Odd-even effect in the formation and extraction performance of ionic-liquid-based aqueous biphasic systems

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Abstract

Ionic-liquid-based aqueous biphasic systems (IL-based ABS) have been extensively investigated in the separation of high-value biomolecules. However, the understanding of the molecular-level mechanisms ruling phase separation and extraction performance of these systems is crucial to successfully design effective separation processes. In this work, IL-based ABS composed of K_2HPO_4 and cholinium carboxylate ILs ([Ch][C_nCO_2] with $n = 1$ to 7, comprising anions with odd and even alkyl chain length) were investigated. The respective ternary phase diagrams, including binodal curves, tie-lengths, tie-line lengths and critical points, as well as the Setschenow salting-out coefficients (k_s) that is a quantitative measure of the two-phase formation ability, were determined at 298 K. The extraction performance of these systems was then evaluated for four amino acids (L-tryptophan, L-phenylalanine, L-tyrosine, L-3,4-dihydroxyphenylalanine/L-dopa). It was found that ILs composed of anions with even alkyl chains display slightly higher k_s values, meaning that these ILs are more easily salted-out or more easily phase separate to form ABS. On the other hand, ABS formed by ILs with anions comprising odd alkyl chains lead to slightly higher partition coefficients of amino acids. Beyond the neat ILs odd-even effect resulting from their nanostructuration, being this a well-known phenomenon occurring in a series of their thermophysical properties, it is here shown the existence of an odd-even effect displayed by the IL anion aliphatic moiety in aqueous solution, visible both in the two-phase formation ability and extraction performance of ABS. These findings contribute to elucidate the molecular-level mechanisms governing ABS formation and partitioning of biomolecules, ultimately allowing the design of effective separation platforms.

1. Introduction

Separation and purification steps within biotechnological processes are still major challenges due to difficulties in purifying and recovering the target products from the original complex media in which they are produced.[1] Furthermore, these should be cost-effective and should be able to keep the products biological activity. Research on alternative separation techniques has been carried out with Aqueous Biphasic Systems (ABS), which correspond to liquid-liquid extraction systems formed by water and two solutes (e.g. two polymers, a polymer and a salt, two salts, etc.) that undergo phase separation above given concentrations.[2-5] In ABS, both phases are mostly composed of water, meaning that such media may be compatible with biologically active molecules.[2]

In addition to more conventional ABS formed by polymers, in the last decade, a large interest has been placed in ionic-liquid-based ABS, initially demonstrated to be formed by mixing ionic liquids and inorganic salts in aqueous media.[6] Ionic liquids (ILs) are poorly coordinated organic salts, and therefore may be liquid at or close to room temperature. In addition to other relevant properties, the major advantage associated to IL-based ABS relies on these compounds designer ability, resulting in a virtually endless opportunity of tuning the ILs chemical structures for specific applications.[7] As a result, IL-based ABS have been successfully used in the separation of a wide number of biomolecules, such as amino acids, proteins, antioxidants, among others.[8] Still, most of the ILs studied up to date are imidazolium-based, most of the times combined with $[BF_4]^-$, which may raise some toxicity and biodegradability concerns.[8] The ample versatility of ILs should however permit the synthesis of new fluids with both an acceptable environmental footprint and enhanced biocompatibility to be used in the creation of novel ABS. Cholinium-based ILs are a promising family of ILs, which can be prepared from natural sources, and may present high

biodegradability and, in some cases, marginal (eco)toxicity.[9-11] In particular, cholinium chloride is an essential nutrient with an important role in living processes, namely as precursor in the synthesis of vitamins (e.g., thiamine) and enzymes that participate in the carbohydrates metabolism.[12-13] Taking into account their advantageous features, several works have been published reporting the use of cholinium-based ILs to form ABS with salts. Xie et al.[14] investigated novel ABS composed of four cholinium-based ILs combined with carboxylate anions and K_3PO_4 to extract tryptophan, phenylalanine, and caffeine. The authors reported novel ABS liquid-liquid phase diagrams and showed their enhanced extraction performance, as well as the presence of liquid crystal structures in ABS formed by ILs with long alkyl chain anions. Shahriari et al.[15] determined the phase diagrams of novel ABS formed by a series of cholinium-based ILs with different anions and K_3PO_4, having evaluated their performance to extract antibiotics. This type of ABS has also been used to extract antibiotics from real fermentation broths,[16] and it was latter demonstrated that they represent a more economical alternative to recover tetracycline from the fermentation broth than more traditional ABS formed by polymers.[17]

