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Ionic liquids as additives to enhance the extraction of antioxidants in aqueous two-phase systems

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ABSTRACT

Aqueous two-phase systems (ATPS) have been proposed as an alternative technique for the extraction, separation and/or purification of diverse biomolecules. Besides the typical polymer–salt ATPS, recently, ionic-liquid-(IL)–salt combinations have been reported to present higher extraction performances than the former systems are able to provide. Therefore, aiming at using the tailoring ability and high extraction efficiencies offered by ILs, yet with lower IL amounts, in this work novel ATPS composed of polyethylene glycol (PEG) and Na_2SO_4, using ILs as additives (at 5 or 10 wt%), were studied. Both the determination of the phase diagrams and their extraction efficiencies for gallic, vanillic and syringic acids were determined at 298 K. Furthermore, the effects of the molecular weight of PEG (200, 300, 400 and 600 g mol^{-1}) and of the IL chemical structure were investigated. The two-phase formation ability increases with the increase of the PEG molecular weight. Moreover, the addition of low amounts of ILs is favorable for the liquid–liquid demixing. The results obtained indicate that all the antioxidants investigated preferentially partition for the PEG-rich phase although depending on the PEG molecular weight and IL employed. The addition of 5 wt% of IL leads to extraction efficiencies ranging between 80% and 99%. These results clearly demonstrate the ability of the IL to tune the polarity of the PEG-rich phase and where the IL chemical structure plays a dominant role in the extraction of phenolic acids. PEG–salt–IL ATPS represent thus an interesting advance in separation processes and open the door for a new range of IL-based extraction processes.

1. Introduction

Aqueous two-phase systems (ATPS) consist on two macroscopic liquid phases formed by the dissolution in water, above certain concentrations, of two incompatible hydrophilic solutes [1]. These solutes can be two polymers, a polymer and a salt, or two salts [2–5]. Due to the existence of their liquid coexisting phases, ATPS can be regarded as a powerful and non-chromatographic process for the separation and/or purification of the most diverse biomolecules. In fact, conventional ATPS have been successfully applied in the purification of different biological materials, such as cells, nucleic acids, lipids, amino acids, proteins, antibodies and enzymes without significant denaturing effects [1,2,6–8]. Both phases in ATPS mainly consist of water (*ca.* 80–90 wt%) and most of the polymers have a stabilizing effect on the proteins tertiary structure [7,9,10]. This technique is relatively simple and inexpensive, of easy operation allowing its scale-up, and further ensures the purification and concentration stages to be integrated in a single step procedure [7,9].

Conventional ATPS are typically formed by polymer–polymer or by polymer–salt mixtures [1]. Polyethylene glycol (PEG) is commonly used as one of the phase-forming polymers in ATPS because it presents high biodegradability, low toxicity, low volatility, low melting temperature, large water miscibility and low cost [6,11]. PEG is a polyether diol that is commercially available in a wide variety of molecular weights. Salt-polymer-type ATPS provide advantages over systems formed by polymer–polymer combinations, such as a low interfacial tension, fast and high phase separation rates and low cost, which makes them practical for downstream processing [6]. Despite all these advantages, the narrow tailoring nature of PEG, which can be achieved only by changes in the molecular weight or by the polymer structural modification, limits its applicability through the complete extraction of several biomolecules to the polymer-rich phase [6]. To overcome this limitation, recent works have introduced ionic

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liquids (ILs) to tune the physicochemical properties of the PEG-rich phase, either by using them as adjuvants or by the synthesis of PEG-functionalized ILs foreseeing high extraction yields [6,12,13]. The reported results [6,12,13] suggest that the use of ILs in conventional ATPS provide tailored and optimized extractions by a proper choice of the chemical structure of the IL.

ILs are salts that are liquid below a conventional temperature of 100 °C and they are usually constituted by a large asymmetric organic cation and either an organic or inorganic anion. Due to their inherent ionic character, most of these fluids present remarkable properties, such as a negligible vapor pressure, null flammability, high ionic conductivity, as well as high thermal and electrochemical stabilities [14–17]. In addition to the ILs negligible volatility and non-flammability – the main features which have contributed to their recurrent classification as "green solvents" – one of the main advantages of ILs as phase-forming components in ATPS is the possibility of tailoring their phases' polarities and affinities by an adequate manipulation of the cation/anion combinations ("designer solvents") [18]. Indeed, ATPS constituted by ILs cover a much wider hydrophilic–lipophilic range allowing for more extensive and selective separations [19]. Due to these outstanding features, ATPS composed of ILs have been intensively investigated in the last decade for the extraction of the most diverse (bio)molecules [2,6,20–23], where results up to complete extraction and concentration factors up to 100 times were achieved.

Antioxidants are phenolic compounds that exhibit relevant properties in the health and nutrition fields. These compounds are widely used in dietary supplements and they have been investigated for the prevention of cancer, coronary heart disease and even altitude sickness due to their antioxidant and radical scavenging properties [24]. Antioxidants are also commonly used and/or added in nutraceutical and cosmetic-related products [25]. Examples of simple antioxidants structures are vanillic, gallic, protocatechuic, ellagic and syringic acids that are typically present in natural sources such as wood, barks, fruits and vegetables [26,27]. In the past few years, there has been a great demand for antioxidants extracted from natural sources to substitute synthetic counterparts that can lead to adverse effects in human health [25,28].

In order to develop new systems for the extraction and concentration of antioxidants, in this work, the ternary phase diagrams of ATPS composed of PEG + $NaSO_4$ were firstly determined at 298 K. The effect of the molecular weight of PEG (200, 300, 400 and 600 g mol^{-1}) was also addressed through the phase diagrams behavior. These ATPS were then evaluated in what concerns their extractive performance for three antioxidants, namely gallic acid (3,4,5-trihydroxybenzoic acid, $C_6H_2(OH)_3COOH$), vanillic acid (4-hydroxy-3-methoxybenzoic acid, $C_6H_3(OH)(OCH_3)COOH$) and syringic acid (4-hydroxy-3,5-dimethoxybenzoic acid, $C_6H_2(OH)(OCH_3)_2COOH$). The molecular structures of the antioxidants investigated are depicted in Fig. 1. Aiming at tailoring the properties of the coexisting phases in the studied polymer–salt ATPS, ILs were additionally evaluated as potential adjuvants to tune the partitioning of the biomolecules for the PEG-rich phase. The effect of eight ILs and their concentration (5 and 10 wt%) in the phase diagrams of the systems constituted by water + PEG + Na_2SO_4 was addressed. Moreover, the influence of the IL chemical structure and pH of the medium on the partition coefficients and extraction efficiencies of gallic, vanillic and syringic acids were evaluated and compared with the results where no IL was added. The chemical structures of the ILs and polymer investigated are shown in Fig. 2.

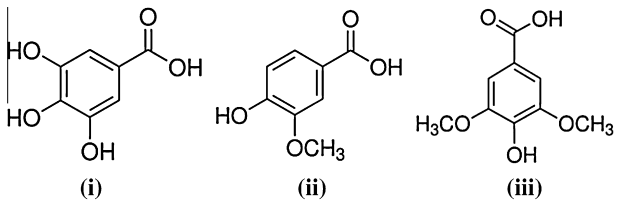

Fig. 1. Chemical structure of the antioxidants studied: (i) gallic acid; (ii) vanillic acid; (iii) syringic acid.

