies of a hydrophobic aromatic amino acid (L-tryptophan). In all the investigated systems the amino acid preferentially partitions for the ionic-liquid-rich phase. The partition coefficients

obtained range between 3 and 67, and extraction efficiencies between 72% and 99% were achieved. The partition coefficients of L-tryptophan in ionic-liquid-based ABS are substantially higher than those observed in conventional polymer-based ABS. Therefore, the application of the systems here investigated in biotechnological approaches, aiming at continuously extracting the amino acid and avoiding competing pathways, is sprightly envisaged.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at <http://dx.doi.org/10.1016/j.bej.2012.05.004>.

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