To design effective ABS for separation processes, it is relevant to determine and characterize their phase diagrams. The respective ternary phase diagrams provide valuable information on the phase-forming components compositions required to form two-phase systems, and on the composition of each phase for a given overall mixture. Accordingly, a significant number of works have focused on the determination of novel IL-based ABS ternary phase diagrams.[8] Furthermore, it is well known that ILs with long alkyl side chains tend to self-aggregate in aqueous media, a trend that was further demonstrated to occur in ABS[18] and that may lead to unfavorable extraction results.[19] In a previous work, the effect of the IL cation alkyl side chain length in the formation of ABS has been comprehensively investigated by employing the 1-alkyl-

3-methylimidazolium chloride series, with both odd and even alkyl side chains.[20] An odd-even

effect on the Setschenow salting-out coefficient (k_s), a quantitative measure of the two-phase

formation ability, along the number of methylene groups of the longest IL cation alkyl side chain

was identified.[20] ILs comprising cations with even alkyl side chains display higher molar volumes

and are thus more easily salted-out and more prone to undergo phase separation in aqueous media.

Although this trend was observed for the IL cation, the effect of the IL anion on ABS formation is

scarcely studied. Some reports exist on the effect of the IL anion alkyl chain length on ABS

formation,[14,21–24] but most of these studies employed ILs with chemically different anions or only

with even alkyl chains at the anion. In general, it is understood that ILs with longer aliphatic chains

at the anion require less inorganic salt to undergo phase separation. However, no studies on the

self-aggregation impact of long alkyl chain anion ILs and on the possibility of an odd-even effect

have been performed. Furthermore, to the best of our knowledge, the impact of the odd-even effect

in the partitioning of target molecules in ABS was never investigated. It should be remarked that

although the odd-even effect is scarcely studied in aqueous solutions, the ILs odd-even effect is an

already well-established phenomenon occurring in a series of neat ILs properties, such as molar

volumes, viscosities and entropy and enthalpy of vaporization.[25-28] Santos and co-workers[26-28], by

studying several series of imidazolium-based ILs with cations of different alkyl side chain length,

demonstrated that the odd-even effect is a result of the ILs nanosegregation and orientation of the

terminal methyl groups to the imidazolium ring cation, with a consequent effect in the ILs cohesive

energy.

Aiming at better understanding the effect of the IL anion alkyl chain length on ABS

formation, in this work, seven cholinium-based ILs with carboxylate anions, from acetate to

octanoate and comprising both even and odd alkyl side chains ([Ch][C_nCO_2], n = 1 to 7), were

synthesized and used in ABS formation with dipotassium hydrogen phosphate (K_2HPO_4). The respective ternary phase diagrams were determined at (298 ± 1) K, including tie-lines and critical points, and further evaluated in terms of their extraction potential for amino acids, namely L-tryptophan, L-phenylalanine, L-tyrosine and L-3,4-dihydroxyphenylalanine/L-dopa. The Setschenow coefficient of each ABS was determined, and the odd-even effect was addressed in both ABS formation and amino acids partitioning between the coexisting phases.

2. Materials and methods

2.1. Materials

The ABS studied in this work were prepared using aqueous solutions of potassium phosphate, K_2HPO_4 (>98% of purity), purchased from Sigma–Aldrich, and aqueous solutions of each IL. The investigated cholinium-based ILs comprise anions derived from carboxylic acids, corresponding to cholinium acetate ([Ch][C_1CO_2], > 98 wt % pure) from Iolitec, and cholinium propanoate ([Ch][C_2CO_2]), cholinium butanoate ([Ch][C_3CO_2]), cholinium pentanoate ([Ch][C_4CO_2]), cholinium hexanoate ([Ch][C_5CO_2]), cholinium heptanoate ([Ch][C_6CO_2]), and cholinium octanoate ([Ch][C_7CO_2]) that were synthetized in our laboratory following protocols previously described.[29] All ILs synthesized showed high purity (>97 wt%), confirmed by ^{1}H and ^{13}C NMR (see the Supporting Information for further details). The required precursors, namely cholinium hydroxide solution (46 wt%, Sigma-Aldrich), propanoic, butanoic, pentanoic, hexanoic and heptanoic acids (99 %, Acros Organics), and octanoic acid (98 %, Sigma-Aldrich), were commercially acquired. Briefly, the corresponding acids were added drop wise into an aqueous solution of [Ch][OH] (1.1 equivalents of acid) at 298 K, and the mixture was continuously stirred

1   at room temperature and under inert atmosphere for at least 12 h to produce the cholinium-based

2   ILs. The mixtures were then dried for 4 h under vacuum using a rotary evaporator. The remaining

3   acid was removed by liquid-liquid extraction with ethyl acetate. The residual solvent was removed

4   under reduced pressure. The obtained ILs were dried under high vacuum for at least 72 h.