2. Experimental section

2.1. Materials

The ATPS studied in this work were established by using an aqueous solution of sodium sulfate, Na_2SO_4 (anhydrous, 100 wt% pure from Prolabo), and several solutions of PEGs. The PEGs studied were of molecular weight 200 g mol^{-1}, 300 g mol^{-1}, 400 g mol^{-1} and 600 g mol^{-1} and are abbreviated as PEG 200, PEG 300, PEG 400 and PEG 600, respectively. All the polymers were acquired from Fluka with the exception of PEG 300 that was from Sigma–Aldrich. Besides the determination of the PEG–salt systems, the effect of ILs through the phase diagrams and partitioning behavior was also investigated. The ILs studied were: 1-butyl-3-methylimidazolium thiocyanate, [C_4mim][SCN] (>98 wt% pure); 1-butyl-3-methylimidazolium tosylate, [C_4mim][TOS] (98 wt% pure); 1-butyl-3-methylimidazolium dicyanamide, [C_4mim][$N(CN)_2$] (>98 wt% pure); 1-butyl-3-methylimidazolium acetate, [C_4mim][CH_3CO_2] (98 wt% pure); 1-butyl-3-methylimidazolium chloride, [C_4mim]Cl (>99 wt% pure); 1-butyl-1-methylpiperidinium chloride, [C_4mpip]Cl (99 wt% pure); and 1-butyl-1-methylpyrrolidinium chloride, [C_4mpyr]Cl (>99 wt% pure). All ILs were purchased from Iolitec and their chemical structures are shown in Fig. 2. To reduce the content of water and other volatile compounds to negligible values, ILs individual samples were dried under constant agitation, at vacuum and moderate temperature (≈323 K), for a minimum of 24 h. After this process, the purity of each IL was further checked by ^1H and ^{13}C NMR spectra and found to be in accordance with the purity levels given by the supplier.

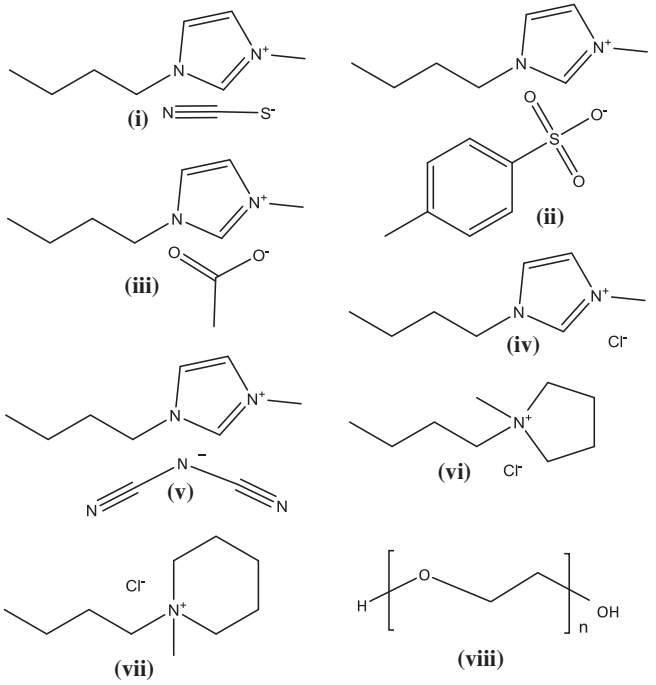

Fig. 2. Chemical structure of the studied ILs and PEG: (i) [C_4mim][SCN]; (ii) [C_4mim][TOS]; (iii) [C_4mim][CH_3CO_2]; (iv) [C_4mim]Cl; (v) [C_4mim][$N(CN)_2$]; (vi) [C_4mpyr]Cl; (vii) [C_4mpip]Cl; (viii) PEG.

Gallic acid (99.5 wt% pure), vanillic acid (97 wt% pure), and syringic acid (>98 wt% pure) were acquired from Merck, Sigma–Aldrich and Alfa Aesar, respectively.

The water employed was double distilled, passed across a reverse osmosis system and finally treated with a Milli-Q plus 185 water purification apparatus.

3. Experimental procedure

3.1. Phase diagrams and tie-lines

The binodal curve of each phase diagram was determined through the cloud point titration method at 298 K (±1 K) and atmospheric pressure [29]. Aqueous solutions of Na_2SO_4 at 17 wt% and pure PEGs were used in the determination of the PEG-salt phase diagrams. To study the effect of each IL in the phase diagrams behavior, aqueous solutions of Na_2SO_4 at 17 wt% and aqueous solutions of the different PEGs at *circa* 70 wt% were used. To these aqueous solutions used to determine the phase diagrams in the presence of IL, each IL was added and kept at a constant concentration during all the experimental procedure (at 5 or 10 wt%). Repetitive drop-wise addition of the aqueous inorganic salt solution to the PEG solution was carried out until the detection of a cloudy biphasic solution, followed by the drop-wise addition of water (or IL aqueous solution) until the detection of a monophasic region. This procedure was carried out under constant stirring. The systems compositions were determined by the weight quantification of all components added within an uncertainty of $\pm 10^{-4}$ g.

The tie-lines (TLs) of each phase diagram were determined by a gravimetric method originally described by Merchuk et al. [30]. A mixture at the biphasic region was gravimetrically prepared with PEG + salt + water or with PEG + salt + water + IL, vigorously stirred, and allowed to reach the equilibrium by the separation of the two phases for at least 12 h at 298 K (±1 K). After the separation of the coexisting phases they were further weighted. Finally, each individual TL was determined by the application of the lever-arm rule to the relationship between the weight of the top and bottom phases and the overall system composition. It should be remarked that the IL concentration was kept constant in the determination of each phase diagram and it was assumed to be part of the solvent (water + IL) in the representation of the phase diagrams shown below.

The experimental binodal curves were fitted using Eq. (1) [30]:

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

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

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

$$[PEG]_{PEG} = A \exp\left[\left(B[salt]_{PEG}^{0.5}\right) - \left(C[salt]_{PEG}^{3}\right)\right] \quad (2)$$

$$[PEG]_{salt} = A \exp\left[\left(B[salt]_{salt}^{0.5}\right) - \left(C[salt]_{salt}^{3}\right)\right] \quad (3)$$

$$[PEG]_{PEG} = \frac{[PEG]_M}{\alpha} - \left(\frac{1-\alpha}{\alpha}\right)[PEG]_{salt} \quad (4)$$

$$[salt]_{PEG} = \frac{[salt]_M}{\alpha} - \left(\frac{1-\alpha}{\alpha}\right)[salt]_{salt} \quad (5)$$

where the subscripts "PEG", "salt" and "M" represent the top and the bottom phases, and the mixture composition, respectively. The parameter α is the ratio between the weight of the top phase and the weight of the total mixture. The solution of the referred system gives the concentration of PEG and Na_2SO_4 in the top and bottom phases. For the examples where IL was added, it was considered as part of the solvent (water + IL at 5 wt% or 10 wt%) for the application of Eqs. (2)–(5).

