Green Chemistry

Accepted Manuscript

ROYAL SOCIETY OF CHEMISTRY

www.rsc.org/greenchem

Journal Name RSCPublishing

ARTICLE

One-step extraction and concentration of estrogens for an adequate monitoring of wastewaters using ionic-liquid-based aqueous biphasic systems

Cite this: DOI: 10.1039/x0xx00000x

Received 00th January 2012,
Accepted 00th January 2012

DOI: 10.1039/x0xx00000x

www.rsc.org/

Teresa B.V. Dinis,‡[a] Helena Passos,‡[a] Diana L. D. Lima,[b,c] Valdemar I. Esteves,[b] João A. P. Coutinho[a] and Mara G. Freire[a*]

Ethinylestradiol (EE2) is a synthetic hormone that has been recognized as one of the most prominent endocrine disruptors found in aqueous environments. Nevertheless, the low content of EE2 in wastewaters makes its identification/quantification unfeasible - a major drawback on the evaluation of its persistence and environmental impact. In this context, a novel extraction/concentration method for EE2 from wastewaters is here proposed based on aqueous biphasic systems composed of ionic liquids (ILs). Aqueous biphasic systems formed by several hydrophilic ILs and $KNaC_4H_4O_6$ were initially screened and optimized, with extraction efficiencies of EE2 for the IL-rich phase ranging between 92 and 100%. Remarkable results were obtained with systems that allow the complete extraction of EE2 in a single-step, and without losses of EE2 or the saturation of the extractive phase. Further, the concentration factors of EE2 attainable with these systems were investigated by a proper manipulation of the composition of the phase-forming components and corresponding volumes of the coexisting phases. An outstandingly concentration of EE2 up to 1000-fold (from $ng \cdot L^{-1}$ to $\mu g \cdot L^{-1}$) in a single extraction and concentration step was achieved for the first time with IL-based aqueous biphasic systems. These systems are straightforwardly envisaged for the monitoring of wastewaters as one-step extraction and concentration routes for a wide array of endocrine disrupting chemicals while allowing an adequate evaluation of their environmental impact.

Introduction

In the past few years, endocrine disrupting compounds (EDCs) have gained a significant relevance due to their association with adverse human health effects and environmental concerns. The United States Environmental Protection Agency (USEPA), defined EDCs as "exogenous agents that interfere with the production, release, transportation, binding, action, or elimination of the natural hormones in the body, responsible for the maintenance of homeostasis and the regulation of the development process".[1]

17α-Ethinylestradiol, EE2 (Fig. 1), is a synthetic steroid hormone classified as an endocrine disruptor. This compound derives from 17β-estradiol (E2) and displays the most potent estrogenic activity amongst the estrogens found in sewage effluents.[2] EE2 is widely used for medical purposes, for instance, in hormone replacement therapy,[3] in the treatment of prostate and breast cancers and in oral contraception,[4] since it mimics the natural estrogens produced by humans (causing endocrine disruption) and it is rapidly absorbed by the organism. The EE2 extensive consumption by humans and further excretion are responsible for its actual presence and persistence in effluents of different sewage treatment plants (up to 64 $ng \cdot L^{-1}$)[5-6] as well as in surface waters (up to 27 $ng \cdot L^{-1}$).[5, 7]

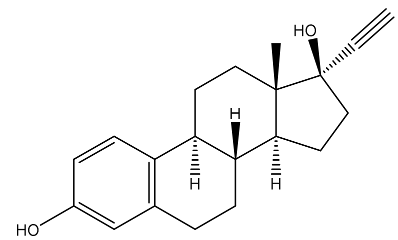


Fig. 1 Chemical structure of EE2.

In 2012, the Legislative Commission of Water Framework Directive[8] classified EE2 as a priority substance with a significant risk to or via the aquatic environment. Its high stability, low volatility and high octanol-water partition coefficient (K_{OW}) are responsible for the EE2 high resistance to degradation and broad bioaccumulation in aquatic organisms.[9] Moreover, the concentration of EE2 has been increasing in sewage effluents, all around the world, due to its widespread use and resistance to degradation.[9-11] Risk assessment bioassays showed the high toxicity of EE2, even at a $ng \cdot L^{-1}$ level, on a wide number of aquatic species.[12-13] All these claims pointed out to the need of finding an effective treatment process for EE2. For instance, physical (sorption, membrane filtration, etc.), biological (activated sludge, etc.) and advanced oxidation (photolysis, strong oxidizers, etc.) processes have been investigated for such a purpose.[9, 14] However, an accurate monitoring of the EE2 content in aqueous samples is crucial to evaluate the efficiency of these processes. Furthermore, a complemented identification and risk assessment of EE2 in the aquatic environment is of vital importance.[12, 15] Several techniques to identify and quantify EE2, such as high performance liquid chromatography (HPLC),[4, 16-18] liquid chromatography (LC)[19-21] and gas chromatography (GC)[22-24], combined with mass spectrometry (MS), have been used. Nevertheless, the presence of unknown EDC in complex matrices of wastewater and the high detection limits of the equipment traditionally used are the major shortcomings in the EE2 identification and quantification.[20, 23] Additional and complex pre-treatment processes, using volatile and hazardous organic solvents, are usually employed to purify and to concentrate EE2 from real samples. This pre-treatment stage is also time consuming and expensive.[25] In this context, the development of alternative methodologies to concentrate EE2 from aqueous media while allowing their proper quantification is a challenging task.

Aqueous biphasic systems fall within the liquid-liquid extraction (LLE) techniques and involve the partitioning of molecules from one aqueous phase to another. Typically, these systems are formed by different pairs of solutes (polymer-polymer, polymer-salt or salt-salt) dissolved in water, and where above specific concentrations the system undergoes a two-phase separation.[26] In addition to their large water content, the non-volatile nature of polymers and salts, allows the phase-forming components to be recovered and recycled, and hence, aqueous biphasic systems are a more benign alternative to traditional liquid-liquid extraction routes which use volatile and hazardous organic compounds. Classical aqueous biphasic systems have already been investigated as extraction/concentration techniques.[27-28]

In addition to the more conventional polymer-based systems, in 2003, Gutowski et al.[29] demonstrated that hydrophilic ionic liquids (ILs) can also form aqueous biphasic systems by the addition of inorganic salts. After this proof of principle, in the following years it was shown that IL-based aqueous biphasic systems can be formed with organic salts, amino acids, carbohydrates or polymers.[30] ILs belong to the molten salts category, and due to the large differences in size and shape of the constituting ions they cannot easily form an ordered crystalline structure, and thus, present melting points below a general temperature of 100 ºC. Due to their ionic nature, most ILs present unique characteristics, such as a negligible vapour pressure, non-flammability, high thermal and chemical stabilities and a high solvation capacity.[30-33] Still, one of the most important features that has attracted both academia and industry is their aptitude as "designer solvents", e.g., the capacity to be synthesized for a given task as a result of their plentiful cations and anions combinations.[34-39]

IL-based aqueous biphasic systems were extensively explored for the extraction and purification of a wide variety of biomolecules.[30, 40-42] The main advantage of using ILs in the formation of aqueous biphasic systems rests on the possibility of tailoring their phases' polarities and affinities by the proper combination of their ions[43] and, therefore, exceptional results were already accomplished with IL-based aqueous biphasic systems.[30] It was recently reported they can be used for the extraction and concentration of alkaloids and bisphenol A from human fluids.[44-45] Yet, only concentration factors up to 100-fold were reported.[44] Furthermore, most of the previously reported systems are composed of ILs and high-charge density inorganic salts[30] which lead to some environmental concerns. Taking this into consideration, and although no enrichment factors were investigated, in recent works we have introduced biodegradable and more biocompatible organic salts in the composition of IL-based aqueous biphasic systems.[46-47]

In this work, we propose the use of novel aqueous biphasic systems composed of ILs and a biodegradable organic salt as a concentration strategy for EE2 from wastewaters. For this purpose, we initially determined the phase diagrams of IL-based aqueous biphasic systems to infer on their formation aptitude. After their evaluation, the ability of IL-based aqueous biphasic systems as a new alternative to the current concentration steps used in the EE2 identification and quantification was investigated.