5   The water used was double distilled, passed by a reverse osmosis system and further treated

6   with a Milli-Q plus 185 water purification apparatus. For the partition studies, four amino acids

7   were investigated, namely L-phenylalanine (99.0 wt% of purity) acquired from Alfa Aesar, L-

8   tyrosine (99.0 wt% of purity) acquired from Fluka, and L-tryptophan (99.0 wt% of purity) and L-

9   3,4-dihydroxyphenylalanine or L-dopa (98.0 wt% of purity) acquired from Sigma–Aldrich. The

10   chemical structure of the amino acids investigated are depicted in Figure 1.
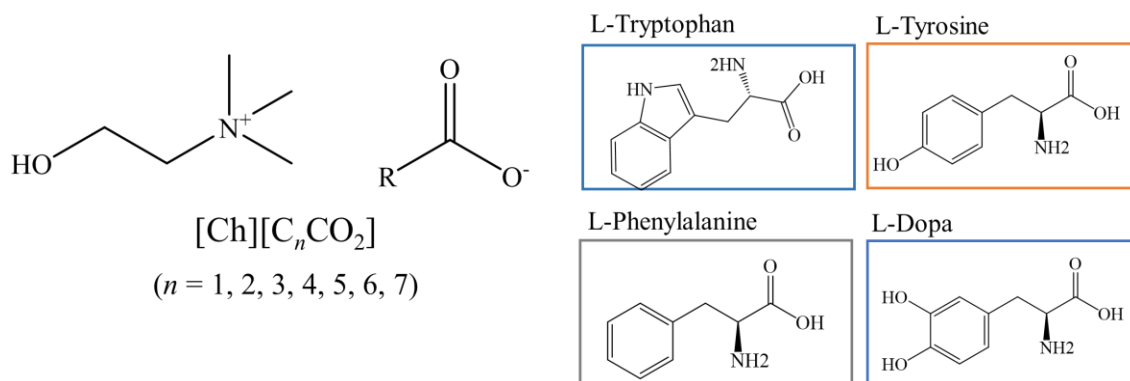
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12   **Figure 1.** Chemical structures of the investigated ILs and amino acids.

13   2.2. Phase diagrams, tie-lines, critical points and Setschenow coefficients

14   The binodal data were determined through the cloud point titration method at (298 ± 1)K and

15   atmospheric pressure. The experimental procedure adopted in this work follows the method

16   previously described by us.[21] Briefly, the salt K_2HPO_4 at 40 wt% in aqueous solution, and aqueous

17   solutions of the different ILs with concentrations ranging from 60 to 80 wt%, were prepared and

1   used for the determination of binodal curves. All additions were carried out under constant stirring,

2   and the ternary system compositions were determined by weight quantification ($\pm10^{-4}$ g). The

3   experimental solubility curves were correlated using the equation proposed by Merchuk et al.[30]:

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

5   where [IL] and [Salt] represent the IL and salt weight fraction percentages, respectively, and A, B,

6   and C are constants obtained by the regression of the experimental data.

7   The tie-lines (TLs) associated to each binodal curve, which give the composition of each

8   phase for a given mixture composition, were determined by the gravimetric method proposed by

9   Merchuk et al.[30]. Several ternary mixtures constituted by IL + K_2HPO_4 + water at the biphasic

10   region were prepared by weight and vigorously agitated. Each mixture was then centrifuged for

11   30 min at 298 K aiming for the separation of the two phases. Both phases were carefully separated

12   and individually weighed. Each TL was determined by the lever-arm rule[30] through the

13   relationship between the top phase and the total system composition, according to the following

14   system of four equations (2-5):

15   $$[IL]_T = A \exp[(B \times [Salt]_T^{0.5}) - (C \times [Salt]_T^3)] \tag{2}$$

16   $$[IL]_B = A \exp[(B \times [Salt]_B^{0.5}) - (C \times [Salt]_B^3)] \tag{3}$$

17   $$[IL]_T = \frac{[IL]_M}{\alpha} - \frac{1-\alpha}{\alpha} \times [IL]_B \tag{4}$$

18   $$[Salt]_T = \frac{[Salt]_M}{\alpha} - \frac{1-\alpha}{\alpha} \times [Salt]_B \tag{5}$$

1   where the subscripts M, T and B designate, respectively, the initial mixture, the top and bottom

2   phases. The value α is the ratio between the mass of the top phase and the total mass of the mixture

3   experimentally determined. In all systems, the top phase corresponds to the IL-rich phase, whereas

4   the salt is majorly enriched in the bottom phase.

