

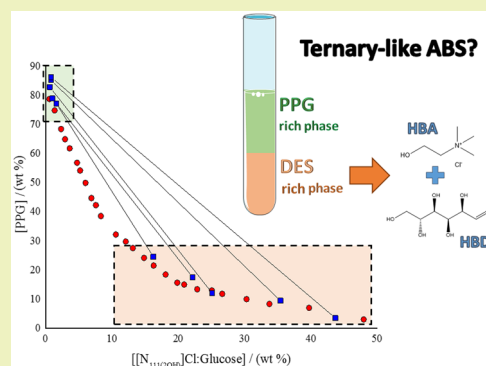
# Is It Possible To Create Ternary-like Aqueous Biphasic Systems with Deep Eutectic Solvents?

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## Supporting Information

**ABSTRACT:** The use of deep eutectic solvents (DES) as phase forming components of aqueous biphasic systems (ABS) has been proposed. However, it was shown that when dissolved in aqueous solutions, the DES complexes are destroyed and a nonstoichiometric partition of the hydrogen-bond acceptor (HBA) and the hydrogen-bond donor (HBD) between the phases of the ABS is observed. Aiming at evaluating the possibility to create ABS in which the DES integrity can be maintained, ABS composed of poly(propylene)glycol and mixtures of cholinium chloride, as HBA, and glucose, as HBD, were investigated. The results obtained suggest that a combination of factors, such as the hydrophobicity/hydrophilicity of the HBD, the nature of the ABS components, as well as the tie-line length, allows the preparation of systems in which the HBA:HBD stoichiometry used in DES preparation is maintained in the phases in equilibrium, thus behaving as *de facto* ternary systems. The partition of a wide range of biomolecules, namely phenolic compounds, amino-acids, and alkaloids, was studied on these systems. It was observed that besides the biomolecules and the DES nature, the HBD concentration, and the tie-line length also influence in the partition of the biomolecules studied, the partition mechanism being dominated by the hydrophobicity difference between the phases, with the exception of L-tryptophan for which specific interactions seem also to play a role.

**KEYWORDS:** Liquid–liquid extraction, Aqueous biphasic systems, Deep eutectic solvents, Partition, Biomolecules



## INTRODUCTION

Deep eutectic solvents (DES) was the designation used by Abbott and co-workers<sup>1</sup> to characterize a new type of solvents resulting from the mixture of two solid starting materials. In general a DES is believed to be created as result of the formation of a complex between a hydrogen-bond donor (HBD) and a hydrogen-bond acceptor (HBA), stabilized by strong hydrogen bonds, resulting in a strong depression of the freezing point of the mixture.<sup>1–3</sup> These solvents share some of the properties of ionic liquids (ILs), while being simpler to prepare and often based in renewable, more sustainable precursors, with low toxicities and good biodegradability.<sup>2–4</sup> The DES properties are dependent on the structure and ratio of the HBA and HBD used in their preparation, as well as, the water content present,<sup>2,4</sup> being thus conceivably possible to tune their properties to fit the requirement of a specific process.

Recently DES have been applied to form aqueous biphasic systems (ABS) for the extraction of biomolecules.<sup>5–8</sup> The ABS are a type of biphasic system constituted by two immiscible aqueous-rich phases, characterized by its high biocompatibility that can be used for liquid–liquid extraction processes.<sup>9,10</sup> Xu

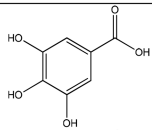
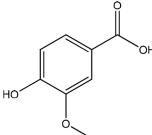
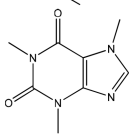
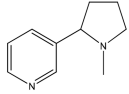
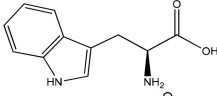
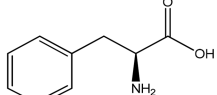
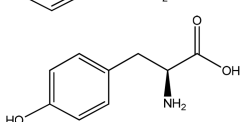
and co-workers<sup>5–8</sup> were the first to use DES as phase forming components of ABS. However, despite the promising results obtained in the extraction of proteins, only phenomenological results were reported and the study of the ABS formation mechanism and the DES stability in the aqueous media were not addressed. Dai et al.<sup>4</sup> presented compelling evidence that the interaction between the HBD and HBA are weakened in the presence of water and, for high water content, a complete disruption of DES complexes can occur. Since ABS are mainly composed of water, Passos et al.<sup>11</sup> focused their efforts in the study of the stability of DES composed of cholinium chloride ( $[N_{111}(2OH)]Cl$ , the HBA) and organic acids (the HBD) when these are used in the preparation of ABS with poly(propylene)-glycol (PPG). The authors<sup>11</sup> observed a nonstoichiometric partition of the HBA and the HBD between the coexisting phases, showing that there is no real DES-based ABS and, in fact, these systems are quaternary mixtures with the HBD and

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**Table 1.** Biomolecules' Chemical Structure, Molecular Weight ( $M_w$ ), Logarithm of Octanol–Water Partition Coefficient ( $\log(K_{OW})$ ),<sup>15</sup> and Solubility in Water ( $s_w$ )<sup>16</sup>

Biomolecule	Chemical structure	$M_w$ (g mol <sup>-1</sup> )	$\log(K_{OW})$	$s_w$ (g L <sup>-1</sup> )
Gallic acid		170.12	0.72	11.9
Vanillic acid		168.14	1.17	1.5
Caffeine		194.19	-0.55	21.6
Nicotine		162.23	1.16	miscible
L-Tryptophan		204.23	-1.09	13.4
L-Phenylalanine		165.19	-1.18	25.9
L-Tyrosine		181.17	-1.49	0.48

the HBA working individually as phase forming components of the ABS. These results were further supported by Farias et al.,<sup>12</sup> who reported the same behavior in ABS composed of an inorganic salt and DES constituted by  $[N_{111}(2OH)]Cl$  and sugars.

Passos et al.<sup>11</sup> also studied the stability of DES composed of  $[N_{111}(2OH)]Cl$  and urea finding different results for these ABS. Independently of the DES HBA:HBD molar ratio, this ratio was always maintained in “DES”-rich phase of polymer-DES-based ABS. The authors<sup>11</sup> suggested that this result could be related with a higher stability of this complex but they did not explore further this unique behavior. Nevertheless this result suggests that, under specific conditions, it may be possible to maintain the DES starting HBA:HBD molar ratio in the ABS phases, creating ABS that behave as true pseudoternary systems. We call these systems here pseudoternary because although the DES in aqueous solution is probably no longer stable and has separated into its precursors, it may nevertheless be treated as a pseudocompound since the HBA:HBD molar ratio is maintained.