Experimental Section

Materials

EE2, (17α)-17-ethinylestra-1,3,5(10)-triene-3,17-diol, was supplied by Sigma, with a purity level ≥ 98 wt % (Fig. 1). The ILs studied were: 1-butyl-3-methylimidazolium bromide, $[C_4mim]Br$ (99 wt %); 1-butyl-3-methylimidazolium trifluoroacetate, $[C_4mim][CF_3CO_2]$ (> 97 wt %); 1-butyl-3-methylimidazolium trifluoromethanesulfonate, $[C_4mim][CF_3SO_3]$ (99 wt %); 1-butyl-3-methylimidazolium thiocyanate, $[C_4mim][SCN]$ (> 98 wt %); 1-butyl-3-methylimidazolium tosylate, $[C_4mim][TOS]$ (98 wt %); 1-ethyl-3-methylimidazolium dicyanamide, $[C_2mim][N(CN)_2]$ (> 98 wt %); 1-butyl-3-methylimidazolium dicyanamide, $[C_4mim][N(CN)_2]$ (> 98 wt %); 1-hexyl-3-methylimidazolium dicyanamide, $[C_6mim][N(CN)_2]$ (> 98 wt %); tetrabutylammonium chloride, $[Bu_4N]Cl$ (≥ 97 wt %); and

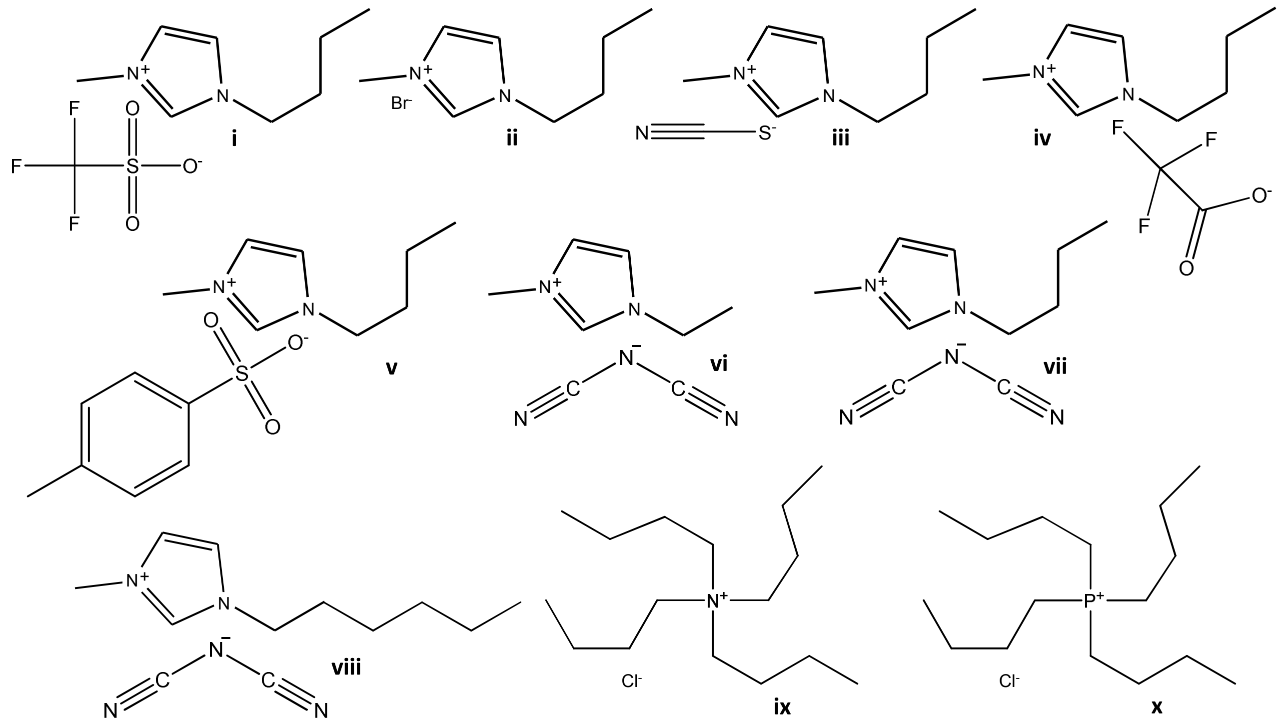


Fig. 2 Chemical structures of the ILs used to form aqueous biphasic systems: (i) $[C_4mim][CF_3SO_3]$; (ii) $[C_4mim]Br$; (iii) $[C_4mim][SCN]$; (iv) $[C_4mim][CF_3CO_2]$; (v) $[C_4mim][TOS]$; (vi) $[C_2mim][N(CN)_2]$; (vii) $[C_4mim][N(CN)_2]$; (viii) $[C_6mim][N(CN)_2]$; (ix) $[Bu_4N]Cl$; (x) $[Bu_4P]Cl$.

tetrabutylphosphonium chloride, $[Bu_4P]Cl$ (98 wt %). All imidazolium-based ILs were purchased from Iolitec. $[Bu_4P]Cl$ was gently offered by Cytec Industries Inc., and $[Bu_4N]Cl$ was purchased from Sigma-Aldrich. The molecular structures of the investigated ILs are illustrated in Fig. 2. Ten more ILs were tested. However, it was not possible to form aqueous biphasic systems with these ILs - their names and acronyms are reported in the ESI†. For the reduction of the water and volatile compounds content to negligible values, ILs individual samples were dried under constant stirring at vacuum and moderate temperature (~ 80 °C) for a minimum of 24 h. After this procedure, the purity of each IL was checked by ^1H, ^{13}C and ^{19}F (whenever possible) NMR spectra and deemed in accordance with the purity given by the suppliers. The organic salt potassium sodium tartrate tetra-hydrated, $KNaC_4H_4O_6{\cdot}4H_2O$ (> 99 wt %) was acquired from Fluka. The water employed was double distilled, passed by a reverse osmosis system and further treated with a Milli-Q plus 185 water purification apparatus. HPLC grade methanol and acetonitrile, 99.9 %, were from Fischer Chemical and HiPerSolv Chromanorm, respectively.

Phase diagrams and tie-lines

The saturation (bimodal) curve of each aqueous biphasic systems, that represents the limit between the monophasic and biphasic regions, was determined through the cloud point titration method at (25 ± 1) °C and atmospheric pressure. Aqueous solutions of $KNaC_4H_4O_6$ at 35 wt % and aqueous solutions of the different hydrophilic ILs at variable concentrations (between 35 and 85 wt %) were prepared gravimetrically (± 10^{-4} g) and used for the determination of the respective binodal curves. Repetitive drop-wise addition of the organic salt solution to each IL aqueous solution was carried out until the detection of a cloudy mixture. Then, repetitive drop-wise of double distilled water was added until the detection of a clear and limpid mixture. Whenever necessary, the addition of the IL aqueous solutions to the salt solutions was also carried out to complete the phase diagrams. Drop-wise additions were performed under constant stirring. The ternary system compositions corresponding to the description of the phase diagrams were determined by weight quantification of all components added to the mixture within ± 10^{-4} g.

The tie-lines (TLs), which are straight lines that describe the composition of the coexisting phases of a given mixture point, were determined by a gravimetric method originally described by Merchuk et al.[48] A mixture composition at the biphasic region was gravimetrically prepared, vigorously stirred, and allowed to reach the equilibrium by the separation of both phases for at least 12 h at (25 ± 1) °C. After the separation step, both top and bottom phases were weighted. Finally, each individual TL was determined by the application of the lever-arm rule.