5   Each tie-line length (TLL) was determined through the application of the following

6   equation:

7    $$\text{TLL} = \sqrt{([\text{Salt}]_T - [\text{Salt}]_B)^2 + ([\text{IL}]_T - [\text{IL}]_B)^2} \tag{6}$$

8   The critical point of each system (the mixture composition at which the composition of the

9   two aqueous phases becomes identical) was estimated through the application of the Sherwood

10   method using the TLs slopes,[31] according to equation (7),

11   $$[IL] = f[salt] + g \tag{7}$$

12   where [IL] and [salt] are the weight fraction percentages of IL and Salt, and f and g are parameters

13   obtained from the fitting.

14   Salting-out effects may be quantified by correlating the solubility data with the empirical

15   equation of Setschenow.[32] In this work the modified version of the original Setschenow equation

16   proposed by Hey et al.[33], previously used in the description of polymer-based ABS[34] and of IL-

17   salt-based ABS[34-35], was applied:

18   $$ln\frac{[\text{IL}]_T}{[\text{IL}]_B} = k_{IL}([\text{IL}]_B - ([\text{IL}]_T) + k_s([\text{Salt}]_B - [\text{Salt}]_T) \tag{8}$$

1   where [IL] and [Salt] represent the molality of IL and salt, T and B designate the top (IL-rich) and

2   bottom (salt-rich) phases, respectively, and k_{IL} and k_s are parameters relating the activity coefficient

3   of the IL to its concentration and the salting-out coefficient, respectively.

4   2.3. Cation-anion interaction energies

5   The cation-anion total interaction energies of each IL, E_{Int}, were determined using the

6   COSMO-RS thermodynamic model. This model combines quantum chemistry, based on the

7   dielectric continuum model known as COSMO (COnductor-like Screening MOdel for Real

8   Solvents), with statistical thermodynamic calculations. The process employed in this work to

9   determine the interaction energies was previously described by Kurnia et al.[36] The quantum

10   chemical COSMO calculations were performed with the TURBOMOLE 6.1 program package at

11   the density functional theory (DFT) level, applying the BP functional B88-P86 with a triple-z

12   valence polarized basis set (TZVP) and the resolution of identity standard (RI) approximation.[37]

13   The COSMOthermX program usin the parameter file BP_TZVP_C20_0111 (COSMOlogic GmbH

14   & Co KG, Leverkusen, Germany) was used in all calculations.[38]

15   2.4. Extraction of amino acids

16   ABS containing aqueous solutions of amino acids at a concentration of 5 g.L^{-1} for L-

17   tryptophan, 15 g.L^{-1} for L-phenylalanine, 2.5 g.L^{-1} for L-tyrosine and 1.5 g.L^{-1} for L-dopa, defined

18   according to the amino acids solubility in water, were prepared and used to study the amino acids

19   partitioning between the ABS coexisting phases. The ternary mixtures compositions used in the

20   partitioning experiments were chosen based on the phase diagrams and TLs determined in this

21   work. To avoid discrepancies in the results, which could arise from the different phase

22   compositions and, with the goal of addressing the odd-even effect in the amino acids partitioning,

all extraction studies were performed at a constant TLL (*ca.* 58 ± 2). Each mixture was vigorously stirred to allow equilibration and then centrifuged for 30 min at 298 K to reach complete separation between the coexisting phases. A careful separation of the phases was performed and the amount of amino acid in each phase was quantified by UV-spectroscopy, using a BioTeck Synergy HT microplate reader, at a wavelength of 280 nm for L-tryptophan, L-tyrosine and L-dopa, and at 260 nm for L-phenylalanine, using calibration curves previously established. At least three independent ABS were prepared, and three samples of each phase quantified.

The ABS performance to extract different amino acids to the IL-rich phase was evaluated by their partition coefficient (K_{AA}), defined as the ratio of the concentration of each amino acid in the IL-rich phase to that in the salt-rich phase, as described by equation (9).

$$K_{AA} = \frac{[AA]_{IL}}{[AA]_{Salt}} \tag{9}$$

where $[AA]_{IL}$ and $[AA]_{Salt}$ are the concentration of each amino acid (AA) in the IL- and in the salt-rich aqueous phases, respectively.

The pH of the IL- and K_2HPO_4-rich aqueous phases containing the amino acids was measured at 298 K using a Mettler Toledo S47 SevenMulti™ dual meter pH/conductivity equipment (data shown in the Supporting Information). The calibration of the pH meter was carried out with two buffers (pH values of 4.00 and 7.00, acquired from Metrohm).