For the calculation of the tie-line lengths (TLLs) it was applied Eq. (6),

$$TLL = \sqrt{\left([salt]_{PEG} - [salt]_{salt}\right)^2 + \left([PEG]_{PEG} - [PEG]_{salt}\right)^2} \quad (6)$$

3.2. Partitioning of antioxidants and ILs in the PEG-salt ATPS

Aqueous solutions of each antioxidant were prepared (0.006 mol dm^{-3} for gallic acid, and 0.030 mol dm^{-3} for vanillic and syringic acids) and used as the aqueous solution required to form the biphasic systems at a given composition. The ternary and quaternary mixtures compositions were chosen based on the phase diagrams determined in this work, either for each PEG–Na_2SO_4 ATPS or for the PEG–Na_2SO_4–IL quaternary systems. To avoid some discrepancies and less accurate interpretations on the partitioning results data, all the partitioning studies were performed at a similar TLL (≈34–37 wt%). The initial mixture compositions are presented in Table 1 while the respective TLs and TLLs are presented in Table 2. In the quaternary systems (with IL) the concentration of IL was maintained at 5 wt% because this lower amount revealed to be enough to almost completely extract all antioxidants for the PEG-rich phase as shown thereinafter. This low amount of IL contributes thus to a lower cost of the overall process when large-scale applications are envisaged.

Each mixture was prepared gravimetrically within $\pm 10^{-4}$ g, vigorously stirred and left to equilibrate for at least 12 h (a time period established in previous optimizing experiments) and at 298 K (±1 K), to achieve the complete partitioning of each antioxidant between the two phases. After the careful separation of the phases, using small glass ampoules designed for the purpose, the amount of a given antioxidant was quantified in each phase. At least three individual experiments were carried out for each ATPS allowing the determination of the average partition coefficients and respective standard deviations. The antioxidant content was quantified through UV-spectroscopy, using a SHIMADZU UV-1700, Pharma-Spec Spectrometer, at a wavelength of 262 nm for gallic acid, 292 nm for vanillic acid and 274 nm for syringic acid, using calibration curves previously established.

The partition coefficients of the studied biomolecules, K_{GA} for gallic acid, K_{VA} for vanillic acid and K_{SA} for syringic acid were determined according to Eq. (7),

Table 1
Initial mixture compositions of the ATPS composed of PEG + Na_2SO_4 and PEG 300 + Na_2SO_4 + 5 wt% IL.

PEG	IL	Weight fraction composition (wt%)		
		PEG	Na_2SO_4	IL
PEG + Na_2SO_4 + water				
600	×	18.02	12.11	0.00
400	×	22.05	12.05	0.00
300	×	23.05	12.05	0.00
200	×	25.00	10.00	0.00
PEG 300 + Na_2SO_4 + 5 wt% IL + water				
300	[C_4mim][TOS]	20.16	12.06	5.12
300	[C_4mim][SCN]	19.06	12.06	5.07
300	[C_4mim][$N(CN)_2$]	19.06	12.05	5.04
300	[C_4mim][CH_3CO_2]	26.52	10.04	4.87
300	[C_4mim]Cl	23.01	12.10	5.05
300	[C_4mpyr]Cl	22.90	12.04	5.08
300	[C_4mpip]Cl	22.99	12.12	5.16

Table 2
Experimental TLs and TLLs of the ATPS composed of PEG + Na_2SO_4 and PEG 300 + Na_2SO_4 + 5 wt% IL.

Weight fraction composition (wt%)							
PEG + Na_2SO_4 + water							
PEG	$[PEG]_{PEG}$	$[salt]_{PEG}$	$[PEG]_M$	$[salt]_M$	$[PEG]_{salt}$	$[salt]_{salt}$	TLL
600	35.26	3.70	18.02	12.11	2.24	19.81	36.75
	40.36	2.23	23.87	10.09	1.62	20.72	42.93
400	35.07	3.93	22.05	12.05	4.92	22.73	35.53
	36.87	3.51	26.99	10.02	3.07	25.81	40.49
300	34.62	4.81	23.05	12.05	5.66	22.91	34.16
	35.52	4.56	24.09	12.04	4.06	25.14	37.59
200	32.34	7.49	25.89	12.43	8.62	25.63	29.86
	30.31	5.72	25.00	10.00	4.81	28.54	34.22
PEG 300 + Na_2SO_4 + 5 wt% IL + water							
IL	$[PEG]_{PEG}$	$[salt]_{PEG}$	$[PEG]_M$	$[salt]_M$	$[PEG]_{salt}$	$[salt]_{salt}$	TLL
[C_4mim][TOS]	31.86	4.69	20.16	12.06	1.16	24.01	36.27
	34.16	4.06	16.94	15.46	0.53	26.31	40.32
[C_4mim][SCN]	30.41	4.85	19.06	12.06	1.30	23.33	34.48
	33.24	4.15	16.08	15.02	0.87	24.65	38.32
[C_4mim][$N(CN)_2$]	32.61	4.20	19.06	12.05	1.13	22.44	36.38
	38.00	2.97	17.16	15.00	0.51	24.61	43.29
	39.86	2.62	10.00	20.15	0.31	25.84	45.87
	43.23	2.30	30.94	10.19	0.01	30.02	51.31
	44.64	2.08	32.97	10.05	0.01	32.54	54.04
[C_4mim][CH_3CO_2]	32.96	4.90	26.52	10.04	5.70	26.67	34.88
	27.70	6.67	19.86	13.55	6.38	25.39	28.37
[C_4mim]Cl	31.94	5.10	23.01	12.10	3.01	27.77	36.75
	32.32	4.99	19.98	14.99	2.41	29.24	38.49
[C_4mpyr]Cl	32.05	4.80	22.90	12.04	3.52	27.38	36.39
	32.91	4.56	20.03	14.97	2.92	28.80	38.57
[C_4mpip]Cl	32.41	4.46	22.99	12.12	3.58	27.92	37.17
	38.70	3.12	29.90	10.02	2.30	31.64	46.24

$$K_{Ant} = \frac{[Ant]_{PEG}}{[Ant]_{salt}} \quad (7)$$

where $[Ant]_{PEG}$ and $[Ant]_{salt}$ are the concentration of each antioxidant in the PEG-rich and in the salt-rich aqueous phases, respectively.

The percentage extraction efficiencies of each biomolecule, $EE_{GA}\%$ for gallic acid, $EE_{VA}\%$ for vanillic acid and $EE_{SA}\%$ for syringic acid, are defined as the percentage ratio between the amount of each antioxidant in the PEG-rich aqueous phase and that in the total mixture, according to Eq. (8),

$$EE_{Ant}\% = \frac{w_{Ant}^{PEG}}{w_{Ant}^{PEG} + w_{Ant}^{Salt}} \times 100 \quad (8)$$

where w_{Ant}^{PEG} and w_{Ant}^{Salt} are the weight of antioxidant in the PEG-rich and in the salt-rich aqueous phases, respectively.

Possible interferences of Na_2SO_4 and the different PEGs or ILs with the analytical method were investigated and found to be not significant at the dilutions carried out for quantification. Only one exception was verified for the ATPS containing [C_4mim][TOS] where the absorbance of the aromatic IL anion interferes with the quantification of the antioxidants. Control or "blank" solutions at the same mixture point used for the extraction studies (with no antioxidant added) were used in this particular system.

For the ATPS containing the IL at a fixed concentration, it was also determined the partition coefficient of the IL itself for a better understanding of the antioxidants migration phenomenon. The concentration of imidazolium and pyridinium-based ILs was determined by UV-spectroscopy using a SHIMADZU UV-1700, PharmaSpec Spectrometer, at a wavelength of 211 nm for the imidazolium ring, while the concentration of pyrrolidinium- and piperidinium-based ILs was determined by conductivity measurements, at room temperature, using a Mettler Toledo S47 SevenMulti™ dual meter pH/conductivity equipment. Since both the top and bottom phases have sodium sulfate, the concentration of pyrrolidinium- and piperidinium-based ILs was determined over "blank" solutions of Na_2SO_4 prepared at accurate concentrations as described by the respective TLs. In the biphasic regime, and after the careful separation of the phases of each ATPS, the experimental TLs presented in Table 2 provide the Na_2SO_4 content in each phase of a given ATPS. All reference solutions were then prepared according to these concentrations.