Aiming at evaluating this hypothesis new ABS composed of PPG with a molecular weight of the 400 g·mol<sup>-1</sup>, and DES obtained from mixtures of  $[N_{111}(2OH)]Cl$  and glucose, which work as HBA and HBD respectively, at 2:1, 1:1, and 1:2 molar ratios, were investigated. PPG with a molecular weight of 400 g·mol<sup>-1</sup> was previously used to study the formation and integrity of this type of systems<sup>11</sup> allowing a direct comparison between the results here obtained and those reported in literature. Furthermore, PPG has shown to be a good phase former,<sup>13,14</sup> and its use prevents a possible ion exchange that can occur when other ionic species (for example, ionic liquids, or salts)

are added in to the system. The partition of the HBA and HBD between the phases in equilibrium was determined at several biphasic mixture points, to evaluate the effect of the system starting composition in the HBA:HBD final stoichiometry. Finally, the potential application of these systems to extract a range of different biomolecules, namely, alkaloids, phenolic compounds and amino-acids, was studied, evaluating the effect of system starting composition and HBD concentration.

## MATERIALS AND METHODS

**Materials.** The liquid–liquid phase diagrams were determined using aqueous solutions of PPG with a molecular weight of 400 g·mol<sup>-1</sup> from Sigma-Aldrich,  $[N_{111}(2OH)]Cl$  (98 wt % pure) from Acros Organics, glucose (99 wt % pure) from Scharlab and urea (99.5 wt % pure) from Sigma-Aldrich. The biomolecules studied were the phenolic compounds vanillic acid (97 wt % pure) from Sigma-Aldrich and the gallic acid (99.5 wt % pure) from Merck, the alkaloids nicotine (99 wt % pure) and caffeine (99 wt % pure) from Fluka, and the amino-acids L-tryptophan (99 wt % pure) and L-phenylalanine (99 wt % pure) from Sigma-Aldrich and L-tyrosine (99 wt % pure) from Fluka. The chemical structures and properties of the biomolecules investigated are presented in the Table 1.

**Methods. Determination of Phase Diagrams and Tie-Lines.** The phase diagrams were obtained by the cloud point titration method<sup>11,12,17</sup> at  $(298 \pm 1)$  K and atmospheric pressure. Aqueous solutions of PPG at 80 wt % and aqueous solutions of  $[N_{111}(2OH)]Cl$  and glucose mixtures at three different molar fractions (2:1, 1:1, and 1:2) and 75 wt % were prepared gravimetrically ( $\pm 10^{-4}$ ) and used to determine the binodal curves. The water content of the ABS constituents was measured using a Metrohm 831 Karl Fischer coulometer and taken into consideration during the preparation of each solution. Repetitive dropwise addition of the aqueous solution of

PPG to  $[N_{111(2OH)}]Cl$  and glucose solution was carried out until the detection of a cloudy solution (biphasic region), followed by the dropwise addition of water until the detection of a clear and limpid solution (monophasic region), always under constant stirring. Whenever necessary the opposite procedure was also applied. The quaternary system composition was determined by weight quantification of all components added with an uncertainty of  $\pm 10^{-4}$  g.

Five different tie-lines were determined for each ABS composed of  $[N_{111(2OH)}]Cl$  + glucose (at different molar ratios), PPG and water. For each tie-line, a quaternary mixture at the biphasic region was gravimetrically prepared within  $\pm 10^{-4}$  g, vigorously stirred and centrifuged at 3500 rpm during 30 min at  $(298 \pm 1)$  K. After reaching the equilibrium, both phases were carefully separated and individually weighted. The coexisting phases composition was analytically determined. The glucose was quantified by a colorimetric method using 3,5-dinitrosalicylic acid. For this, 1 mL of 3,5-dinitrosalicylic acid standard solution was added to 1 g of each properly diluted sample, stirred and placed in a water bath at 373 K for 10 min. To stop the reaction, the samples were immersed in ice for few minutes and diluted in water up to a final volume of 10 mL. The quantification of the reduced product (3-amino-5-nitrosalicylate) was carried by UV-visible spectroscopy (Shimadzu UV-1700) at 540 nm and using a calibration curve previously established. The water content was determined by Karl Fischer titration (Metrohm 831 Karl Fischer coulometer), using the Hydranal-Coulomat AG reagent from Riedel-de Haën as the analyte. The  $[N_{111(2OH)}]Cl$  was quantified using a chloride ion selective electrode (Metrohm 904 Titrando). The PPG mass fraction was determined by weight balance. The pH values ( $\pm 0.02$ ) of PPG- and  $[N_{111(2OH)}]Cl$ -rich phases were measured at  $(298 \pm 1)$  K, using a pH Meter (SevenExcellence, Mettler Toledo).

Passos et al.<sup>11</sup> observed that the HBA:HBD stoichiometry of DES composed of  $[N_{111(2OH)}]Cl$  and urea could be kept in DES-rich phases of PPG-based ABS. However, this result was not further explored and this behavior was not fully understood. Thus, in this work a study on the application of these DES in ABS formation was also performed. Three tie-lines for each molar mixture of  $[N_{111(2OH)}]Cl$  and urea proposed by Passos et al.<sup>11</sup> (2:1, 1:1, and 1:2) were gravimetrically prepared and quantified by  $^1H$  NMR using a Bruker Avance 300 at 300.13 MHz, with deuterated dimethyl sulfoxide as solvent and tetramethylsilane (TMS) as the internal reference. The water content was quantified by Karl Fischer titration (Metrohm 831 Karl Fischer coulometer) and the PPG mass fraction was determined by weight balance. The tie-line length (TLL) of each tie-line was calculated according to eq 1. Three components were taken into consideration to the TLL calculate, considering that in quaternary systems the tie-lines are in the space.

$$TLL = \frac{1}{\sqrt{3}} \sqrt{([X]_Y - [X]_X)^2 + ([Y]_Y - [Y]_X)^2 + ([Z]_Y - [Z]_X)^2} \quad (1)$$

where  $[X]$ ,  $[Y]$ , and  $[Z]$  are  $[N_{111(2OH)}]Cl$ , PPG 400, and glucose (or urea) weight fraction percentages, respectively, while the subscripts  $X$  and  $Y$  designate the  $[N_{111(2OH)}]Cl$ - and the PPG-rich phases, respectively.

The critical point of each pseudoternary ABS was determined by extrapolating the TL sloped of individual systems (cf. the Supporting Information) followed by the fitting using eq 2.

$$[PPG] = f[[N_{111(2OH)}]Cl:glucose] + g \quad (2)$$

where  $f$  and  $g$  are the fitting parameters.