3. Results and Discussion

3.1. Phase diagrams, tie-lines and critical points

Novel ternary phase diagrams of ABS composed of water, K_2HPO_4 and cholinium-based ILs with carboxylate anions of variable alkyl chain length were determined in this work at (298 ±

1) K. Their orthogonal representations, both in percentage weight fraction and in molality units,

are shown in Figure 2. The detailed weight fraction data are provided in the Supporting Information

(Table S1-S7). The experimental binodal data were fitted using the empirical relationship

described by Equation (1), also shown in Figure 2. The regression parameters A, B and C were

estimated by the least squares regression, and their values and corresponding standard deviations

are provided in the Supporting Information (Table S8). At least 4 TLs and respective length (TLL)

were determined for each system by Equations (2) to (6), with the detailed results shown in the

Supporting Information (Table S9). In addition, the critical point of each system was determined,

being provided in Figure 2 (*cf.* the Supporting Information for detailed data - Table S10).

Since the cholinium cation is common to all ILs, the results depicted in Figure 2 reflect the

effect of the IL anion on the formation of ABS with K_2HPO_4. The biphasic region is located above

the solubility curve; the larger this region, the higher is the ability of the IL to be salted-out by

K_2HPO_4 and to form ABS. It is well documented[39] that small IL anions derived from organic acids

are excellent hydrogen bond acceptors and exhibit high capability to establish hydrogen bonds

with water. Furthermore, the hydroxyl group at the end of the ethyl group of the cholinium cation

is able to establish strong hydrogen bonds or dipole-dipole interactions with water molecules.[40,41]

Therefore, cholinium carboxylate ILs display a high affinity for water and only moderate to strong

salting-out salts able to create ABS. Accordingly, it was previously shown that these ILs form

ABS with K_3PO_4,[14] and in this work we show that they form ABS with K_2HPO_4. However, the

studied cholinium carboxylate ILs do not form ABS with weaker salting-out salts, such as KH_2PO_4

or K_2HPO_4/KH_2PO_4 at pH 7 (as experimentally attempted by us in this work - data not shown).

The ability of the investigated ILs to form ABS at ~ 22 wt% of K_2HPO_4 follows the order:

$[Ch][C_1CO_2] \approx [Ch][C_7CO_2] < [Ch][C_6CO_2] < [Ch][C_2CO_2] < [Ch][C_5CO_2] < [Ch][C_3CO_2] \approx$

[Ch][C_4CO_2]. When weight fraction is used [Figure 2(a)], a direct correlation of the ILs' aptitude to be salted-out cannot be straightforwardly assessed. When using molality units, the differences arising from different molecular weights of ILs are avoided, allowing for a more comprehensive analysis of the effect of individual solutes on promoting liquid−liquid demixing. The binodal curves represented in molality units, shown in Figure 2(b), illustrate that the ILs ability for ABS formation, e.g. at 2.0 mol·kg^{-1} of K_2HPO_4, follows the order: [Ch][C_1CO_2] < [Ch][C_2CO_2] < [Ch][C_7CO_2] ≈ [Ch][C_6CO_2] < [Ch][C_3CO_2] < [Ch][C_4CO_2] ≈ [Ch][C_5CO_2]. This order shows that the capability to form ABS (or of the IL to be salted-out) increases with the increase of the alkyl chain length of the IL anion, at least up to [Ch][C_5CO_2], in which the ILs self-aggregation is not playing a role yet. When self-aggregation of ILs occurs, there is a decrease in their ability to create ABS, as observed for [Ch][C_6CO_2] (cholinium heptanoate) and [Ch][C_7CO_2] (cholinium octanoate). Both ILs have alkyl chains sufficiently long to self-aggregate in aqueous media at 298 K (Critical micellar concentration (CMC) values: (410 ± 13) mM for [Ch][C_6CO_2] and 383.0 mM for [Ch][C_7CO_2][42]). Details on the CMC determination are given in the Supporting Information. This trend in ABS formation is in agreement with what has been previously observed for the IL cation effect[20] and seems to be independent of the salt used.
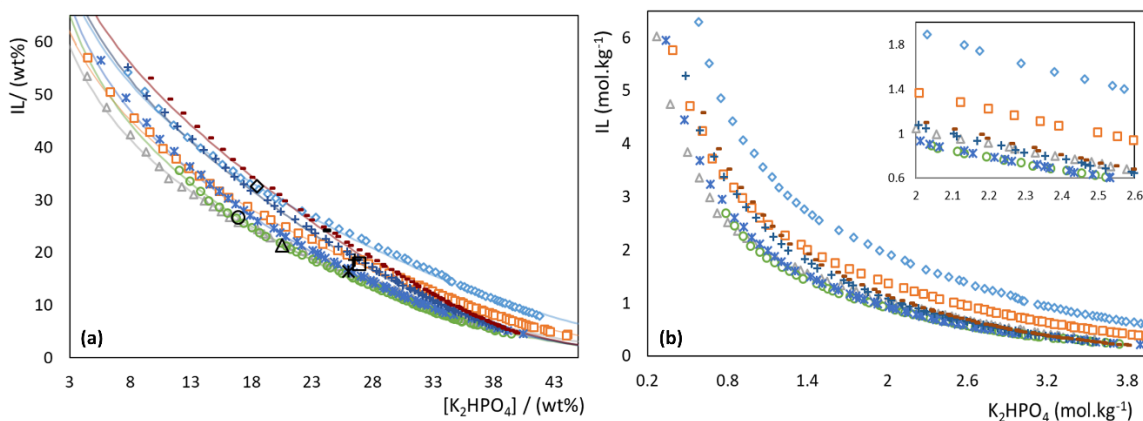
Figure 2. Phase diagrams in an orthogonal representation in **(a)** weight fraction and **(b)** molality units for the ABS formed by cholinium-based ILs + K_2HPO_4 + water at (298 ± 1) K and atmospheric pressure. ILs: $[Ch][C_1CO_2]$ ($\diamond$), $[Ch][C_2CO_2]$ ($\square$), $[Ch][C_3CO_2]$ ($\triangle$), $[Ch][C_4CO_2]$ ($\bigcirc$), $[Ch][C_5CO_2]$ ($\ast$), $[Ch][C_6CO_2]$ ($+$), $[Ch][C_7CO_2]$ (-). The lines correspond to the fitting by Equation (1) and larger symbols correspond to the critical point of each system.