The partition coefficient of IL, K_{IL}, was determined according to Eq. (9),

$$K_{IL} = \frac{[IL]_{PEG}}{[IL]_{salt}} \quad (9)$$

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

3.3. pH measurements

The pH of the PEG- and Na_2SO_4-rich aqueous phases was measured at 298 K (±1 K) using a Mettler Toledo S47 SevenMulti™ dual meter pH/conductivity equipment within ±0.02. The calibration of the pH meter was carried out with two buffers (pH values of 4.00 and 7.00).

4. Results and discussion

4.1. Phase diagrams and tie-lines

Novel ternary phase diagrams were determined for several PEGs (PEG 200, 300, 400 and 600) + water + Na_2SO_4, at 298 K and at atmospheric pressure. The respective ternary phase diagrams are illustrated in Fig. 3. The experimental weight fraction data of

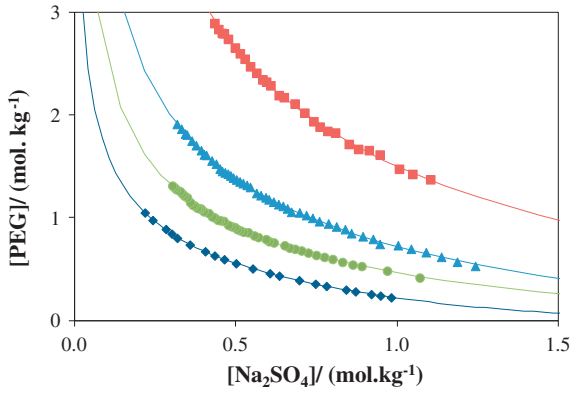

Fig. 3. Phase diagrams for the systems composed of PEG + Na_2SO_4 + H_2O: PEG 200 (■); PEG 300 (▲), PEG 400 (●); PEG 600 (◆). The lines correspond to the respective correlations derived from Eq. (1).

each phase diagram are reported in the Supporting Information. In all the studied ATPS, the top phase corresponds to the aqueous PEG-rich phase while the bottom phase is mainly composed of Na_2SO_4 and water.

The system composed of PEG 600, Na_2SO_4 and water has already been reported in literature and the results obtained in this work are in close agreement with literature data [6,31] – *cf.* Supporting Information.

Fig. 3 depicts the effect of the molecular weight of PEG in the formation of ATPS. The solubility curves are presented in molality units for a better understanding of the impact of each species on the phase diagrams behavior. In Fig. 3, the biphasic or two-phase region is localized above the solubility curve. The larger this regime, the higher is the ability of PEG to undergo liquid–liquid demixing in the presence of Na_2SO_4 aqueous solutions.

The influence of the PEG molecular weight on the phase diagrams is notorious. For polymers of lower molecular weight the phase separation only occurs at higher concentrations of PEG and Na_2SO_4. In general, the ability of PEG to form ATPS in the presence of a fixed inorganic salt decreases in the following order: PEG 600 > PEG 400 > PEG 300 > PEG 200. Similar trends have been observed in other ATPS composed of polymer/salt or PEG/IL pairs [7,32]. This behavior is a consequence of the higher hydrophobicity displayed by PEGs of higher molecular weight, *i.e.*, they present a lower affinity for water, and are more easily excluded for a second liquid phase [32].

Fig. 4 shows the experimental phase diagrams, at 298 K and atmospheric pressure, for each system constituted by PEG 300 + Na_2SO_4 + H_2O + $[C_4mim][N(CN)_2]$, at various concentrations of IL (0, 5 and 10 wt%), as well as the system constituted by $[C_4mim][N(CN)_2]$ + Na_2SO_4 + H_2O (where no PEG is present) previously reported in literature [29]. The binodal curves are reported in molality units to better evaluate the impact of the IL in the formation of ATPS. The respective experimental data and the representation of the phase diagrams in weight fraction are given in the Supporting Information.

The ability of each ATPS to form two liquid phases is as follows: PEG 300 + Na_2SO_4 + H_2O + 10 wt% $[C_4mim][N(CN)_2]$ > PEG 300 + Na_2SO_4 + H_2O + 5 wt% $[C_4mim][N(CN)_2]$ > $[C_4mim][N(CN)_2]$ + Na_2SO_4 + H_2O (no PEG) > PEG 300 + Na_2SO_4 + H_2O (no IL). Overall, lower amounts of inorganic salt (in molality units) are needed to form the PEG–Na_2SO_4 when compared with the IL–Na_2SO_4 system. This pattern could be a direct consequence of the higher hydrophobicity and neutral character of PEG compared to hydrophilic ILs, being thus the polymer more easily excluded for a second liquid phase. However, surprising results are obtained with the addition of ILs as adjuvants. ATPS formed by ILs added in small amounts display a better phase separation than the systems formed with either the PEG or the IL and salt. This trend suggests that mixtures of IL–PEG may be more "hydrophobic" than their pure components and are more easily salted-out by Na_2SO_4 in aqueous media. These results clearly reveal two advantages associated to the polymer–salt type ATPS using ILs as adjuvants: better performance for phase separation, requiring therefore lower amounts of each solute to form an ATPS, and comparatively more benign and cheaper than IL–salt–ATPS since lower amounts of IL are used. It should be remarked that the systems formed by IL as adjuvants also display a better phase separation when compared in weight fraction, and as revealed in the Supporting Information.

Figs. 5 and 6 depict the experimental phase diagrams, at 298 K and atmospheric pressure, for the systems constituted by PEG 300 + Na_2SO_4 + H_2O + 5 wt% IL, and allow the comparison of the IL cation *versus* anion effects on the phase separation ability. The respective experimental weight fraction data are given in the Supporting Information. It should be pointed out that the IL concentration was kept constant during the determination of all phase diagrams. The binodal curves are also reported in molality units for an enhanced understanding on the impact of the distinct ILs in the formation of ATPS.

Fig. 5 depicts the influence of 5 wt% of $[C_4mim]$-based ILs, and hence of the IL anion nature, on the phase diagrams behavior. Results for $[C_4mim][TOS]$, $[C_4mim][SCN]$, $[C_4mim][CH_3CO_2]$, $[C_4mim]Cl$ and $[C_4mim][N(CN)_2]$ are presented. The phase diagram

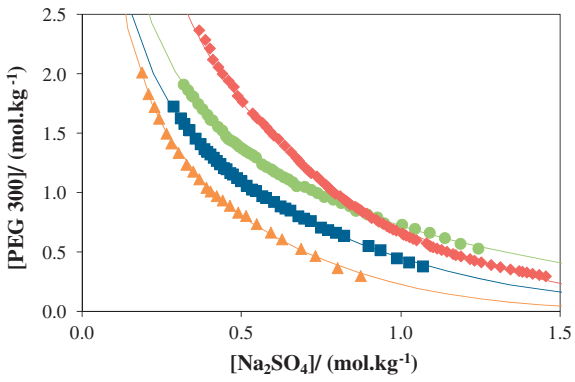

Fig. 4. Phase diagrams for the systems composed of PEG + Na_2SO_4 + H_2O + $[C_4mim][N(CN)_2]$ at 298 K: no IL (●); 5 wt% of IL (■); 10 wt% of IL (▲); no PEG (◆). The lines correspond to the respective correlations derived from Eq. (1).