**Biomolecules' Partition.** Aqueous solutions of each biomolecule were prepared at the following concentrations:  $0.5 \text{ g}\cdot\text{L}^{-1}$  for gallic acid ( $2.94 \times 10^{-3} \text{ mol}\cdot\text{L}^{-1}$ ) and vanillic acid ( $2.97 \times 10^{-3} \text{ mol}\cdot\text{L}^{-1}$ ),  $0.1 \text{ g}\cdot\text{L}^{-1}$  for L-tyrosine ( $5.52 \times 10^{-4} \text{ mol}\cdot\text{L}^{-1}$ ),  $1.0 \text{ g}\cdot\text{L}^{-1}$  for caffeine ( $5.15 \times 10^{-3} \text{ mol}\cdot\text{L}^{-1}$ ), nicotine ( $6.16 \times 10^{-3} \text{ mol}\cdot\text{L}^{-1}$ ) and L-tryptophan ( $4.90 \times 10^{-3} \text{ mol}\cdot\text{L}^{-1}$ ), and  $3.0 \text{ g}\cdot\text{L}^{-1}$  for L-phenylalanine ( $1.82 \times 10^{-2} \text{ mol}\cdot\text{L}^{-1}$ ). The quaternary mixture compositions were chosen based on the phase diagrams determined before for  $[N_{111(2OH)}]Cl$  + glucose + PPG +  $H_2O$  systems, and the mixture points prepared were the following:

14.5 wt % of  $[N_{111(2OH)}]Cl$ :glucose + 33.0 wt % of PPG + 52.5 wt % of biomolecule aqueous solution, 17.5 wt % of  $[N_{111(2OH)}]Cl$ :glucose + 37.0 wt % of PPG + 45.5 wt % of biomolecule aqueous solution, and 22.5 wt % of  $[N_{111(2OH)}]Cl$ :glucose + 45.0 wt % of PPG + 32.5 wt % of biomolecule aqueous solution. All phase-forming components were weighted within  $\pm 10^{-4}$  g, vigorously stirred and centrifuged at 3500 rpm during 30 min at  $(298 \pm 1)$  K, to achieve the complete partition of each biomolecule between the two coexisting phases. The biomolecule concentration in each phase was determined by UV spectroscopy, using a BioTeck Synergy HT microplate reader, at a wavelength of 262 nm for gallic acid, 259 nm for vanillic acid, 273 nm for caffeine, 260 nm for nicotine, 279 nm for L-tryptophan, 258 nm for L-phenylalanine, and 275 nm for L-tyrosine and using calibration curves previously established. Possible interferences of the phase forming components with the analytical method were taken into account, and control samples were prepared at the same weight fraction composition, using pure water instead of biomolecule aqueous solutions. At least two individual experiments were carried out in order to determine the average in the partition coefficient ( $K$ ) and percentage extraction efficiency (EE%), as well as the respective standard deviations ( $\sigma$ ). The biomolecules' partition coefficients were determined as the ratio between the concentration of each biomolecule in the  $[N_{111(2OH)}]Cl$ -rich phase and that in PPG-rich phase, according to eq 3:

$$K = \frac{[Biom]_{[N_{111(2OH)}]Cl}}{[Biom]_{PPG}} \quad (3)$$

where  $[Biom]_{[N_{111(2OH)}]Cl}$  and  $[Biom]_{PPG}$  are the concentration of biomolecule in the  $[N_{111(2OH)}]Cl$ - and in the PPG-rich phase, respectively. The percentage extraction efficiencies are defined as the percentage ratio between the amount of each biomolecule in a  $[N_{111(2OH)}]Cl$ -rich phase and that in the total mixture, according to eq 4:

$$EE\% = \frac{w_{Biom}^{[N_{111(2OH)}]Cl}}{w_{Biom}^{[N_{111(2OH)}]Cl} + w_{Biom}^{PPG}} \times 100 \quad (4)$$

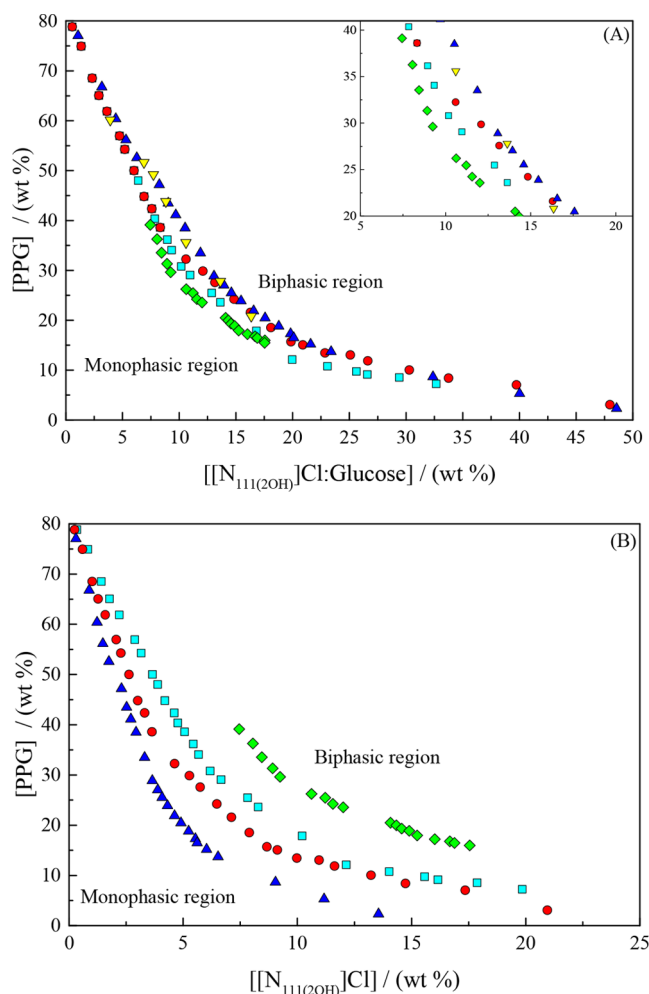
where  $w$  are the weights of the extracted biomolecules in a specific phase ( $[N_{111(2OH)}]Cl$ -rich phase or PPG-rich phase).

## RESULTS AND DISCUSSION

**Phase Diagrams.** The phase diagrams of ABS composed of  $[N_{111(2OH)}]Cl$ , glucose, PPG, and water, measured at different molar ratios of  $[N_{111(2OH)}]Cl$ :glucose, are presented in Figure 1. The detailed experimental weight fraction data are reported in the Supporting Information.

We have previously shown that due to the preferential solvation of DES individual components by water,<sup>11,12</sup> DES-based ABS are true quaternary systems and should not be treated as pseudoternary systems. Thus, for a correct interpretation of the phase diagram and the effect of the HBD and HBA in the formation of the system, they should be represented in a three-dimensional diagram, in which all the four components of the system are individually considered—cf. the Supporting Information. However, it is quite difficult to read and interpret the phase diagrams on a 3D representation. Moreover, since the HBD presents a minor effect in the ABS formation for the systems studied in this work, the representation of DES-based ABS as pseudoternary systems, as depicted in Figure 1, remains the best option.