The phase separation in ABS is a result of the salting-out of the salt over the IL in aqueous media, which can be quantified by the Setschenow salting-out coefficient, k_s. The Setschenow constant of each IL-based ABS was determined by the simultaneous regression of at least four experimentally determined TLs, at initial mixture compositions falling within the biphasic region. The respective values are reported in the Supporting Information (Table S11). Figure 3 depicts the behavior of the salting-out coefficient, k_s, as a function of the IL anion alkyl chain length. Although there is a continuous increase of the salting-out coefficient with the size of the IL anion aliphatic moiety, as expected due to IL anion hydrophobicity increase, a subtle odd-even effect is identified in these results. A slightly higher value of the salting-out coefficient is visible for ILs comprising anions with even alkyl chains, meaning that these are more easily salted-out by K_2HPO_4 or are

14

1   more prone to undergo phase separation in aqueous media. The current results are in good

2   agreement with our previous findings on the effect of the IL cation alkyl chain in ABS formation

3   using the $[C_nC_1im]Cl$ ($n = 2$-12) IL series and K_2CO_3.[20] In this previous work,[20] the odd-even effect

4   in the k_s values was however more significant, and particularly in ILs up to n=6 where the

5   nanostructuration/nanosegregation of ILs plays a less relevant role. This difference in intensity is

6   thus connected to the cation vs. anion effect towards the ILs nanostructuration. The odd-even effect

7   has been previously identified in several ILs properties, such as viscosities, molar volumes and

8   entropy and enthalpy of vaporization.[25-28] It is here demonstrated that it also occurs in aqueous

9   solution, indicating that the ILs organization and their nanostructuration in aqueous media

10   contribute to the observed pehnomenon. For the ILs pure properties, the odd-even effect has been

11   explained based on the orientation of the terminal methyl groups and further influence on the ILs

12   cohesive energy[26-28], which is here shown to have a direct impact on their behaviour in aqueous

13   solution and visible also as a result of the IL anion aliphatic moiety.

14   Contrarily to the IL cation effect on the formation of ABS, which is mostly governed by

15   steric and entropic contributions[21], the IL anion influence on the formation of biphasic systems is

16   a main result of favorable (or non-favorable) interactions with water. Due to their more diffuse

17   valence electronic configuration, anions are typically more polarizable than cations; thus, their

18   hydration is usually stronger than that of the cations.[43] The anions tendency to form hydration

19   complexes is directly connected to their cation-anion interaction strength. Accordingly, there is a

20   close correlation between the salting-out coefficient and the total cation-anion energy of each IL,

21   which in this work was determined by COSMO-RS, and as shown in Figure 3. The odd-even effect

22   observed is a direct result of the k_s odd-even effect itself. It should be remarked that the cation-

23   anion total interaction energies of each IL is a good indicator of the complexity of the interactions

1 taking place in ABS, particularly van der Waals, electrostatic and hydrogen-bonding interactions,