The experimental binodal curves were fitted according to Eq. 1:

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

where [IL] and [$salt$] correspond to IL and salt weight fraction percentages, respectively; and A, B and C are constants achieved by the least-squares regression of the experimental data. Their values and corresponding standard deviations (σ) are provided in the ESI†.

For the determination of TLs, it was used the following system of four equations (Eqs. 2 to 5) with four unknown variables ($[IL]_{\text{IL}}$, $[salt]_{\text{IL}}$, $[IL]_{\text{salt}}$, $[salt]_{\text{salt}}$):

$$[IL]_{\text{IL}} = Aexp\left[\left(B \times [salt]_{\text{IL}}^{0.5}\right) - \left(C \times [salt]_{\text{IL}}^{3}\right)\right] \tag{2}$$

$$[IL]_{\text{salt}} = Aexp\left[\left(B \times [salt]_{\text{salt}}^{0.5}\right) - \left(C \times [salt]_{\text{salt}}^{3}\right)\right] \tag{3}$$

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

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

where the indexes M, IL and salt correspond to the mixture, IL-rich phase and salt-rich phase, respectively. The parameter α is the ratio between the IL rich-phase and the total mixture weight. The solution of the referred system gives the concentration of IL and organic salt in the top and bottom phases. Some TLs were also analytically determined and as described below.

The tie-line length (TLL) denotes the distance, *i.e.*, the differences in composition, between the salt-rich phase and the IL-rich phase and was calculated according to Eq. 6:

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

Extraction of ethinylestradiol

For the screening of improved IL-based aqueous biphasic systems for the one-step extraction and concentration of EE2, several ternary (biphasic) systems (IL + $KNaC_4H_4O_6$ + water) were prepared. The weight fraction percentage of each component was established taking into account a constant weight ratio between the coexisting phases of approximately 1. EE2, *ca.* 2.0×10^{-3} g, was added to each ternary mixture or individual experiments. The ternary mixture was vigorously stirred and left to achieve the equilibrium for at least 12 h, at (25 ± 1) °C, aiming at allowing the complete partitioning of EE2 between the two phases. After the separation of the top and bottom phases, the detection and quantification of EE2 was carried out through UV-spectroscopy, using a Shimadzu UV-1700, Pharma-Spec Spectrometer, at a wavelength of 284 nm. Blank controls of the ternary mixture were always prepared to eliminate possible interferences of the IL and $KNaC_4H_4O_6$. Three samples of each aqueous phase were analysed, in at least three individual systems, in order to determine the extraction efficiencies of EE2 and the respective standard deviations.

The percentage extraction efficiencies of EE2, $EE_{\text{EE2}}\%$, were estimated by Eq. 7:

$$EE_{\text{EE2}}\% = \frac{Abs_{\text{EE2}}^{\text{IL}} \times w_{\text{IL}}}{Abs_{\text{EE2}}^{\text{IL}} \times w_{\text{IL}} + Abs_{\text{EE2}}^{\text{salt}} \times w_{\text{salt}}} \tag{7}$$

where $Abs_{\text{EE2}}^{\text{IL}}$ and $Abs_{\text{EE2}}^{\text{salt}}$ are the absorbance values of EE2 in the IL-rich and $KNaC_4H_4O_6$-rich aqueous phases (taking into account the respective dilution factors – in weight) and w_{IL} and w_{salt} are the weight obtained for the IL-rich and $KNaC_4H_4O_6$-rich phases, respectively.

Concentration of ethinylestradiol in the [C$_4$mim][N(CN)$_2$]-based aqueous biphasic systems

The larger TL of the liquid-liquid aqueous biphasic systems composed of [C$_4$mim][N(CN)$_2$] + $KNaC_4H_4O_6$ + H_2O was determined by means of the preparation of several ternary systems and further addition of $KNaC_4H_4O_6$ until the detection of a solid phase. Once the equation commonly applied to describe the binodal data is not able to correctly describe the regions of the solubility curve for high IL and salt concentrations,[49] the concentration of each compound on both phases of this TL was analytically determined. [C$_4$mim][N(CN)$_2$] was quantified through UV-spectroscopy, using a Shimadzu UV-1700, Pharma-Spec Spectrometer, at a wavelength of 212 nm. Blank controls were prepared to eliminate interferences caused by the salt in the $KNaC_4H_4O_6$-rich phase. The water content in each phase was determined by evaporation, by means of an air oven at ~ 60 °C, until a constant weight of the non-volatile mixture [C$_4$mim][N(CN)$_2$] + $KNaC_4H_4O_6$ was achieved. The $KNaC_4H_4O_6$ amount was determined by the weight difference. This process was carried out in duplicate to ascertain on the associated standard deviations. In general, shorter TLs obtained by the quantification of all phase-forming components agree well with those obtained by the mass-balance method proposed by Merchuk et al.[48]

The concentration factor of EE2 along the characterized TL was evaluated through the preparation of ternary systems at different compositions and thus at different weight ratios (weight of water added to the system *per* weight of IL-rich phase): 100 and 1000. It should be noted that along the same TL the composition of each phase is maintained while varying only the volume or weight ratio of the phases. In these two situations, and where higher lower detection limits are required, EE2 was quantified by a Shimadzu High-Performance Liquid Chromatograph (HPLC) Prominence system equipped with a fluorescence detector. This device consists of a degasser DGU-20A5, a bomb LC-20AD, a column oven CTO-10ASVP. An ACE® C18 column-PFP (5 µm, 150 mm x 4.6 mm) connected to an ACE® 5 C18 4.6 mm i.d. guard column was used for the separation. The mobile phase consisted in a water:acetonitrile mixture (55:45, *v/v*), at a flow rate of 0.8 mL·min^{-1}, with an injection volume of 20 µL. The detection/quantification of EE2 was performed using a Shimadzu Prominence RF-20A XS fluorescence detector at an excitation wavelength of 280 nm and an emission wavelength of 310 nm.[25] Both the column and cell temperature were maintained at 25 °C. Three individual samples of the IL-rich phase were analyzed in order to determine the recovery of EE2 and the respective standard deviations. Individual standard stock solutions of EE2 were prepared in methanol at a concentration of 100 mg·L^{-1} and were further diluted at appropriate concentrations (between 2.5 and 100 μ.L^{-1}) using ultrapure water to obtain the calibration curve. Water and acetonitrile used in the mobile phase were pretreated by filtration through a 0.2 μm polyamide membrane filters from Whatman.

pH determination

The pH values of both the IL-rich and salt-rich phases were measured at (25 ± 1) °C using a Mettler Toledo SevenMulti pH meter within ± 0.02.

Results and discussion

Phase diagrams and tie-lines

New ternary phase diagrams were determined for the several ILs + $KNaC_4H_4O_6$ + H_2O aqueous biphasic systems, at (25 ± 1) °C and at atmospheric pressure. The obtained liquid-liquid phase diagrams are depicted in Fig. 3 (the detailed weight fraction data are provided in the ESI†). For all the phase diagrams the biphasic region is localized above the solubility curve while the monophasic region is presented below. In general, the larger the biphasic region the higher is the capacity of the IL to undergo liquid-liquid demixing and to form an aqueous biphasic systems, *i.e.*, the easier the IL is salted-out by the salt.