It was previously demonstrated<sup>18,19</sup> that the formation of an ABS by mixing  $[N_{111(2OH)}]Cl$  and PPG in an aqueous solution, is driven by a salting-out mechanism.  $[N_{111(2OH)}]Cl$  presents a salt-like behavior and works as the salting-out agent due to its higher ability to form hydration complexes. It is important to highlight that this mechanism is totally different from that



**Figure 1.** Phase diagrams at 298 K and atmospheric pressure of ABS composed of PPG, water, and mixtures of  $[N_{111(2OH)}]Cl$  and glucose at different molar ratios—2:1 (■), 1:1 (●), and 1:2 (▲)— $[N_{111(2OH)}]Cl$ , PPG, and water (◆) and glucose, PPG, and water (▼). (A) Representation of the binodal curves as a function of  $[N_{111(2OH)}]Cl$ :glucose concentration. (B) Representation of the binodal curves as a function of  $[N_{111(2OH)}]Cl$  concentration.

observed in ionic-liquid-polymer-based ABS, which are controlled by the interactions established between the IL and polymers.<sup>19</sup>

Through the analysis of Figure 1A, it is possible to observe that the binodal curves for the DES at the various  $[N_{111(2OH)}]Cl$ :glucose ratio studied are very similar with only a slight reduction of the biphasic region as the glucose concentration increases. However, when the binodal curves are represented as a function of  $[N_{111(2OH)}]Cl$  concentration it is possible to observe a very distinct pattern. Contrary to what was previously reported by us in the literature for ABS composed of  $[N_{111(2OH)}]Cl$ :carboxylic-acids + PPG +  $H_2O$  and  $[N_{111(2OH)}]Cl$ :carbohydrates +  $K_2HPO_4$  +  $H_2O$ ,<sup>11,12</sup> the binodal curves are strongly affected by the glucose concentration (Figure 1B). As the glucose concentration increases, a lower amount of  $[N_{111(2OH)}]Cl$  is necessary to induce the phase separation (the binodal curves became closer to the origin). This result suggests that glucose is also acting as a salting-out agent, which was further demonstrated by the determination of the liquid–liquid equilibrium existent in the ternary mixture glucose + PPG +  $H_2O$ —cf. Figure 1A. The systems' representation as a function of glucose concentration is presented in the Supporting Information.

Several experimental tie-lines and the respective length (TLL) were determined for the studied systems. The obtained results are presented in Table 2.

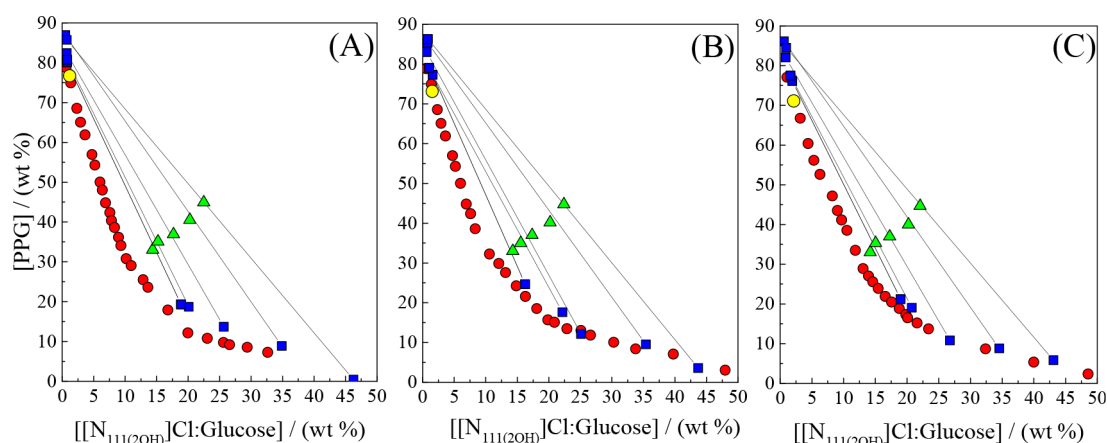
For all the quaternary mixtures prepared, independent of the  $[N_{111(2OH)}]Cl$ :glucose molar ratio and the length of the tie-line prepared, the top phase is always composed by a large amount of PPG (>76 wt %), corresponding to the PPG-rich phase, while the bottom phase is mainly composed of water (>50 wt %), presenting a higher concentration of  $[N_{111(2OH)}]Cl$  and glucose than polymer—cf. Table 2.

The binodal curves and respective tie-lines of ABS composed of a fixed  $[N_{111(2OH)}]Cl$ :glucose molar ratio are represented in Figure 2. The three-dimensional representation of all the phase diagram including the tie-lines is presented in the Supporting Information.

The representations presented in Figure 2 are cuts of the tetrahedral diagram (cf. the Supporting Information) in the

**Table 2. Liquid–Liquid Equilibrium Experimental Data of  $[N_{111(2OH)}]Cl$  (HBA) + glucose (HBD) + PPG +  $H_2O$  Systems at 298.15 K and Atmospheric Pressure**

HBA:HBD molar/mass ratio	overall composition/wt %		PPG-rich phase composition/wt %			$[N_{111(2OH)}]Cl$ -rich phase composition/wt %			TLL
	[HBA:HBD]	[PPG]	[HBA]	[HBD]	[PPG]	[HBA]	[HBD]	[PPG]	
2:1/0.6:0.4			0.3	0.4	80.1	9.8	9.1	19.3	62.2
1:1/0.4:0.6	14.0	33.0	0.5	1.1	77.3	6.6	9.6	24.6	53.7
1:2/0.3:0.7			0.3	1.6	76.1	4.4	14.6	21.2	56.6
2:1/0.6:0.4			0.3	0.5	80.7	11.2	8.9	18.7	63.5
1:1/0.4:0.6	15.0	35.0	0.3	0.7	79.0	8.4	14.7	17.6	63.3
1:2/0.3:0.7			0.4	1.2	77.5	4.3	16.4	19.0	60.6
2:1/0.6:0.4			0.3	0.4	82.4	14.3	11.4	13.6	71.0
1:1/0.4:0.6	17.5	37.0	0.2	0.4	83.0	10.3	14.8	12.1	73.0
1:2/0.3:0.7			0.2	0.7	82.1	6.9	19.9	10.8	74.1
2:1/0.6:0.4			0.2	0.3	86.9	20.4	14.5	8.8	81.9
1:1/0.4:0.6	20.0	40.0	0.3	0.4	85.3	14.4	21.0	9.5	79.8
1:2/0.3:0.7			0.1	0.5	86.2	9.9	24.6	8.8	81.7
2:1/0.6:0.4			0.4	0.4	85.7	25.9	20.4	0.4	91.3
1:1/0.4:0.6	22.5	45.0	0.3	0.5	86.3	17.9	25.8	3.6	88.3
1:2/0.3:0.7			0.2	0.8	84.5	11.6	31.5	5.8	85.3