2 since the same type of interactions occur in the neat ILs contributing to their cohesive energy.
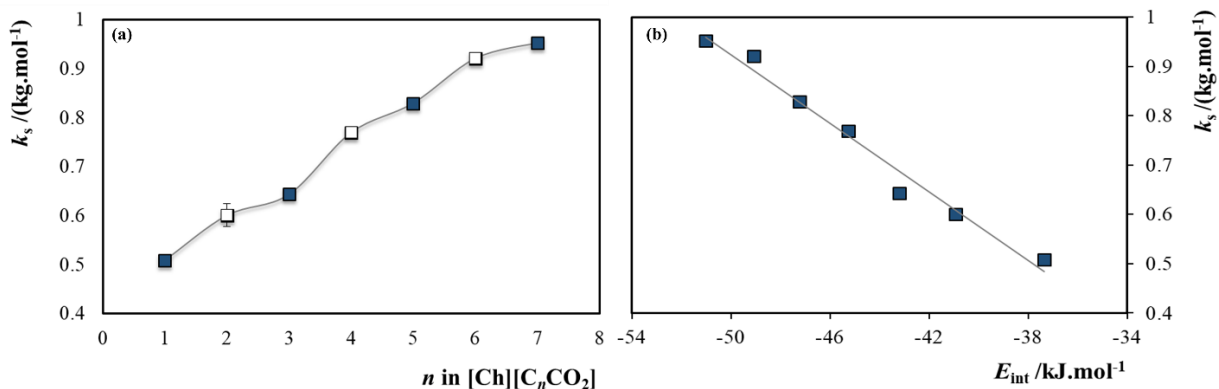
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4 **Figure 3.** Salting-out coefficients (k_s) as a function of the IL anion alkyl chain length, n, in

5 [Ch][C$_n$CO$_2$] ILs, and of the cation-anion total interaction energies of each IL (E_{int}).

6 3.2. Extraction of amino acids

7 The potential of ABS for extracting target compounds depends on the ability to manipulate

8 the properties of the coexisting phases, which can be achieved by changing the IL used in ABS

9 formulation. In this work, the potential of ABS composed of cholinium carboxylate ILs and

10 K$_2$HPO$_4$ to act as separation platforms for hydrophobic amino acids, namely L-tryptophan, L-

11 phenylalanine, L-tyrosine and L-dopa, was evaluated by their partition coefficients. These

12 aromatic amino acids, and thus hydrophobic amino acids, were chosen to identify differences in

13 partitioning that could mainly arise from dispersive interactions established with the IL anion

14 aliphatic moieties. The results obtained are given in Figure 4. The extraction efficiencies of these

15 systems are provided in the Supporting Information, as well as the respective detailed results for

16 both parameters. All partition studies were performed at a constant TLL (*ca.* 58 ± 2) to prevent the

effects that could result from the TLL effect or differences in the composition between the coexisting phases.

ABS formed by cholinium carboxylate ILs and K_2HPO_4 present a satisfactory extraction ability for amino acids. In all systems, amino acids preferentially partition to the most hydrophobic phase, *i.e.* the IL-rich phase, with extraction efficiencies ranging from 66.8% to 99.9% (cf. the Supporting Information). The partition coefficients of amino acids (K_{AA}), depicted in Figure 4, range between 1.88 and complete extraction. In Figure 4, a K_{AA} of 100 is shown for the cases where complete extraction was achieved. These high values in both partition coefficients and extraction efficiencies result from the salting-out effect exerted by K_2HPO_4 and favorable interactions established between the IL-rich phase-forming components and the amino acids. Zafarani-Moattar and Hamzehzadeh[44] investigated the separation of several amino acids (L-tryptophan, L-phenylalanine, L-tyrosine, L-leucine, and L-valine) using IL-based ABS formed by [C₄mim]Br and potassium citrate. The authors[44] concluded that the dispersive interactions are the main driving force for the amino acids partition. The results obtained for a given IL or ABS agree with the findings of Zafarani-Moattar and Hamzehzadeh[44] since the extraction efficiencies (Table S12 and Figure A3 in the Supporting Information) and the partition coefficients (Table S13 in the Supporting Information and Figure 4) follow the amino acids octanol-water partition coefficients ($\log(K_{ow})$: L-tryptophan = -1.06; L-phenylalanine = -1.38; L-tyrosine= -2.26; L-dopa = -2.74).[45] In general, more hydrophobic amino acids are better extracted to the most hydrophobic IL-rich phase. However, ABS composed of ILs with shorter alkyl chains have a better performance at extracting amino acids to the IL-rich phase, meaning that hydrophobic interactions are not the only factor ruling the partition of a given amino acid along the [Ch][C$_n$CO₂] series of ILs. This trend shows that the partitioning results are related with the affinity of each amino acid for each phase,

which is ruled by multiple effects, including salting-out effects, hydrogen-bonding, dispersive and electrostatic interactions.