The ILs investigated allow addressing the effect of their chemical structures (cation alkyl side chain length and cation and anion nature) on the phase diagrams behavior or aqueous biphasic systems formation aptitude. Fig. 3A depicts the effect of the imidazolium cation alkyl side chain length on the formation of aqueous biphasic systems, and which follows the order: $[C_2mim][N(CN)_2]$ << $[C_4mim][N(CN)_2]$ << $[C_6mim][N(CN)_2]$. Longer aliphatic chains at the cation enhance the IL hydrophobicity and lead to a wider biphasic region.[50-51] Fig. 3B presents the influence of the cation core, with the $[Bu_4N]Cl$ and $[Bu_4P]Cl$ ILs, on the liquid-liquid demixing. The phase diagram for $[C_4mim][N(CN)_2]$ is also depicted as one imidazolium-based fluid reference. Both $[Bu_4N]$- and $[Bu_4P]$-based ILs have an identical ability for the formation of aqueous biphasic systems due their similar chemical structure (four butyl chains at the cation which are responsible for their high hydrophobicity). The effect of the IL anion nature on the aqueous biphasic systems formation is shown in Fig. 3C, where the following order was observed: Br^- ≈ $[CF_3CO_2]^-$ < $[TOS]^-$ < $[N(CN)_2]^-$ < $[SCN]^-$ << $[CF_3SO_3]^-$. This rank is in close agreement with previous works using other inorganic or organic salts[46, 52-55] and it is related with the ability of the IL anion to hydrogen-bond with water: ILs anions with lower hydrogen bond basicity values (β) present a higher ability to form aqueous biphasic systems.[56]

Besides its biodegradable and biocompatible features, $KNaC_4H_4O_6$ presents a significant salting-out effect. This is an important characteristic when foreseeing the use of aqueous biphasic systems for the one-step extraction and concentration of a target analyte since it will permit to obtain considerably long tie-lines, and thus, high concentration factors.

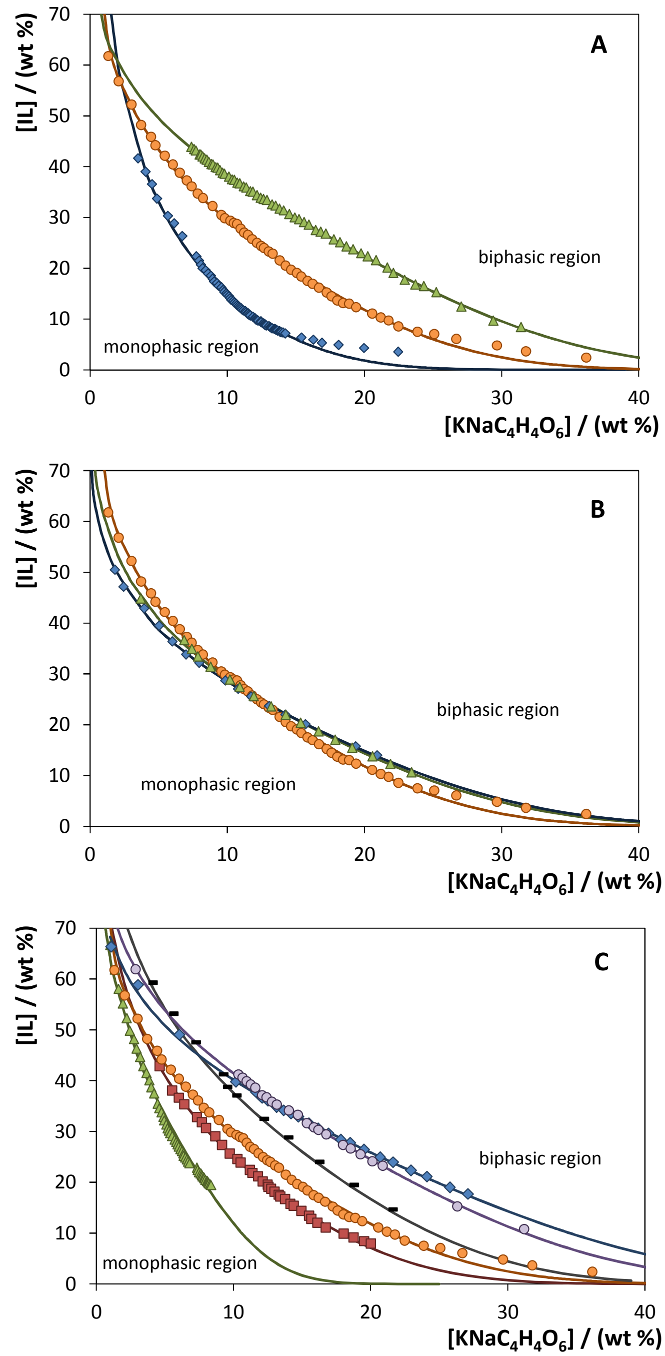


Fig. 3 Evaluation of the **(A)** cation alkyl side chain length, **(B)** cation core and **(C)** anion nature in the ternary phase diagrams composed of IL + water + $KNaC_4H_4O_6$ at (25 ± 1) °C: **(A)** $[C_2mim][N(CN)_2]$ (▲), $[C_4mim][N(CN)_2]$ (●), $[C_6mim][N(CN)_2]$ (◆); **(B)** $[Bu_4P]Cl$ (▲), $[Bu_4N]Cl$ (◆), $[C_4mim][N(CN)_2]$ (●); **(C)** $[C_4mim][CF_3SO_3]$

(▲)[57], $[C_4mim][SCN]$ (■), $[C_4mim][N(CN)_2]$ (●), $[C_4mim][TOS]$ (-), $[C_4mim][CF_3CO_2]$ (●), $[C_4mim]Br$ (◆). Adjusted binodal data by Eq.1 (–).

The experimental TLs, *i.e.*, the composition of the phases for a given mixture point, along with their respective length (TLL), are reported in Table 1. The pH values of both phases in each aqueous biphasic systems are also shown in Table 1. The pH values of the coexisting phases range from neutral to slightly alkaline (6.23 – 9.24) and are useful to explore the possibility of using these aqueous biphasic systems as extractive platforms for specific compounds and/or compounds that may suffer speciation.

Extraction of ethinylestradiol

For a successful extraction, it is always required an appropriate manipulation of the phases' properties, which control the selectivity and the partition of the solute of interest. This approach was taken into consideration by scanning systems with different ILs and by manipulating their phases' compositions and volumes (*cf.* the ESI†), as presented and discussed below.

Effect of the IL type. The extraction efficiencies of EE2, and respective standard deviations, in aqueous biphasic systems formed with different ILs, $KNaC_4H_4O_6$ and H_2O are depicted in Fig. 4 (*cf.* the ESI† with the detailed values). To avoid dissimilarities in the extraction efficiencies that could result from differences in the compositions of the coexisting phases, all the partitioning experiments were carried out at a fixed TLL (≈ 43) – Table 1.

EE2 preferentially migrates for the IL-rich phase in all investigated aqueous biphasic systems, with extraction efficiencies higher than 92 %. The higher affinity of EE2 for the IL-rich phase correlates well with its octanol-water partition coefficient (K_{OW}) value. The log(K_{OW}) of EE2 is 4.15,[9] meaning that the synthetic hormone has a preferential affinity for more hydrophobic phases, that in these systems corresponds to the IL-rich phase. Since the pH values of each phase range between 6.2 and 8.6, and therefore EE2 is mostly present in its neutral form, possible electrostatic interactions between the salt or IL ions and the charged EE2 species are not significant neither responsible for the solute preferential migration.

Table 1. Experimental data for TLs and TLLs of IL + $KNaC_4H_4O_6$ aqueous biphasic systems and respective pH values of the coexisting phases.