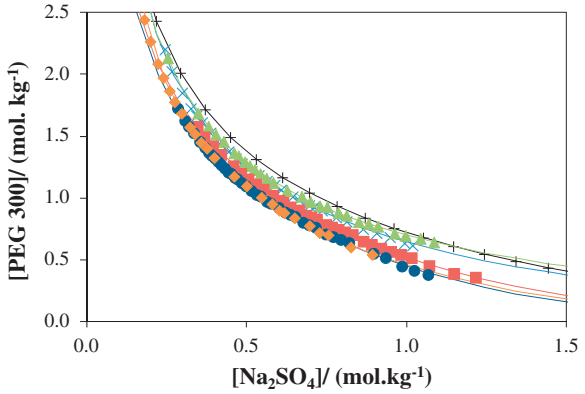

Fig. 5. Phase diagrams for the $[C_4mim]$-based quaternary systems composed of PEG 300 + Na_2SO_4 + H_2O + 5 wt% IL at 298 K: no IL (+); $[C_4mim][CH_3CO_2]$ (▲); $[C_4mim]Cl$ (×); $[C_4mim][TOS]$ (■); $[C_4mim][N(CN)_2]$ (●); $[C_4mim][SCN]$ (◆). The lines correspond to the respective correlations derived from Eq. (1).

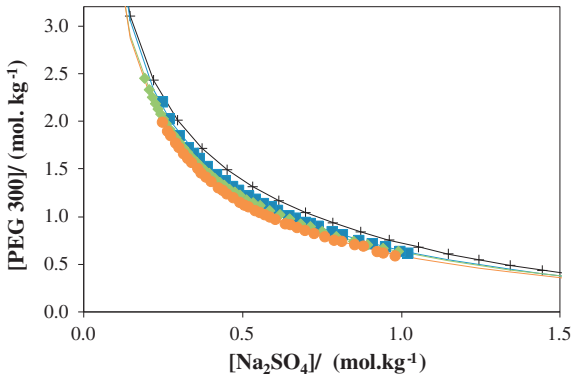

Fig. 6. Phase diagrams for the chloride-based quaternary systems composed of PEG 300 + Na_2SO_4 + H_2O + 5 wt% of IL at 298 K: no IL (+); [C_4mim]Cl (■); [C_4mpyr]Cl (◆); [C_4mpip]Cl (●). The lines correspond to the respective correlations derived from Eq. (1).

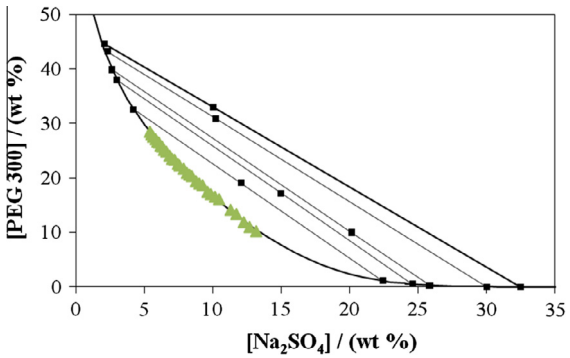

Fig. 7. Phase diagram for the quaternary system composed of PEG 400 + Na_2SO_4 + H_2O + 5 wt% of [C_4mim][$N(CN)_2$]: binodal curve data (▲); TL data (■); adjusted binodal data using Eq. (1) (—).

for the polymer–salt system without IL is also represented in Fig. 5 for comparison purposes. In all the examples, the presence of 5 wt% of IL promotes the phase separation since the biphasic region of the IL-containing systems is always larger than those of the control (no IL) ATPS. The IL anion ability to form ATPS is as follows: $[CH_3CO_2]^- \approx Cl^- < [TOS]^- < [SCN]^- \approx [N(CN)_2]^-$. This pattern closely follows the IL anion affinity for water or the anion hydrogen-bond basicity [29]. The hydrophobicity of the IL anion or its ability to hydrogen-bond with water largely controls the ATPS formation as previously demonstrated [6]. More hydrophobic ILs, or those composed of anions with a lower ability to accept protons from water, are more easily excluded to a second liquid phase. This pattern further prove that the inorganic salt is also inducing the salting-out of the low amount of IL present in the aqueous medium towards the PEG-rich phase as will be demonstrated and discussed below.

Fig. 6 shows the influence the IL cation ability to form ATPS at a common concentration of 5 wt% of [C_4mim]Cl, [C_4mpyr]Cl and [C_4mpip]Cl. As previously observed with the IL anion influence, also in all the studied systems where the Cl^- anion was kept constant, the presence of 5 wt% of IL facilitates the phase separation. The IL cation ability to form ATPS is as follows: $[C_4mim]^+ < [C_4mpyr]^+ < [C_4mpip]^+$. This trend reflects the capacity of the IL cation to be solvated by water (since the chloride counterion is common to all ILs) and which is regulated by steric and entropic contributions [33–35]. In general, the aromatic cations, with higher affinity for water, present a lower ability for phase separation when compared with their saturated counterparts ($[C_4mim]^+ < [C_4mpyr]^+$). Among the non-aromatic ILs, the 6-sided ring ILs are more able for undergo liquid–liquid demixing when compared with the 5-sided ring fluids ($[C_4mpip]^+ > [C_4mpyr]^+$). In fact, this trend is also confirmed by results previously reported for IL-based ATPS and where the IL is salted-out by an inorganic/organic salt [34,36]. However, it should be remarked that the IL cation effect is less relevant in the phase diagrams behavior when compared with the IL anion influence shown before. Anions are typically more polarizable and their hydration is usually stronger than that of cations and, therefore, their salting-in/salting-out effects are more pronounced [37].

All the experimental binodal curves were fitted by the empirical relationship described by Eq. (1). The regression parameters were estimated by the least-squares regression method, and their values and corresponding standard deviations (σ) are provided in the Supporting Information. Figs. 3–6 depict the corresponding binodal curves (description by Eq. (1)) in addition to the experimental data.

The experimental TLs, along with their respective length (TLLs), are reported in Table 2. An example of the TLs obtained is shown in Fig. 7.

4.2. Effect of the molecular weight of PEG in the gallic acid partitioning

The partition coefficients or preferential migration of solutes/biomolecules in ATPS are dependent on specific and favorable interactions and/or their affinity for a given phase, electrostatic forces, molecular size, solubility, among others. The magnitude of the partition coefficient parameters further depends on the two-phase compositions and temperature [6,38,39].