In addition to the two-phase formation ability, the occurrence of an even-odd effect is also observed in the partition coefficient results along the size of the anion aliphatic moiety – Figure 4. The odd-even effect occurs in the partition coefficients of all amino acids. In general, ABS formed by ILs with odd alkyl chains at the anion lead to slightly higher partition coefficients. These results suggest that the salting-out effect exerted by the salt is not the main factor ruling the amino acids partition since higher k_s values are observed for ABS composed of IL with even alkyl chains at the anion (Figure 3). Taking into account the previous discussion, there is thus a multitude of simultaneous effects governing the amino acids partitioning to the IL-rich phase, where salting-out effects and non-covalent interactions occurring between the amino acids and the ABS phase-forming components play a major role. As observed in several properties of pure ILs[26-28], the orientation of the terminal methyl groups of the carboxylate anion and their influence on the ILs cohesive energy directly influences the ILs ability to interact with amino acids, which may thus be reponsible for the observed odd-even effect.
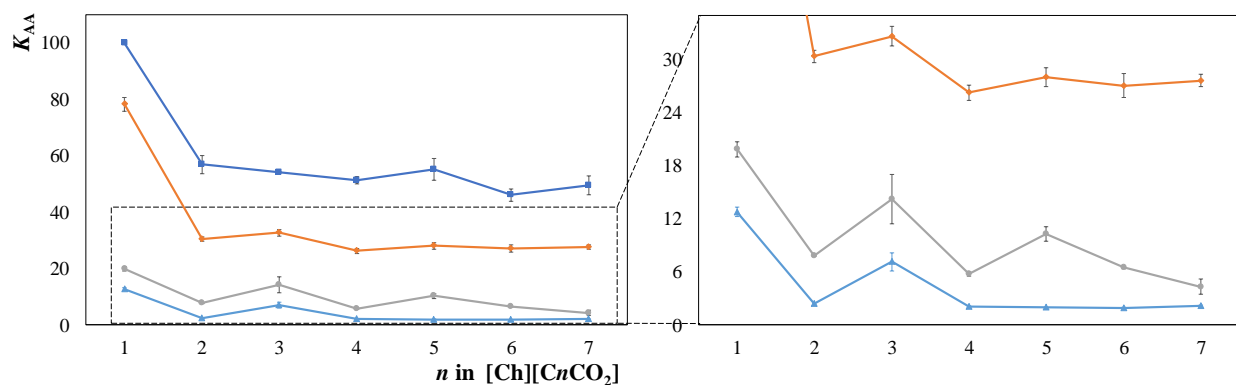
Figure 4. Partition Coefficients (K_{AA}) of the studied ABS at 298 K for L-tryptophan (■), L-phenylalanine (◆), L-tyrosine (●), and L-dopa (▲).

In addition to previously reported odd-even effects in several thermophysical and thermodynamic properties of pure ILs[26-28], a similar phenomenon is here shown to occur in aqueous solutions of ILs, namely in ABS formed by ILs and salts, and that it is visible also as a result of the IL anion. The gathered data allow to confirm the existence of an odd-even effect derived from the IL anion alkyl chain length, with impact on both the two-phase or ABS formation ability and partitioning behavior of biomolecules.

4. Conclusions

IL-based ABS have been largely studied as separation strategies, demonstrating their outstanding performance on the extraction and purification of a variety of biomolecules. However, to achieve optimal extraction and separation performance, *a priori* characterization of the respective ABS ternary phase diagrams is required. In this work, we determined the phase

diagrams of ABS formed by cholinium carboxylate ILs and K_2HPO_4, and evaluated their performance to extract hydrophobic amino acids. The $[Ch][C_nCO_2]$ IL series, with $n = 1$ to 7, was chosen to appraise the existence of odd-even effects. It is here demonstrated that this phenomenon occurs in both the ABS formation ability and extraction performance. ILs comprising even alkyl chains display slightly higher k_s values, meaning that these ILs are more easily salted-out or more easily phase separated. The phase separation ability in ABS formed by ILs and salts is largely controlled by the IL anion capability to form hydration complexes, largely depending on the interactions established between the IL anion and water, as demonstrated by the correlation found between k_s and the total cation-anion IL interaction energy. On the other hand, ABS formed by ILs with odd alkyl chains at the anion lead to slightly higher partition coefficients, demonstrating that the salting-out effect does not play the major role on defining the partitioning trend. In addition to the previously reported odd-even effect in several properties of pure ILs, these results confirm an odd-even effect induced by the IL anion alkyl chain length in aqueous media, valuable to a better understanding of the molecular-level mechanisms ruling the ABS formation ability and their extraction performance.

Acknowledgments

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