IL	Weight fraction composition / wt %								
	$[IL]_{IL}$	$[salt]_{IL}$	pH_{IL}	$[IL]_M$	$[salt]_M$	$[IL]_{salt}$	$[salt]_{salt}$	pH_{salt}	TLL
$[C_4mim]Br$	35.41	12.98	6.93	34.13	13.92	26.80	19.27	7.11	10.67
$[C_4mim][CF_3SO_3]$	62.09	1.16	6.23	40.82	4.57	19.28	8.03	6.29	43.36
$[C_4mim][CF_3CO_2]$	41.56	9.95	9.10	27.12	19.87	8.75	32.50	8.99	39.82
	37.86	11.87	9.24	25.72	19.98	13.16	28.37	8.88	29.71
$[C_4mim][SCN]$	59.72	1.98	6.63	27.25	15.19	2.55	25.22	6.68	61.71
	55.66	2.45	6.64	23.05	14.99	5.11	21.88	6.70	54.15
	49.31	3.37	6.40	31.23	10.28	8.29	19.05	6.44	43.92
$[C_4mim][TOS]$	57.71	4.24	7.21	38.20	14.93	1.72	34.90	7.34	63.84
	49.84	6.06	7.10	32.91	14.98	4.49	29.94	6.96	51.25
	45.17	7.40	7.10	30.38	15.05	6.96	27.16	7.11	43.02
	41.37	8.64	7.01	29.64	14.59	9.83	24.66	6.93	35.37
$[C_2mim][N(CN)_2]$	45.89	6.39	8.69	33.90	15.04	4.22	36.46	8.71	51.39
	43.05	7.64	8.78	32.00	14.98	9.97	29.61	8.68	39.71
$[C_4mim][N(CN)_2]$	61.75	1.32	8.64	19.91	30.01	2.39	36.17	8.69	68.84
	54.78	2.55	8.76	29.86	15.09	3.37	28.41	8.63	57.55
	50.69	3.28	8.52	29.92	13.50	5.66	25.41	8.55	50.18
	46.37	4.22	8.61	29.88	12.29	8.23	22.87	8.74	42.44
$[C_6mim][N(CN)_2]$	49.51	2.87	7.99	22.86	2.87	7.00	14.25	8.30	44.01
	44.99	3.33	8.04	20.04	10.06	8.66	13.12	7.99	37.62
$[Bu_4P]Cl$	41.58	4.75	6.60	28.09	14.93	2.44	34.29	6.72	49.03
	37.16	6.30	6.65	24.84	15.58	3.93	31.34	6.73	41.61
	35.20	7.10	6.65	24.80	15.20	4.40	30.56	6.82	38.72
$[Bu_4N]Cl$	46.95	2.58	8.06	30.01	15.04	2.41	35.34	7.94	55.29

	36.61	5.87	8.11	25.17	15.03	4.12	31.88	8.24	41.62

.

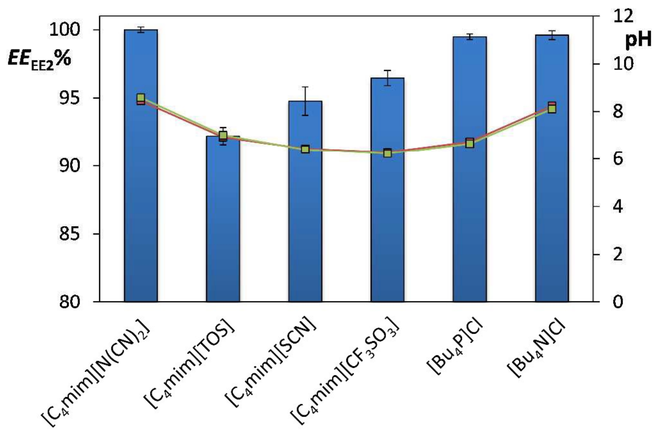


Fig. 4 Extraction efficiencies of EE2, EE_{EE2}%, in several aqueous biphasic systems at (25 ± 1) °C: IL-rich phase pH (■); salt-rich phase pH (■).

As depicted in Fig. 4, the EE2 extraction efficiency is strongly related with the hydrophobicity of the IL that forms a given aqueous biphasic systems. The extraction efficiencies of EE2 decrease in the order: $[C_4mim][N(CN)_2]$ > $[Bu_4P]Cl$ ≈ $[Bu_4N]Cl$ > $[C_4mim][CF_3SO_3]$ > $[C_4mim][SCN]$ > $[C_4mim][TOS]$. Outstandingly, with the aqueous biphasic systems composed of $[C_4mim][N(CN)_2]$ and $KNaC_4H_4O_6$ it was possible to achieve a complete extraction of EE2 for the IL-rich phase in a single-step.

TLL effect. Fig. 5 depicts the impact of the TLL on the EE2 extraction with $[C_4mim][N(CN)_2]$-based aqueous biphasic systems (detailed data in the ESI†). $[C_4mim][N(CN)_2]$ was chosen since this IL led to the best extraction efficiency (100% of extraction achieved in a single-step) and allows working with long tie-lines that will further provide the highest concentration factors. This screening was performed since a reduction on the TLL would allow a reduction on the amount of the phase-forming components used, and thus a reduction in the overall costs of the process.

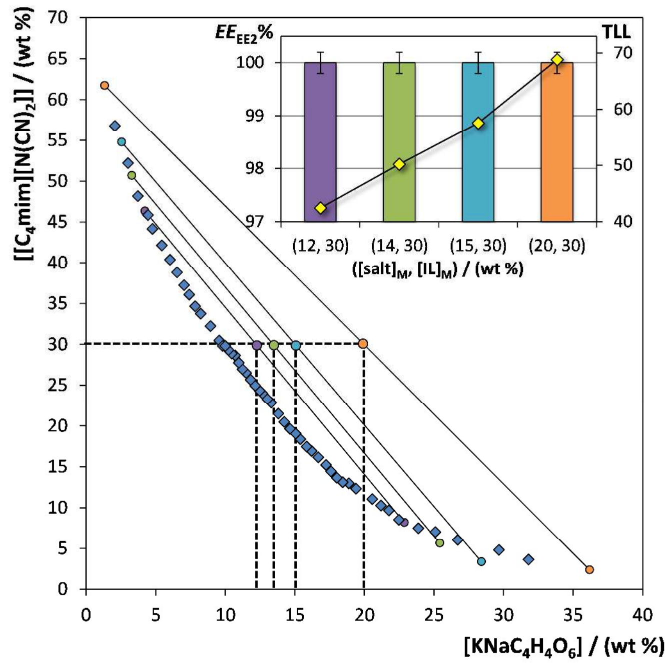


Fig. 5 Evaluation of the TLL in the extraction efficiencies of EE2, EE_{EE2}%, in the $[C_4mim][N(CN)_2]$ + $KNaC_4H_4O_6$ + H_2O aqueous biphasic systems, at (25 ± 1) °C: binodal curve data (◆); TL data (●); TLL values (◆).

From Fig. 5, it is possible to conclude that the TLL (at least in the range studied) does not affect the extraction capacity for EE2 and a complete extraction is always attained. The TLL values range from 68.8 to 46.7, which means that it is possible to optimize the process to be more economical and environmentally benign by decreasing the concentration of IL without losing the complete extraction efficiency.

Concentration of ethinylestradiol in $[C_4mim][N(CN)_2]$-based aqueous biphasic systems

The main characteristic of aqueous biphasic systems to be used as concentration platforms comprises the presence of very long tie-lines. Indeed, the longer the TL the higher the concentration factor that can be achieved. For a one-stage extraction-concentration step, the complete extraction of the target analyte for the IL-rich phase is also required. At this stage, we should also guarantee that the IL-rich phase doesn't saturate with the extracted biomolecule; otherwise, the accurate quantification of EE2 in the IL-rich phase will be not accomplished leading to underestimated results. The aqueous biphasic system composed of $[C_4mim][N(CN)_2]$ + $KNaC_4H_4O_6$ + H_2O was here selected since it led to a single-step complete extraction of EE2, without saturation of the IL-rich phase, and allows working with long TLs.

Fig. 6 depicts the solubility curve of the $[C_4mim][N(CN)_2]$ + $KNaC_4H_4O_6$ + H_2O system, as well as the TLs and mixture points investigated. The extraction efficiency values, and respective standard deviations, for different initial compositions along the same and largest TL, are also shown in Fig. 6.