The effect of the molecular weight of the polymer in the partitioning of gallic acid in PEG + Na_2SO_4 systems was evaluated using four PEGs with distinct molecular weights (PEG 600, 400, 300 and 200). The partition coefficients and extraction efficiencies of gallic acid, at 298 K and in the several PEG + Na_2SO_4 ATPS, at a common TLL ≈ 34–37, are shown in Fig. 8. The mixture compositions used in partitioning experiments are presented in Table 1 whereas the respective phases' compositions and TLLs are presented in Table 2. In addition, the pH values of both top and bottom phases are also shown due to the possible speciation of gallic acid. The dissociation curves of gallic acid (p*K*a = 4.0; 9.4; 11.0) [40] as a function of pH are presented in the Supporting Information. The partition

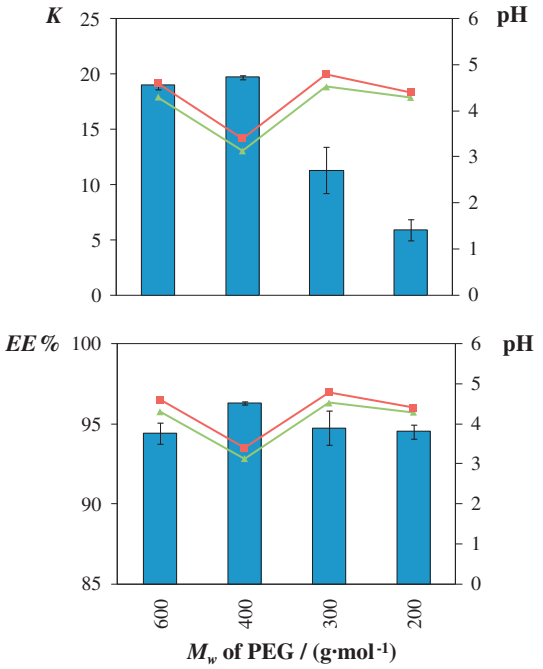

Fig. 8. Partition coefficients (*K*) and extraction efficiencies (*EE*%) of gallic acid and pH of the top (squares) and bottom (triangles) phases for the systems composed of PEG + Na_2SO_4 at 298 K according to the molecular weight of PEG.

coefficients and extraction efficiencies of gallic acid and respective standard deviations are presented in Table 3.

In all systems it is observed a preferential partitioning of gallic acid for the PEG-rich aqueous phase with partition coefficients larger than 1. The preferential migration of gallic acid observed in this work follows a similar pattern to that previously reported with a clear preference of the antioxidant for the most hydrophobic phase [28]. The partitioning coefficients of gallic acid range between 5.9 and 19.7 and are highly dependent on the molecular weight of the PEG used. Lower partition coefficients of gallic acid occur for PEGs of lower molecular weight supporting the preferential migration of the antioxidant for the most hydrophobic and less charged phase. However, one exception is observed with PEG 400 where the partitioning coefficient of gallic acid is slightly higher than the partitioning coefficient observed in the ATPS composed of PEG 600. This tendency can be a result of the lower pH of the aqueous medium afforded by the PEG 600 samples which lead to a higher amount of non-charged gallic acid in solution, and thus, to a preferential affinity for less ionic and more hydrophobic polymer-rich phases. This pattern is in close agreement with previous partitioning results of phenolic acids in IL–salt ATPS [28]. Previously we have demonstrated that the partition behavior of phenolic acids is strongly pH dependent [28]. Charged species tend to migrate into the salt-rich phase whereas neutral molecules preferentially partition into the most hydrophobic and less charged layer. This pH-driven phenomenon is indeed useful in the development of back-extraction processes as previously shown [40].

The extraction efficiencies of gallic acid, depicted in Fig. 8, are in a narrower range if compared with the partition coefficient values, and vary between 94% and 96%. With the exception of PEG 400, that presents the highest extraction efficiency, all PEGs have similar extraction efficiencies and around 94%. The exception showed by PEG 400 suggests that not only the molecular weight of the polymer influences the partitioning of gallic acid but also other factors are acting in opposite directions, such as the pH of the medium, and as discussed before. Finally, it should be highlighted that the extraction efficiencies obtained here are higher than those previously reported for similar systems yet with other inorganic salt, such as PEG 6000/$(NH_4)_2SO_4$ [41], or even with IL–salt-based ATPS [28].

4.3. Effect of the IL chemical structure in the gallic acid partitioning

With the goal of improving the extractive performance of the studied ATPS, several ILs were studied as adjuvants at a fixed 5 wt% concentration. Both the IL anion and cation effects were evaluated with combinations of the $[C_4mim]^+$ cation with $[CH_3CO_2]^-$, $[TOS]^-$, $[SCN]^-$, $[N(CN)_2]^-$ and Cl^-, and by keeping the Cl^- anion while changing the cations $[C_4mim]^+$, $[C_4mpyr]^+$ and $[C_4mpip]^+$. The effect of the ILs on the extraction of gallic acid was investigated with the PEG 300 + Na_2SO_4 ATPS. Besides its lower partitioning coefficient that further permits a better inspection of the IL impact, ATPS with lower molecular weight PEGs are by far less viscous meaning that they are more advantageous for scale-up since they require lower energy consumption.

The detailed partition coefficients and extraction efficiencies of gallic acid at 298 K are reported in Table 3. The respective TL data corresponding to the biphasic systems where the extractions were carried out are listed in Table 2. The K_{GA} and EE_{GA}% dependence on the IL anion and cation, at 298 K, is displayed in Figs. 9 and 10,

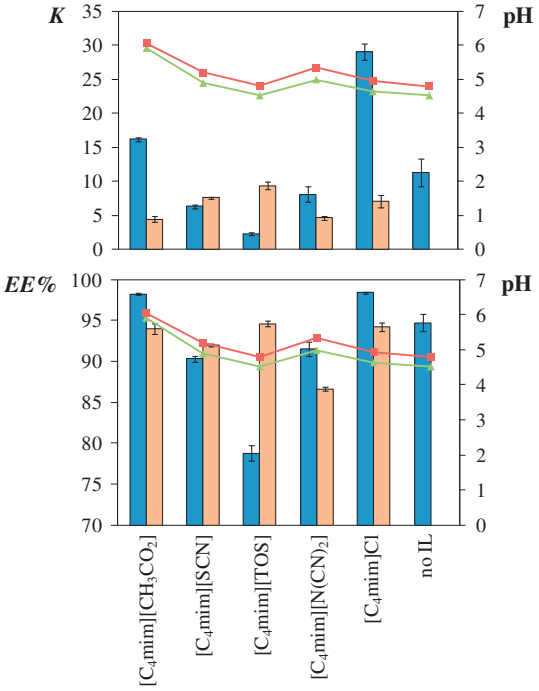

Fig. 9. Partition coefficients (K) and extraction efficiencies (EE%) of gallic acid (blue) and of each IL (orange), and pH of the top (squares) and bottom (triangles) phases, for the [C_4mim]-based systems composed of PEG 300 + Na_2SO_4 + 5 wt% IL at 298 K. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

Table 3
Partition coefficients of each antioxidant (K_{Ant}) and IL (K_{IL}), and extraction efficiencies of each antioxidant (EE_{Ant}%) and IL (EE_{IL}%), in the ternary and quaternary systems at 298 K.