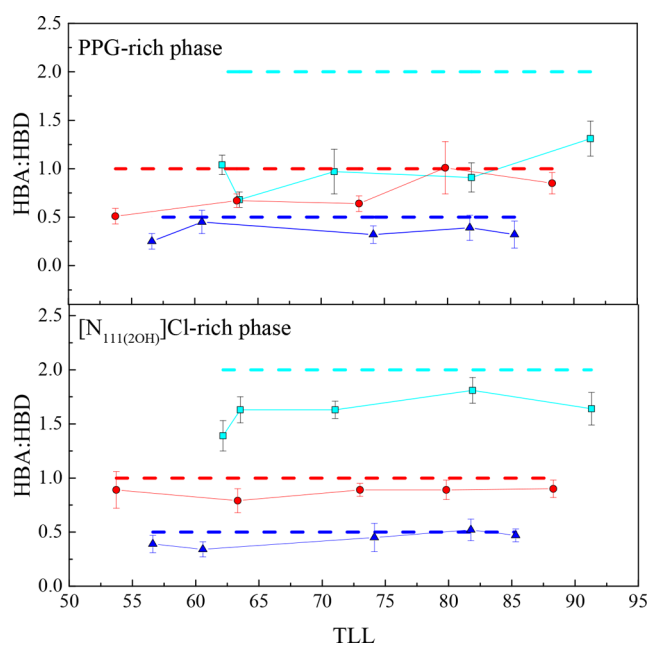
**Figure 2.** Phases diagram for the pseudoternary system  $[N_{111(2OH)}]Cl + \text{glucose} + \text{PPG} + \text{H}_2\text{O}$  at 298 K and different  $[N_{111(2OH)}]Cl$ :glucose molar ratios: (A) 2:1; (B) 1:1; (C) 1:2; binodal curve (● red), tie-line overall composition (▲), tie-line phase composition (■), and critical point (● yellow).

plans where  $[N_{111(2OH)}]Cl$ :glucose is equal to 2:1, 1:1 and 1:2. It is possible to observe that, independently of the HBA:HBD molar ratio considered, the increase of the TLL presents a high impact on the  $[N_{111(2OH)}]Cl$ -rich phase, with a significant decrease of the water amount and increase of the  $[N_{111(2OH)}]Cl$  and glucose concentration. On the other hand the composition of the PPG-rich phase is only slightly affected by the change of the starting mixture point, presenting an amount of PPG larger than 76 wt % (cf. Table 2) and less than 2 wt % of  $[N_{111(2OH)}]Cl + \text{glucose}$ . This behavior is also well described in the critical points of these systems. The critical points (cf. Figure 2) present a very high content of PPG (>70 wt %) and almost no  $[N_{111(2OH)}]Cl$  and glucose (<2.1 wt %)—cf. Supporting Information.

The  $[N_{111(2OH)}]Cl$ :glucose molar ratio was calculated for both phases of all the tie-lines studied, to infer on the final stoichiometry of the HBA and HBD in the system. The results obtained are given in the Figure 3. The experimental data of molar ratio between the HBA and HBD in each phase are presented in the Supporting Information.

By the analysis of the data presented in Figure 3, it is possible to observe a good agreement between the HBA:HBD initial mixture molar ratio (dashed lines) and the ratio measured in  $[N_{111(2OH)}]Cl$ -rich phases, where it is also clear that an increase on the TLL improves these results. Furthermore, the lower the HBA:HBD ratio, the better the results obtained for PPG-rich phases. These results are well supported by the behavior observed for the tie-lines presented in the Figure 2. It is clear, that as the PPG concentration in the top phase increases, DES-components solubility in PPG-rich phase is reduced and its partition to the bottom phase favored. Furthermore, the significant differences observed in PPG-rich phases at 2:1 molar ratio are probably related with the almost complete partition of DES components to the  $[N_{111(2OH)}]Cl$ -rich phases. Consequently, only vestigial amounts of the DES components are presented in PPG-rich phases at this molar ratio (cf. Table 2) introducing a significant deviation in the final molar ratio.

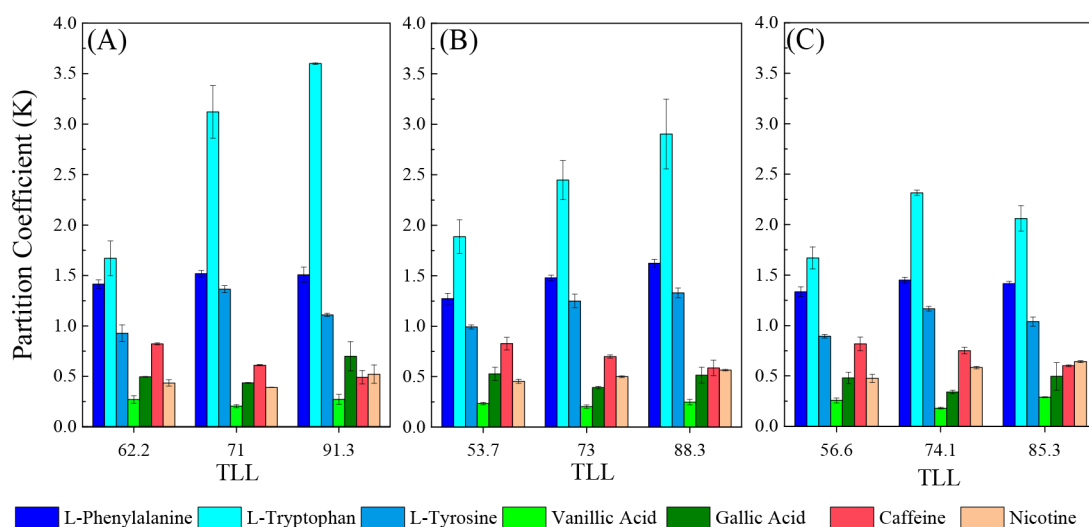
Farias et al.<sup>12</sup> showed that when DES composed of  $[N_{111(2OH)}]Cl$  and glucose are used in the formation of  $K_2HPO_4$ -based ABS, the salt induces the salting-out of the  $[N_{111(2OH)}]Cl$ , with glucose acting as an additive with a low impact in the phase diagram. Due to its high hydrophilic character ( $\log(K_{OW}) = -2.93$ ),<sup>15</sup> glucose preferentially