Different initial compositions (*cf.* the ESI†) along the same TL, and with a TLL value *circa* 69, lead to different weight ratios of the coexisting phases (IL-rich and salt-rich phases). Nevertheless, since the aim of this work is the concentration of an aqueous sample containing EE2 in wastewater, it should be taken into account that the amount of the real sample added to the system will be the same amount of water required to create the initial mixture point. Thus, in this situation, the concentration factor is equal to the total amount of water added to the mixture point divided by the IL-rich phase amount (phase for which EE2 is completely extracted). Yet, even if the IL-rich phase and water present similar densities ($[C_4mim][N(CN)_2]$-rich phase density at 25 ºC ≈ 1.04 $g \cdot cm^{-3}$ as experimentally determined by us), for higher concentration factors this difference becomes significant and should be considered. Therefore, in the following results regarding the concentration factors, volumes are considered instead of the weights of water and of the coexisting phases.

The complete extraction of EE2 was always attained for all the mixture compositions evaluated – Fig. 6. The weight ratio between the water (containing EE2) added in the mixture and the IL-rich phase ranges from 1.12 to 10.03, which means that it is possible to concentrate the synthetic hormone by reducing the volume of the IL-rich phase without losing the complete extraction performance. Indeed, this possibility of concentrating EE2 may overcome the main problem on its detection derived from its low concentrations in sewage treatment plants and wastewaters. For the mixture point with a concentration factor of 10.03, it was experimentally possible to concentrate EE2 up to 200-fold regarding its saturation solubility in water (4.8 $mg \cdot L^{-1}$).[9]

In a wastewater real sample, EE2 is present in concentrations in the order of $ng \cdot L^{-1}$. Therefore, when dealing with real samples, a concentration factor of 10 is still not enough. Thus, the $[C_4mim][N(CN)_2]$-based aqueous biphasic systems was further tested for higher concentration factors using an HPLC with a fluorescence detector for that purpose. Concentration factors of 100 and 1000 can be obtained in the largest TL of the ternary system (Fig. 8). The mixture points required to create these conditions are 2.69 wt % of $[C_4mim][N(CN)_2]$ + 36.00 wt % of $KNaC_4H_4O_6$ and 2.43 wt % of $[C_4mim][N(CN)_2]$ + 36.15 wt % of $KNaC_4H_4O_6$, respectively.

Standard samples with initial concentrations of ~ 450 and 90 $ng \cdot L^{-1}$ were used to evaluate the EE2 extraction efficiency and the concentration factors of 100 and 1000 afforded by the $[C_4mim][N(CN)_2]$-based aqueous biphasic systems. The major goal is to reach a final concentration of EE2 at the IL-rich phase higher than the HPLC lower limit detection (3.1 $\mu g \cdot L^{-1}$). The results obtained were 98 ± 8 % and 102 ± 10 % for a concentration factor of 100 and 1000, respectively. Therefore, the concentration of EE2 in wastewaters can be increased at least up to 1000-fold, in a single-step, without losses in EE2 and

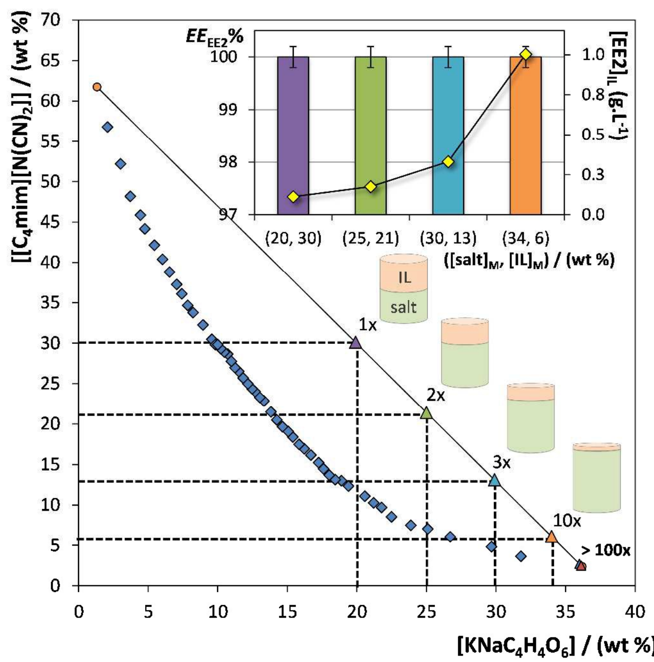


Fig. 6 Extraction efficiencies of EE2, EE_{EE2}%, for different initial compositions along the same TL in the $[C_4mim][N(CN)_2]$ + $KNaC_4H_4O_6$ + H_2O aqueous biphasic systems, at (25 ± 1) °C: binodal curve data (◆); TL data (●); initial compositions (▲); EE2 final concentration in the IL-rich phase, $[EE2]_{IL}$ (◆).

without saturating the IL-rich phase, simple by the tuning of the mixture point composition for a minimum IL-rich phase volume. Furthermore, the phase-forming components of the phase containing EE2, mainly IL and water, don't interfere with the HPLC quantification. In summary, the proposed methodology allows to increase the EE2 concentration by, at least, three orders of magnitude (from $ng \cdot L^{-1}$ to $\mu g \cdot L^{-1}$), and its proper identification and quantification.

The economical and sustainable viability of the proposed method to detect EE2 in sewage treatment plants is also ensured. The amount of IL used for aqueous biphasic systems formation is inversely proportional to the concentration factor required. For instance, for a real water sample of ≈ 61 mL (aqueous biphasic systems with a total volume of 100 mL) only 2.69 g or 2.43 g of $[C_4mim][N(CN)_2]$ are required to reach a concentration factor of 100 and 1000, respectively.

The analysis of effluent samples requires analytical techniques with high sensitivity because of their complex matrices and low concentration of marker pollutants. LC-MS and LC-MS/MS, due to high sensitivity and selectivity, have been selected as the techniques of choice for environmental analysis of steroid hormones.[58] However, they display several drawbacks, namely the use of expensive devices, high maintenance costs and still require skilled analysts. Therefore, these techniques are unaffordable for many analytical laboratories. Compared with LC-MS and LC-MS/MS, HPLC coupled either to UV or fluorescence detector is a simpler, cheaper, easy-to-use and extensively available technique. Yet, due to its lower detection limits, a pre-concentration step is always required. To this end, HPLC conjugated with solid-phase extractions (SPE) or liquid-liquid extractions (LLE) are regularly used.[59-61] Both of these pre-concentration techniques require large quantities of toxic and volatile organic solvents and are time-consuming. Low-cost dispersive liquid-liquid micro extraction (DLLME) have also been proposed[44,45] and successfully applied for the concentration of steroid hormones, with an enrichment factor of 178 and an extraction efficiency of 89% for EE2.[25] Compared to these methods, IL-based aqueous biphasic systems do not require the use of volatile organic solvents and allow obtaining a complete extraction of EE2 (with no losses of solute) and significantly higher concentration factors. Furthermore, it is possible to tune the aqueous biphasic systems properties through the change of the IL nature creating therefore a new plethora of concentration systems for other target analytes present in wastewaters. The concentration factors here obtained are also far superior to those previously reported with IL-based aqueous biphasic systems with maximum enrichment factors of 10 in the extraction of opium alkaloids or steroid hormones from biological fluids,[62-63] and up to 100 in the extraction of bisphenol A from human fluids.[44]

Conclusions

Aiming at overcoming one of the major limitations in the analysis and monitoring of wastewaters, a novel methodology is here proposed to concentrate EE2 by the application of IL-based aqueous biphasic systems. As a first approach, their ternary phase diagrams were determined and the single-step extraction capacity of several IL-based aqueous biphasic systems was addressed. Extraction efficiencies ranging between 92 % and 100 % were obtained for the IL-rich phase revealing the high affinity of EE2 to the most hydrophobic phase. In particular, outstanding extraction efficiencies, *i.e.*, the complete extraction of EE2 in a single-step, were attained with $[C_4mim][N(CN)_2]$-based aqueous biphasic systems. Therefore, this type of systems were further tested to optimize the concentration factors of EE2. It was found that by tuning the mixture point composition for a minimum IL-rich phase volume, the EE2 concentration in wastewaters can be increased at least up to 1000-fold in a single-step. The proposed methodology allows the increase of the EE2 concentration by three orders of magnitude (from $ng\cdot L^{-1}$ to $\mu g\cdot L^{-1}$) and thus to overcome the detection limits of conventional analytical equipment commonly used in the analysis and monitoring of wastewaters. These systems are straightforwardly envisaged for the monitoring of wastewaters as potential one-step extraction and concentration routes for a wide array of endocrine disrupting chemicals or other trace pollutants.