PEG	IL	Antioxidant[a]	$K_{Ant} \pm \sigma$	$K_{IL} \pm \sigma$	$EE_{Ant}\% \pm \sigma$	$EE_{IL}\% \pm \sigma$
PEG + Na_2SO_4 + water						
600	×	GA	19.0 ± 0.4	×	94.4 ± 0.7	×
400	×	GA	19.7 ± 0.2	×	96.3 ± 0.1	×
300	×	GA	11.3 ± 2.1	×	94.7 ± 1.0	×
300	×	VA	15.5 ± 0.3	×	96.2 ± 0.1	×
300	×	SA	16.9 ± 2.3	×	96.5 ± 0.5	×
200	×	GA	5.9 ± 1.0	×	94.5 ± 0.5	×
PEG 300 + Na_2SO_4 + 5 wt% IL + water						
300	[C_4mim][TOS]	GA	2.3 ± 0.3	9.4 ± 0.5	78.8 ± 1.0	94.6 ± 0.3
300	[C_4mim][SCN]	GA	6.3 ± 0.3	7.6 ± 0.2	90.4 ± 0.4	92.0 ± 0.2
300	[C_4mim][$N(CN)_2$]	GA	8.1 ± 1.1	4.6 ± 0.3	91.5 ± 0.8	86.6 ± 0.2
300	[C_4mim][CH_3CO_2]	GA	16.2 ± 0.3	4.4 ± 0.4	98.3 ± 0.2	94.0 ± 0.6
300	[C_4mim]Cl	GA	29.0 ± 1.2	7.1 ± 0.9	98.4 ± 0.1	94.2 ± 0.5
300	[C_4mpyr]Cl	GA	26.0 ± 0.4	0.9 ± 0.2	97.0 ± 1.8	66.0 ± 5.5
300	[C_4mpip]Cl	GA	27.2 ± 1.3	1.9 ± 0.1	98.2 ± 0.1	79.1 ± 0.6
300	[C_4mim]Cl	VA	46.0 ± 3.2	7.1 ± 0.9	99.0 ± 0.1	94.2 ± 0.5
300	[C_4mim]Cl	SA	50.2 ± 3.5	7.1 ± 0.9	99.0 ± 0.1	94.2 ± 0.5

[a] GA: Gallic acid; VA: Vanillic acid; SA: Syringic acid.

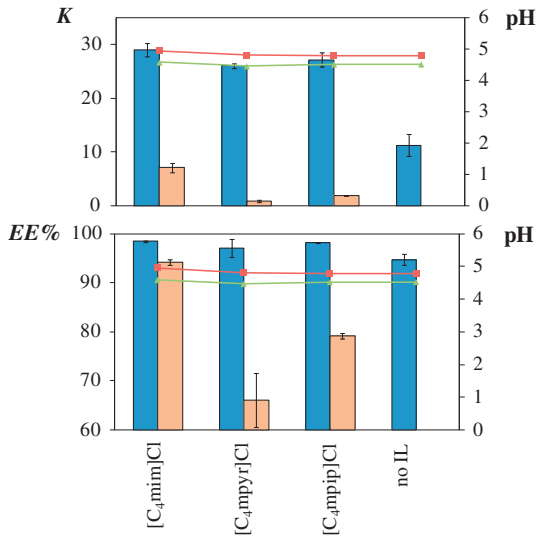

Fig. 10. Partition coefficients (K) and extraction efficiencies (EE%) of gallic acid (blue) and of each IL (orange), and pH of the top (squares) and bottom (triangles) phases, for the chloride-based systems composed of PEG 300 + Na_2SO_4 + 5 wt% IL at 298 K. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

respectively. In all the situations, K_{GA} is larger than 1.0, confirming the gallic acid preferential partitioning for the polymer-rich phase (more hydrophobic phase when compared with the inorganic-salt-enriched phase).

Besides the antioxidants partitioning, the ILs added to the ATPS also partition themselves between the coexisting phases according to their physical and chemical nature [6]. For a better understanding of the extraction results, the partition coefficients and extraction efficiencies of the IL, K_{IL} and EE_{IL}%, are presented in Table 3 and in Figs. 9 and 10 combined with the antioxidant partitioning results.

The K_{IL} values range between 0.94 and 9.65. With the exception of [C_4mpyr]Cl, all the ILs investigated display partition coefficients larger than 1.0 and a preferential migration for the PEG-rich phase. The K_{IL} values vary according to the following order: [C_4mim][CH_3CO_2] < [C_4mim][$N(CN)_2$] < [C_4mim]Cl < [C_4mim][SCN] < [C_4mim][TOS] and [C_4mpyr]Cl < [C_4mpip]Cl < [C_4mim]Cl. Amongst the studied anions, higher partition coefficients for the PEG-rich phase are observed for the IL [C_4mim][TOS] which presents an extra aromatic anion in addition to the imidazolium ring. In the same line, the presence of π electrons in the aromatic imidazolium cation corresponds to a higher preferential partitioning for the PEG-rich phase if the results for the non-aromatic piperidium- and pyrrolidinium-based ILs are taken into account. Within the non-aromatic ILs, the IL with 6-sided ring cation, piperidinium, is more hydrophobic and leads to a higher partitioning of gallic acid for the polymer-rich phase. In fact, these trends are closely related with the binary PEG-IL miscibility behavior and the respective ATPS formation ability [32].

The preferential IL migration for the top phase will naturally change the chemical and physical properties of the polymer-rich phase and, as will be discussed below, the IL chemical nature and its content are the main features controlling the extraction ability of antioxidants observed in the several ATPS.

From Table 3 and Fig. 9, the K_{GA} and EE_{GA}% for the systems using the more hydrophobic ILs anions ($[TOS]^-$, $[SCN]^-$ and $[N(CN)_2]^-$) are smaller than those obtained in the reference ATPS without IL. On the other hand, the ATPS composed of ILs with the anions $[CH_3CO_2]^-$ and Cl^- lead to higher partition coefficients and extraction efficiencies than those observed when no IL was added. The amount of the most hydrophobic anions is lower in the PEG-rich phase meaning that the IL content is not the main factor ruling the partitioning of gallic acid for the polymer-rich phase. Indeed, those that are at lower amounts, such as the [CH_3CO_2]- and Cl-based ILs, are the ILs which provide the highest partitioning of gallic acid for the polymer-rich phase. Albeit gallic acid preferentially migrates for most hydrophobic and less charged phases it seems that the high ability of the former anions to accept protons from gallic acid is ruling the partitioning behavior. Amongst the studied IL anions, Cl^- and $[CH_3CO_2]^-$ display the higher hydrogen-bond basicity values, $\beta = 0.84$ and 1.20 [42], respectively, or the enhanced ability to accept protons. In summary, the addition of ILs as adjuvants in typical polymer–salt ATPS allows specific interactions to occur, such as hydrogen-bonding, and where the chemical nature of the IL is shown to be more relevant than its content.

A high partition coefficient of 29.0 and an enhanced extraction efficiency of 98.4% is observed in the ATPS composed of [C_4mim]Cl. Although it would be expected a higher partition coefficient of gallic acid in the system with [C_4mim][CH_3CO_2], due to its higher hydrogen-bond basicity as discussed before, the structure and size of the anion may also have some impact. Among the two anions, the partition coefficient of [C_4mim]Cl for the polymer-rich phase is higher than the [C_4mim][CH_3CO_2] and a higher amount of the IL at the PEG-rich phase which also presents a high hydrogen-bond basicity seems to favor the antioxidant migration.

The influence of the IL cation, depicted in Fig. 10, reveals that narrower deviations in the partitioning coefficients and extraction efficiencies are obtained if compared with the IL anion effect. This result is a direct consequence of the common IL anion whereas differences in specific interactions are now only observed in the hydrogen-bond acidity (hydrogen-bond donor ability) of the different IL cations. In all the systems studied, the K_{GA} and EE_{GA}% values in the systems with ILs are higher than those obtained with the reference ATPS without IL. The best results are obtained with [C_4mim]Cl that is indeed the most hydrophilic IL displayed in Fig. 10 and in agreement with the discussion presented before for the IL anion effect. [C_4mim]Cl is an aromatic IL with high tendency for hydrogen-bonding, contrarily to the more hydrophobic and non-aromatic [C_4mpyr]Cl and [C_4mpip]Cl ILs. Again, it is verified that the ability of the IL for hydrogen-bonding with gallic acid is more relevant than its content at the PEG-rich phase.