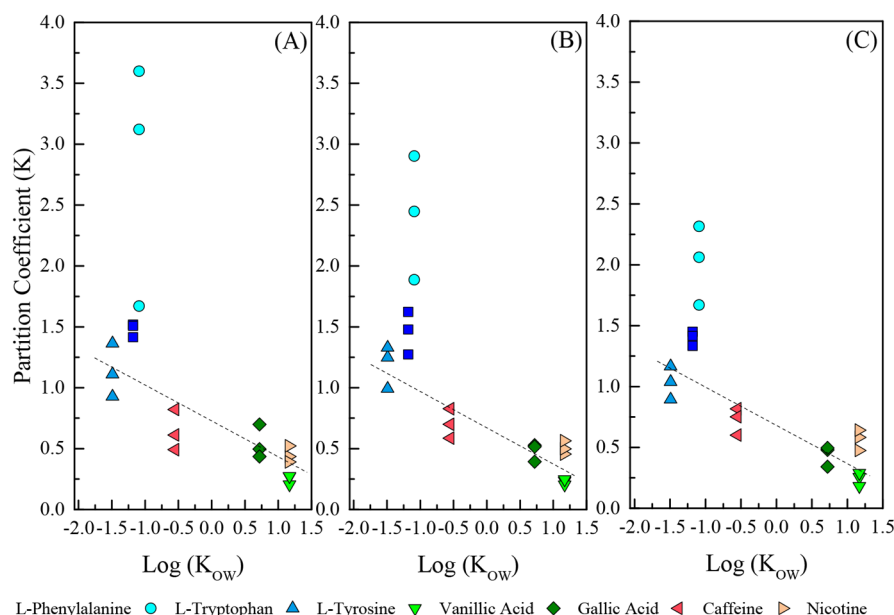
**Figure 3.** Molar ratio between the HBA ( $[N_{111(2OH)}]Cl$ ) and the HBD (glucose) in the coexisting phases of ABS composed of  $[N_{111(2OH)}]Cl$ :glucose + PPG +  $\text{H}_2\text{O}$  (solid lines) and in the initial mixture composition (dashed line): 2:1 (■); 1:1 (●); 1:2 (▲).

partitioned to the salt-rich phase, and consequently the DES initial molar ratio is not maintained in the ABS phases.<sup>12</sup> However, in the ABS here prepared, both  $[N_{111(2OH)}]Cl$  and glucose induce the salting-out of the polymer, with the formation of a very hydrophobic polymer-rich phase. Both HBA and HBD present a low solubility in this polymer-rich phase and thus preferentially remain in the opposite phase, as showed in Figure 1 and Table 2, being possible to maintain the DES initial composition in the phases of the ABS. Furthermore, and as discussed above, with the increase of the TLL, the ABS moves from the critical point (cf. Figure 2), where the two coexisting phases present similar compositions, further decreasing the solubility of the DES in the PPG-rich phase and confining the  $[N_{111(2OH)}]Cl$  and glucose to the same phase and consequently the final molar ratio value—cf. Figure 3.

Passos et al.<sup>11</sup> reported similar results to those here presented, when the DES  $[N_{111(2OH)}]Cl$ :urea were used in



**Figure 4.** Biomolecules' partition coefficients ( $K$ ) to the  $[N_{111(2OH)}]Cl$ -rich phase of ABS composed of  $[N_{111(2OH)}]Cl$ :glucose + PPG +  $H_2O$  at different  $[N_{111(2OH)}]Cl$ :glucose molar ratios: (A) 2:1; (B) 1:1; (C) 1:2.

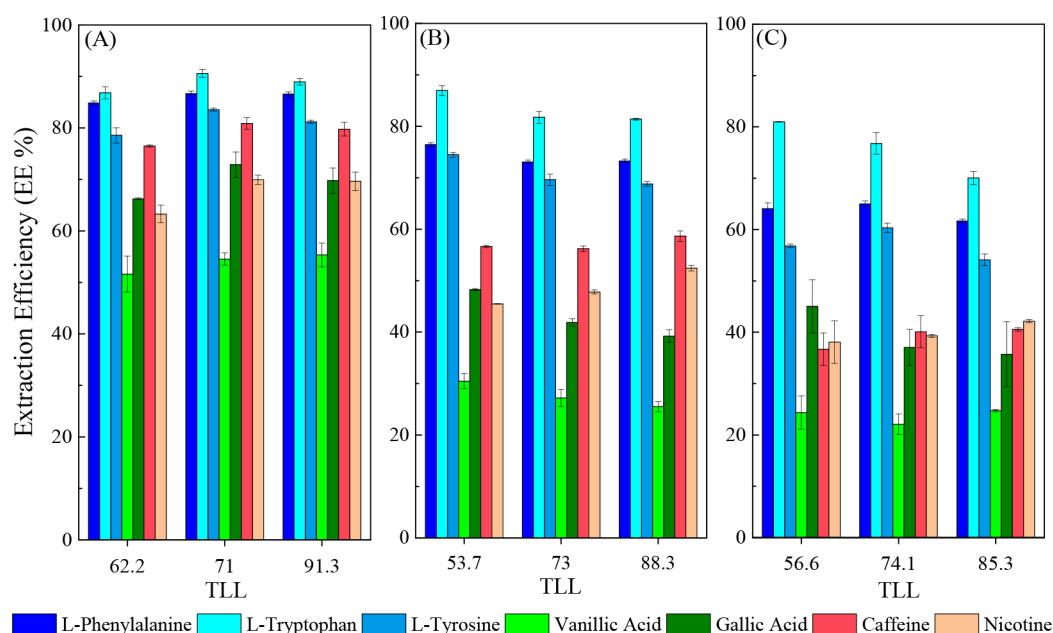


**Figure 5.** Partition coefficient ( $K$ ) in function of the  $\log(K_{OW})$  for each biomolecule studied in ABS composed of  $[N_{111(2OH)}]Cl$ :glucose + PPG +  $H_2O$  at different  $[N_{111(2OH)}]Cl$ :glucose molar ratios: (A) 2:1; (B) 1:1; (C) 1:2.

the formation of PPG-based ABS, while DES composed of  $[N_{111(2OH)}]Cl$ :carboxylic-acids lose their integrity in the same type of systems. The results here reported indicate that, unlike suggested by Passos et al.<sup>11</sup> the stability of the DES is not related with a superior stability of this complex but with the poor solubility of its compounds in the polymer-rich phase. This was confirmed here by the determination of three tie-lines for each molar mixtures of the  $[N_{111(2OH)}]Cl$ :urea proposed by Passos et al.<sup>11</sup> The obtained data for the tie-lines, critical points, and HBA:HBD stoichiometry in the coexisting phases of these systems are given in the [Supporting Information](#). As expected, and due to its strong hydrophilic character ( $\log(K_{OW}) = -1.36$ ),<sup>15</sup> the urea-based ABS present the same behavior here reported for the quaternary systems composed of  $[N_{111(2OH)}]Cl$  + glucose + PPG +  $H_2O$  systems (cf. the [Supporting Information](#)).