Acknowledgements

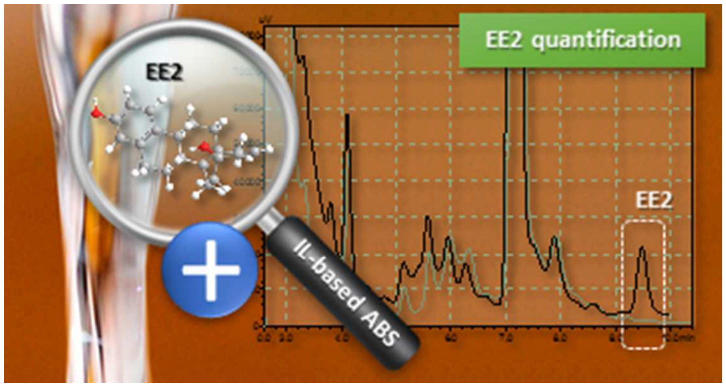
This work was developed in the scope of the project CICECO-Aveiro Institute of Materials (Ref. FCT UID/CTM/50011/2013), financed by national funds through the FCT/MEC and when applicable co-financed by FEDER under the PT2020 Partnership Agreement. D.L.D. Lima and H. Passos acknowledge FCT for the post-doctoral and doctoral grants SFRH/BPD/80315/2011 and SFRH/BD/85248/2012, respectively. M. G. Freire acknowledges the European Research Council (ERC) for the Starting Grant ERC-2013-StG-337753.

Notes and references

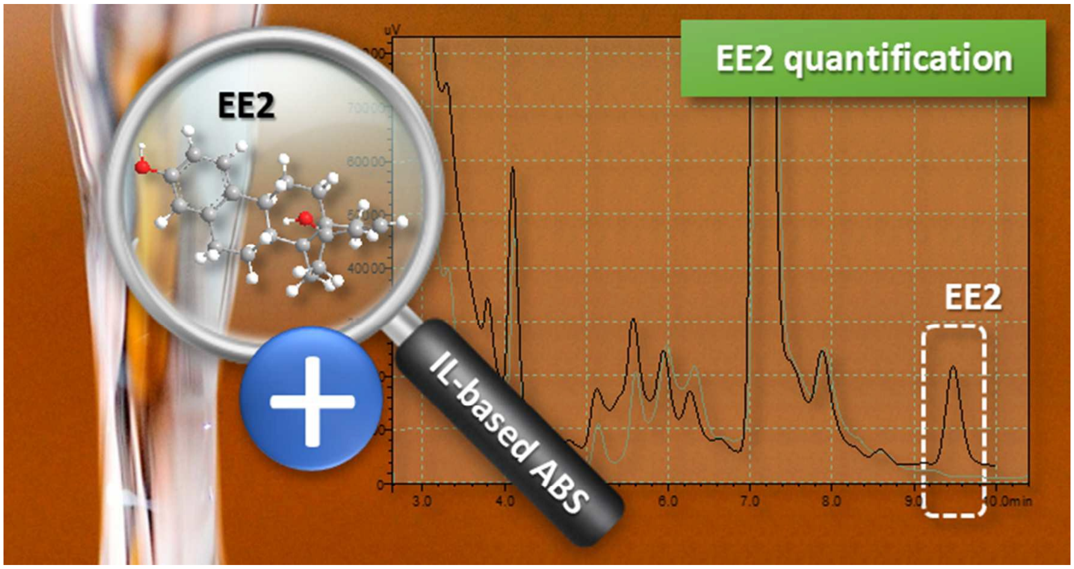
[a]CICECO - Aveiro Institute of Materials, Department of Chemistry, University of Aveiro, 3810-193 Aveiro, Portugal Tel: +351 234370200; Fax: +351 234370084; E-mail address: maragfreire@ua.pt.
[b]Chemistry Department, CESAM, University of Aveiro, 3810-193 Aveiro, Portugal.
[c]Department of Complementary Sciences, Superior School of Health Technology of Coimbra, 3040-854 Coimbra, Portugal.
†Electronic Supplementary Information (ESI) available: List of ILs that were not able to form aqueous biphasic systems, binodal curves experimental data, Merchuck' correlation parameters, extraction efficiencies and mixture compositions. See DOI: 10.1039/b000000x/
‡Equally contributing authors.

1. USEPA, Endocrine Disruptors Research, www.epa.gov/research/endocrinedisruption/index.htm, Accessed 09.14.
2. A. C. Johnson and J. P. Sumpter, *Environ. Sci. Technol.*, 2001, **35**, 4697–4703.
3. D. Nasuhoglu, D. Berk and V. Yargeau, *Chem. Eng. J.*, 2012, **185**, 52–60.
4. D. Matejicek and V. Kuban, *Anal. Chim. Acta*, 2007, **588**, 304–315.
5. G.-G. Ying, R. S. Kookana and Y.-J. Ru, *Environ. Int.*, 2002, **28**, 545–551.
6. Z. Yu, B. Xiao, W. Huang and P. A. Peng, *Environ. Toxicol. Chem.*, 2004, **23**, 531–539.
7. A. K. Sarmah, G. L. Northcott and F. F. Scherr, *Environ. Int.*, 2008, **34**, 749–755.
8. EWA, www.ewaonline.de, Accessed 01.13.
9. C. P. Silva, M. Otero and V. Esteves, *Environ. Pollut.*, 2012, **165**, 38–58.
10. R. J. Williams, A. C. Johnson, J. J. L. Smith and R. Kanda, *Environ. Sci. Technol.*, 2003, **37**, 1744–1750.
11. O. Braga, G. A. Smythe, A. I. Schäfer and A. J. Feitz, *Environ. Sci. Technol.*, 2005, **39**, 3351–3358.
12. T. Frische, J. Bachmann, D. Frein, T. Juffernholz, A. Kehrer, A. Klein, G. Maack, F. Stock, H.-C. Stolzenberg, C. Thierbach and S. Walter-Rohde, *Toxicol. Lett.* , 2013, **223**, 306–309.
13. A. Lange, G. C. Paull, T. S. Coe, Y. Katsu, H. Urushitani, T. Iguchi and C. R. Tyler, *Environ. Sci. Technol.*, 2009, **43**, 1219–1225.
14. H. M. Maes, S. X. Maletz, H. T. Ratte, J. Hollender and A. Schaeffer, *Environ. Sci. Technol.*, 2014, **48**, 12354–12361.