The extraction efficiencies obtained here range between 80% and 98% in the presence of 5 wt% of IL. Extraction efficiencies of 98 wt% with ATPS composed of ILs and salts (no PEG) require the use of *circa* 25 wt% IL + 15 wt% Na_2SO_4 [28]. These results confirm thus the possible substitution of high amounts of IL by the less expensive and benign PEG without losing the extractive performance of the studied ATPS and opening a new range of potential extraction-related strategies.

In summary, the overall results suggest that gallic acid has a greater affinity for the most hydrophobic phase (PEG-rich phase over the highly charged phase) in conventional ATPS formed by PEGs and inorganic salts. However, when dealing with PEG–salt–IL ATPS, the most hydrophilic ILs are those that enhance the partition coefficient due to a possible increase on the hydrogen-bonding ability between the IL and the antioxidant. Furthermore, the effect of the IL anion is more relevant than the effect of the IL cation due to its higher ability to accept protons from gallic acid. The preferential partitioning of gallic acid is strongly controlled by the IL chemical structure whereas a minor effect of the IL content was also observed.

4.4. Comparison on the partitioning of gallic, vanillic and syringic acids

After the previous investigations carried out with different ILs, the best IL to be used as adjuvant in the PEG–Na_2SO_4 system was

further tested in the extraction of other antioxidants. The results obtained for the partition coefficients and extraction efficiencies of gallic, vanillic and syringic acids in the system composed of 23 wt% PEG 300 + 12 wt% Na_2SO_4 + 5 wt% [C_4mim]Cl are presented in Table 3 and depicted in Fig. 11. In addition, the pH of the top and bottom phases is also shown due to the possible speciation of gallic, vanillic and syringic acids. The dissociation curves of gallic acid (p*K*a = 4.0; 9.3; 11.0), vanillic acid (p*K*a = 4.2; 10.2) and syringic acid (p*K*a = 4.0; 9.6) as a function of pH are presented in the Supporting Information [43]. The partition coefficients and extraction efficiencies of the three phenolic acids in the ATPS without IL are also presented for comparison purposes.

For the 3 antioxidants, the presence of 5 wt% of [C_4mim]Cl leads to a large increase on the partition coefficient. The K_{Ant} and EE_{Ant}% increases in the following order: gallic acid < vanillic acid < syringic acid. According to Fig. 1, that presents the chemical structures of the three antioxidants, it is patent that all antioxidants have a similar structure that only differs in the substituents of the aromatic ring. Syringic and vanillic acids are more hydrophobic than gallic acid and with a lower number of hydroxyl groups able to hydrogen-bonding. Therefore, they present a higher partition coefficient or a preferential migration for the polymer-rich phase. This hint is also confirmed by their octanol–water partition coefficients (K_{ow}): gallic acid (K_{ow} = 0.72), vanillic acid (K_{ow} = 1.17) and syringic acid (K_{ow} = 1.01) [43]. However, an inversion on the pattern exists between the vanillic and the syringic acids meaning that others factors must be ruling their partitioning. From Supporting Information, their speciation curves are similar supporting that this is not a result of a pH effect. Syringic acid is a larger molecule due to its extra –OCH_3 group and that may be responsible for a lower partition coefficient due to steric effects.

In all examples, the addition of [C_4mim]Cl leads to higher K_{Ant} and EE_{Ant}% values. The system composed of 23 wt% PEG 300 + 12 wt% Na_2SO_4 + 5 wt% [C_4mim]Cl provides extraction efficiencies ranging between 98% and 99%. Extraction efficiencies between 93 and 99 wt% for the 3 phenolic acids with ATPS composed of ILs and inorganic salts (no PEG) require the use of *circa* 25 wt% IL + 20 wt% Na_2SO_4 or 20 wt% IL + 10 wt% Na_2CO_3 [40]. These results once again confirm the possible substitution of high amounts of IL by the less expensive and benign PEG without losing the extractive performance of the studied ATPS. Other ATPS composed of acetonitrile (40 wt%) and carbohydrates (20 wt%) led to recovery efficiencies of vanillin up to 90% [44].

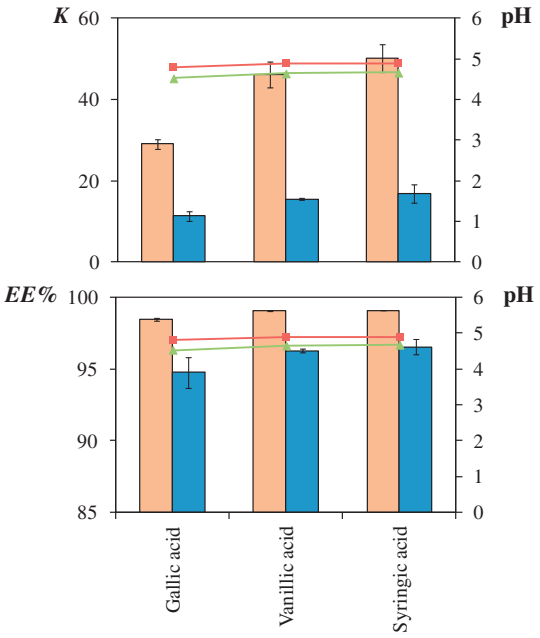

Fig. 11. Partition coefficients (*K*) and extraction efficiencies (*EE*%) of antioxidants for the systems composed of 23 wt% PEG 300 + 12 wt% Na_2SO_4 (blue) and 23 wt% PEG 300 + 12 wt% Na_2SO_4 + 5 wt% [C_4mim]Cl (orange), and pH of the top (squares) and bottom (triangles) phases at 298 K. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

5. Conclusions

The use of ILs as adjuvants in conventional PEG + Na_2SO_4 ATPS to improve the extraction of added-value products, such as antioxidants, is here proposed. As a first approach, the ternary PEG (200, 300, 400 and 600) + Na_2SO_4 phase diagrams and quaternary PEG 300 + Na_2SO_4 + 5/10 wt% IL were determined at 298 K. The respective TLs and TLLs were also ascertained. After the characterization of these systems they were finally evaluated, at a fixed TLL, for the extraction of antioxidants. The optimization investigations were carried out with gallic acid whereas the best results were achieved with [C_4mim]Cl. An increase in the partition coefficient from 11.3 to 29.0 was observed with 5 wt% of IL. To support the preferential migration of the antioxidant it was also determined the partition coefficient of each IL. It was shown that aromatic ILs preferentially migrate for the PEG-rich phase. However, better extraction efficiencies are obtained with more hydrophilic ILs or those with a higher ability to hydrogen-bond with gallic acid. These results confirm that the chemical nature of the IL is more important than its content to control the preferential migration of the phenolic acid for a given phase. Nevertheless, a moderate effect of the IL content is also observed. Extraction efficiencies of gallic acid ranging between 80% and 98% were obtained with the ATPS containing 5 wt% of IL. Finally, the quaternary PEG–salt–IL–water system (with [C_4mim]Cl) was tested in the extraction of two additional antioxidants (vanillic and syringic acids). Extraction efficiencies up to 99% were attained.

The results here reported indicate that the use of ILs as adjuvants in conventional polymer–salt ATPS can provide enhanced extraction efficiencies, and these can be maximized by a correct selection of the chemical structure of the IL employed, opening thus a new route for less "conventional" ATPS.

Acknowledgments

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Appendix A. Supplementary material

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.seppur.2014.03.004.

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