The results here reported show that the formation of DES-based ABS that behave as pseudoternary mixtures depends not only on the nature of the phase forming components of ABS—salt or polymer—but also on the nature of the HBD. The DES initial molar ratio can be maintained in DES-based ABS if the HBD and HBA both present a highly hydrophilic character and are poorly soluble in the aqueous phase rich in the other hydrophobic ABS former. This is the case for the DES of  $[N_{111(2OH)}]Cl$ :urea or  $[N_{111(2OH)}]Cl$ :glucose with PPG. We further postulate that the same can also be achieved with opposite polarities, i.e., with highly hydrophobic DES in the presence of strong salting-out inducing salts should also be able to form pseudoternary DES-based ABS.

**Biomolecules' Partition.** The potential application of ABS composed of DES in the extraction and separation of value-added compounds, such as proteins, phenolic compounds, and textile dyes, has already been demonstrated.<sup>5–8,11,12</sup> In this



**Figure 6.** Biomolecules extraction efficiencies (EE%) to the  $[N_{111(2OH)}]Cl$ -rich phase of ABS composed of  $[N_{111(2OH)}]Cl$ :glucose + PPG +  $H_2O$  at different  $[N_{111(2OH)}]Cl$ :glucose molar ratios: (A) 2:1; (B) 1:1; (C) 1:2.

work, the systems composed of  $[N_{111(2OH)}]Cl$ , glucose, PPG, and water were used in the extraction of seven different biomolecules: gallic acid, vanillic acid, nicotine, caffeine, L-tyrosine, L-tryptophan, and L-phenylalanine. The partition coefficients ( $K$ ) as well as the partition coefficient in function of the  $\log(K_{OW})$  of each biomolecule are presented in Figures 4 and 5, respectively. The percentage extraction efficiencies (EE%) obtained for all the biomolecules are represented in Figure 6.

The results obtained for biomolecules partition coefficients in Figure 4 show that most biomolecules were partitioned to the  $[N_{111(2OH)}]Cl$ -rich phase, with the exception of gallic acid, vanillic acid, and nicotine ( $K \ll 1$ ). Independently of the initial  $[N_{111(2OH)}]Cl$ :glucose molar ratio and the TLL considered, the following trend is usually observed:  $K(L\text{-tryptophan}) > K(L\text{-phenylalanine}) > K(L\text{-tyrosine}) > K(\text{caffeine}) > K(\text{nicotine}) > K(\text{gallic acid}) > K(\text{vanillic acid})$ . The partition coefficients have a clear dependency with the octanol–water partition coefficient ( $\log(K_{OW})$ ) as highlighted in Figure 5. The partition toward the more hydrophilic phase,  $[N_{111(2OH)}]Cl$ -rich phase, decreases with increasing  $\log(K_{OW})$  (cf. Table 1). This behavior was observed for all biomolecules with exception of the L-tryptophan, probably due to specific interactions with the  $[N_{111(2OH)}]Cl$ , considering that the difference between the observed partition coefficients for this biomolecule, and those expected from the  $\log(K_{OW})$  dependency decrease with the  $[N_{111(2OH)}]Cl$  concentration.

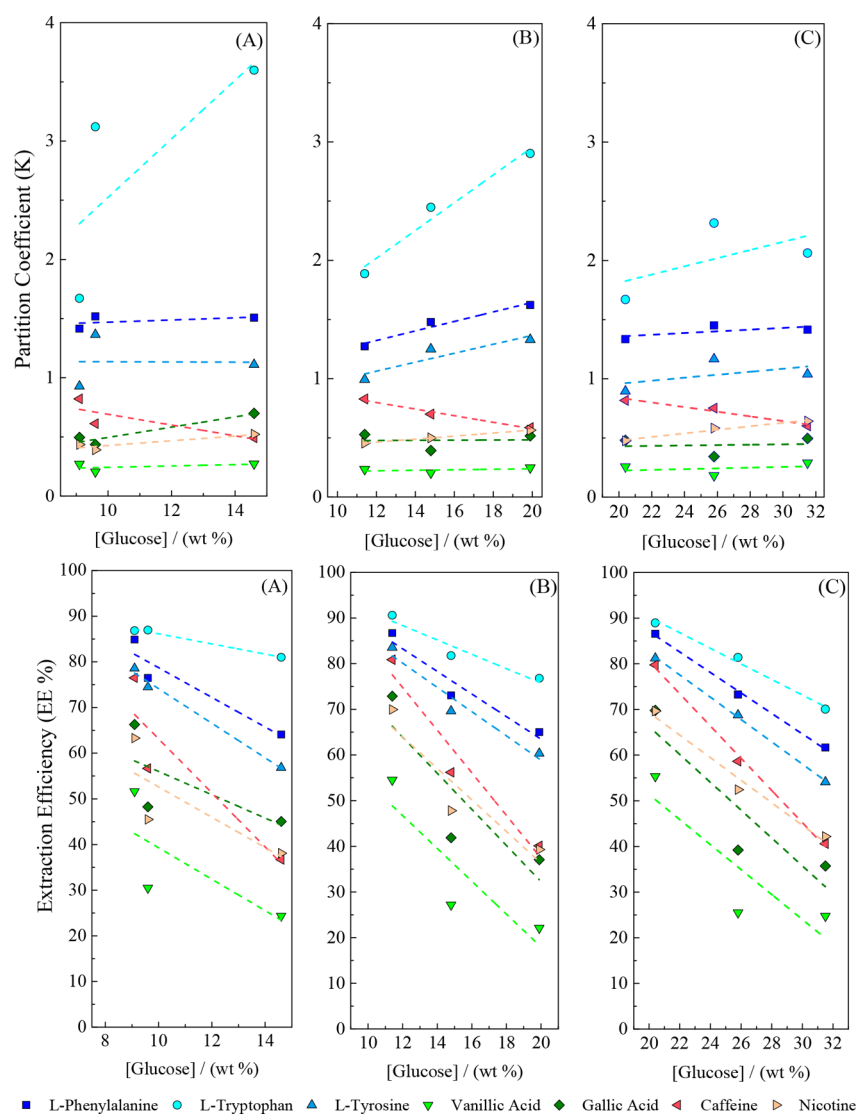
The data presented in Figure 4 shows that the increase of the TLL favors the partition of the biomolecules that presents higher affinity to the  $[N_{111(2OH)}]Cl$ -rich phase, i.e., with  $K$  higher than 1. For the compounds with  $K$  lower than 1, preferentially partitioning to the PPG-rich phase, when the TLL increases the  $K$  value further decreases, by their higher partition to the more hydrophobic phase. However, this effect is less pronounced at lower HBA:HBD molar ratios. Independently of the HBA:HBD molar ratio, the increase of the TLL increases the amount of DES components in the  $[N_{111(2OH)}]Cl$ -rich phase (cf. Table 2) inducing a more pronounced hydrophilic