15. T. A. Ternes, M. Stumpf, J. Mueller, K. Haberer, R. D. Wilken and M. Servos, *Sci. Total Environ.*, 1999, **225**, 81–90.
16. T. Benijts, W. Lambert and A. De Leenheer, *Anal. Chem.* , 2004, **76**, 704–711.
17. T. Vega-Morales, Z. Sosa-Ferrera and J. J. Santana-Rodriguez, *J. Hazard. Mater.*, 2010, **183**, 701–711.
18. N. Yoshioka, Y. Akiyama and K. Teranishi, *J. Chromatogr. A*, 2004, **1022**, 145–150.
19. H. C. Chen, H. W. Kuo and W. H. Ding, *Chemosphere*, 2009, **74**, 508–514.
20. M. Henriques, V. Cardoso, A. Rodrigues, E. Ferreira, M. Benoliel and C. Almeida, *J. Water Resource Prot.*, 2010, **2**, 818–829.
21. B. J. Vanderford, R. A. Pearson, D. J. Rexing and S. A. Snyder, *Anal. Chem.*, 2003, **75**, 6265-6274.
22. H. G. J. Mol, S. Sunarto and O. M. Steijger, *J. Chromatogr. A*, 2000, **879**, 97–112.
23. G. Saravanabhavan, R. Helleur and J. Hellou, *Chemosphere*, 2009, **76**, 1156–1162.
24. S. D. Richardson, *Anal. Chem.*, 2002, **74**, 2719–2741.
25. D. L. D. Lima, C. P. Silva, M. Otero and V. I. Esteves, *Talanta*, 2013, **115**, 980–985.
26. P. A. Albertsson, *Partitioning of Cell Particles and Macromolecules*, Wiley-Interscience, New York, 1986.
27. M. Milosevic, K. J. J. Staal, B. Schuur and A. B. de Haan, *Desalination*, 2013, **324**, 99–110.
28. X. Xie, Y. Wang, J. Han and Y. Yan, *Anal. Chim. Acta*, 2011, **687**, 61–66.
29. K. E. Gutowski, G. A. Broker, H. D. Willauer, J. G. Huddleston, R. P. Swatloski, J. D. Holbrey and R. D. Rogers, *J. Am. Chem. Soc.*, 2003, **125**, 6632–6633.
30. M. G. Freire, A. F. M. Cláudio, J. M. M. Araujo, J. A. P. Coutinho, I. M. Marrucho, J. N. Canongia Lopes and L. P. N. Rebelo, *Chem. Soc. Rev.*, 2012, **41**, 4966–4995.
31. J. F. Brennecke and E. J. Maginn, *AIChE J.*, 2001, **47**, 2384–2389.
32. S. Aparicio, M. Atilhan and F. Karadas, *Ind. Eng. Chem. Res.*, 2010, **49**, 9580–9595.
33. T. Kakiuchi, *Anal. Chem.*, 2007, **79**, 6442–6449.
34. T. D. Ho, C. Zhang, L. W. Hantao and J. L. Anderson, *Anal. Chem.*, 2013, **86**, 262–285.
35. H. Passos, M. G. Freire and J. A. P. Coutinho, *Green Chem.*, 2014, **16**, 4786–4815.
36. D. K. Bwambok, S. K. Challa, M. Lowry and I. M. Warner, *Anal. Chem.*, 2010, **82**, 5028–5037.
37. J. F. B. Pereira, A. S. Lima, M. G. Freire and J. A. P. Coutinho, *Green Chem.*, 2010, **12**, 1661–1669.
38. C. D. Tran, D. Oliveira and S. Yu, *Anal. Chem.*, 2006, **78**, 1349–1356.
39. K. R. Seddon, *J. Chem. Technol. Biot.*, 1997, **68**, 351–356.
40. A. F. M. Claudio, C. F. C. Marques, I. Boal-Palheiros, M. G. Freire and J. A. P. Coutinho, *Green Chem.*, 2014, **16**, 259–268.
41. M. Taha, F. A. e Silva, M. V. Quental, S. P. M. Ventura, M. G. Freire and J. A. P. Coutinho, *Green Chem.*, 2014, **16**, 3149–3159.
42. F. J. Deive, A. Rodriguez, A. B. Pereiro, J. M. M. Araujo, M. A. Longo, M. A. Z. Coelho, J. N. C. Lopes, J. M. S. S. Esperanca, L. P. N. Rebelo and I. M. Marrucho, *Green Chem.*, 2011, **13**, 390–396.
43. J. F. B. Pereira, L. P. N. Rebelo, R. D. Rogers, J. A. P. Coutinho and M. G. Freire, *Phys. Chem. Chem. Phys.*, 2013, **15**, 19580–19583.
44. H. Passos, A. C. A. Sousa, M. R. Pastorinho, A. J. A. Nogueira, L. P. N. Rebelo, J. A. P. Coutinho and M. G. Freire, *Anal. Methods*, 2012, **4**, 2664–2667.
45. M. G. Freire, C. M. S. S. Neves, I. M. Marrucho, J. N. Canongia Lopes, L. P. N. Rebelo and J. A. P. Coutinho, *Green Chem.*, 2010, **12**, 1715–1718.
46. H. Passos, A. R. Ferreira, A. F. M. Cláudio, J. A. P. Coutinho and M. G. Freire, *Biochem. Eng. J.*, 2012, **67**, 68–76.
47. J. Han, C. Yu, Y. Wang, X. Xie, Y. Yan, G. Yin and W. Guan, *Fluid. Phase Equilibr.*, 2010, **295**, 98–103.
48. J. C. Merchuk, B. A. Andrews and J. A. Asenjo, *J. Chromatogr. B*, 1998, **711**, 285–293.
49. E. Alvarez-Guerra, S. P. M. Ventura, J. A. P. Coutinho and A. Irabien, *Fluid. Phase Equilibr.*, 2014, **371**, 67–74.
50. S. P. M. Ventura, S. G. Sousa, L. S. Serafim, Á. S. Lima, M. G. Freire and J. A. P. Coutinho, *J. Chem. Eng. Data*, 2011, **56**, 4253–4260.
51. C. M. S. S. Neves, S. P. M. Ventura, M. G. Freire, I. M. Marrucho and J. A. P. Coutinho, *J. Phys. Chem. B*, 2009, **113**, 5194–5199.
52. S. P. M. Ventura, C. M. S. S. Neves, M. G. Freire, I. M. Marrucho, J. Oliveira and J. A. P. Coutinho, *J. Phys. Chem. B*, 2009, **113**, 9304–9310.
53. A. F. M. Cláudio, A. M. Ferreira, S. Shahriari, M. G. Freire and J. A. P. Coutinho, *J. Phys. Chem. B*, 2011, **115**, 11145–11153.
54. C. Li, J. Han, Y. Wang, Y. Yan, J. Pan, X. Xu and Z. Zhang, *J. Chem. Eng. Data*, 2009, **55**, 1087–1092.
55. S. P. M. Ventura, S. G. Sousa, L. S. Serafim, Á. S. Lima, M. G. Freire and J. A. P. Coutinho, *J. Chem. Eng. Data*, 2012, **57**, 507–512.
56. A. F. M. Cláudio, L. Swift, J. P. Hallett, T. Welton, J. A. P. Coutinho and M. G. Freire, *Phys. Chem. Chem. Phys.*, 2014, **16**, 6593–6601.
57. S. Shahriari, C. M. S. S. Neves, M. G. Freire and J. A. P. Coutinho, *J. Phys. Chem. B*, 2012, **116**, 7252–7258.
58. M. Petrović, M. D. Hernando, M. S. Díaz-Cruz and D. Barceló, *J. Chromatogr. A*, 2005, **1067**, 1–14.
59. S. Rodriguez-Mozaz, M. J. Lopez de Alda and D. Barceló, *Anal. Chem.*, 2004, **76**, 6998–7006.
60. X. Du, X. Wang, Y. Li, F. Ye, Q. Dong and C. Huang, *Chromatographia*, 2010, **71**, 405–410.
61. M. Rezaee, Y. Yamini and M. Faraji, *J. Chromatogr. A*, 2010, **1217**, 2342–2357.
62. C. He, S. Li, H. Liu, K. Li and F. Liu, *J. Chromatogr. A*, 2005, **1082**, 143–149.
63. S. Li, C. He, H. Liu, K. Li and F. Liu, *J. Chromatogr. B*, 2005, **826**, 58–62.



94x50mm (96 x 96 DPI)



Aqueous biphasic systems composed of ionic liquids allow a single-step extraction-concentration of EE2 from wastewaters up to 1000-fold.