character to this phase and consequently improving the partition of the biomolecules that present higher affinity to this phase ( $K > 1$ ) and decreasing the partition of those that preferentially migrate to the more hydrophobic phase ( $K < 1$ ). However, as the HBA:HBD molar ratio decreases it is possible to observe that with the increase of the TLL the increment in  $[N_{111(2OH)}]Cl$  concentration at this phase is progressively lower. Despite the increase of glucose ( $\log(K_{OW}) = -2.93$ )<sup>15</sup> concentration,  $[N_{111(2OH)}]Cl$  is the component that most contributes to the hydrophilicity of this phase ( $\log(K_{OW}) = -4.66$ ).<sup>15</sup> Thus, at 1:2 ratio the increase of the TLL presents a lower effect on the partition coefficient of the biomolecules under study independently of their affinity.

The results obtained for the extraction efficiencies (Figure 6) follow the same trends observed for the partition coefficients. Compounds with high  $K_{OW}$  values (cf. Table 1), such as the phenolic compounds, partition preferentially to the PPG-rich phase, while the remaining compounds present extraction efficiencies larger than 50% and up to ~90% for the more hydrophilic phase ( $[N_{111(2OH)}]Cl$ -rich phase).

The TLL effect in biomolecules extraction efficiency is highly dependent on the volume of the coexisting phases. The ratio between the PPG- and the  $[N_{111(2OH)}]Cl$ -rich phases, the parameter  $\alpha$ , is given in the Supporting Information. Independently of the initial  $[N_{111(2OH)}]Cl$ :glucose molar ratio, as the TLL increases, the volume of  $[N_{111(2OH)}]Cl$ -rich phase decreases, while that of the PPG-rich phase increases. Thus, the extraction efficiency of the biomolecules with preferential partition to the more hydrophilic phase will decrease due to the phase saturation and consequent higher partition of the biomolecules to the PPG-rich phase.

Considering the tie-lines with the same overall composition (Table 2) and prepared with different  $[N_{111(2OH)}]Cl$ :glucose molar ratios, it is possible to evaluate the glucose concentration effect in the partition coefficients and extraction efficiencies of the biomolecules under study. The results obtained for the partition coefficient and extraction efficiencies in function of



**Figure 7.** Effect of glucose concentration in biomolecules partition coefficient ( $K$ ) and extraction efficiency (EE%) to the  $[N_{111(2OH)}]Cl$ -rich phases of the following pseudoternary systems: (A) 14.5 wt % of  $[N_{111(2OH)}]Cl$ :glucose + 33.0 wt % of PPG; (B) 17.5 wt % of  $[N_{111(2OH)}]Cl$ :glucose + 37.0 wt % of PPG, and (C) 22.5 wt % of  $[N_{111(2OH)}]Cl$ :glucose + 45.0 wt % of PPG.

the glucose mass fraction percentage in the  $[N_{111(2OH)}]Cl$ -rich phase are represented in Figure 7.

The increase of glucose concentration in the  $[N_{111(2OH)}]Cl$ -rich phase has a negligible effect in the partition coefficients ( $K$ ) of the most biomolecules. However, a slight increase of the  $K$  to the amino-acids extraction was observed and L-tryptophan partition was the most affected. Concerning the alkaloids partition coefficients, caffeine and nicotine presents opposite behaviors: while  $K$  of caffeine slightly decreases with the increase of glucose concentration in the  $[N_{111(2OH)}]Cl$ -rich phase,  $K$  of nicotine slightly increases. This behavior is in good agreement with the octanol–water partition coefficient ( $\log(K_{OW})$ ) of the studied alkaloids—cf. Table 1. As previously referred, the addition of glucose to the  $[N_{111(2OH)}]Cl$ -rich phase decreases its hydrophilic character. Thus, since caffeine presents a hydrophilic character, the increase of glucose concentration in the  $[N_{111(2OH)}]Cl$ -rich phase results on a decrease of its partition, while nicotine partition is enhanced due to its hydrophobic character. On the other side, the extraction efficiencies (EE%) of the more hydrophobic compounds (vanillic acid and gallic acid) are the most affected by the

increase of glucose concentration, with an enhancement in the extraction of these biomolecules to the PPG-rich phase. Furthermore, to the more hydrophilic biomolecules a higher glucose content reduce the extraction to the  $[N_{111(2OH)}]Cl$ -rich phase. These results can be related with the parameter  $\alpha$  as discussed above (cf. the Supporting Information).

The results show that the phase formers concentrations can be used to maintain the stoichiometry of the HBA and HBD at the coexisting phases, and simultaneously used to tune the partition behavior of a target molecule. Considering the huge capability of DES for application in different extraction processes,<sup>2,3,20–25</sup> the possibility of using them as phase forming components of ABS in which it is possible to keep the HBA:HBD stoichiometry in the phases in equilibrium, make of DES-based ABS a potential downstream purification processes in the extraction of value-added compounds, allowing the purification of the target biomolecules and facilitating the recovery of DES-based components.



## CONCLUSION

Aqueous biphasic systems composed of  $[N_{111}(2OH)]Cl$ :glucose mixtures at different molar ratios, PPG and water were here studied. It was here shown, for the first time, that DES HBA:HBD molar ratio can be maintained in ABS coexisting phases. Depending on the nature of the HBA and HBD used, the nature of the other phase forming components, and even their concentration in the starting mixture, it is possible to manipulate the HBA:HBD molar ratio in ABS phases. The DES initial molar ratio can be maintained in DES-polymer-based ABS if the HBD and HBA both present a highly hydrophilic character and are poorly soluble in the polymer-rich phase. These same factors can be used to tune the partition of several biomolecules in this type of systems, as was here demonstrated using seven biomolecules from three different families. It was observed that besides the biomolecules and the DES nature, the HBD concentration and the TLL also influence the partition of the biomolecules studied, the partition mechanism being dominated by the hydrophobicity difference between the phases, with exception of L-tryptophan for which specific interactions seem also to play a role.

## ASSOCIATED CONTENT

### Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: [10.1021/acssuschemeng.7b02514](https://doi.org/10.1021/acssuschemeng.7b02514).

Detailed experimental data of binodal curves, critical points, pH of the phases, and biomolecules extraction efficiencies and partition coefficients; 3D representation of the quaternary systems (PDF)

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### Notes

The authors declare no competing financial interest.

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