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Effective separation of aromatic and aliphatic amino acids mixtures using ionic-liquid-based aqueous biphasic systems

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Based on the particular ability of aliphatic amino acids to form aqueous biphasic systems with ionic liquids, it is here shown how these systems can be used to selectively and efficiently separate mixtures of aliphatic and aromatic amino acids usually present in protein hydrolysates or in fermentation broths.

Amino acids are the "building blocks" of proteins and play an important role in physiological phenomena, such as metabolism, gene expression, signal transduction, and in cellular and extracellular structures. Therefore, amino acids are critical compounds in animal and human nutrition, being also employed as food additives, feed supplements and artificial sweeteners.[1] In particular, aromatic amino acids, such as L-Phenylalanine (L-Phe), L-Tryptophan (L-Trp) and L-Tyrosine (L-Tyr), are amongst the most important for nutritional applications. L-Phe is a major component of the artificial sweetener aspartame, L-Trp is used in large quantities as an animal feed,[2] and L-Tyr is used in diet supplements.[3] These compounds can be obtained by the hydrolysis of proteins, fermentation or synthesis.[4] With the exception of the latter, the remaining processes result in complex mixtures of amino acids, thus requiring additional separation and purification steps to obtain the target product with an adequate purity level. In spite of these difficulties, natural amino acids are still the preferred choice and seen as the more safe option for use in human nutrition.

The hydrolysis of natural proteins, such as casein or whey proteins, results in a wide variety of essential amino acids – Phe, Trp, Treonine (Thr), Valine (Val), Lysine (Lys), among others – and non-essential amino acids – Alanine (Ala), Asparagine (Asn), Serine (Ser), Proline (Pro) and Tyr.[5] The same type of amino acids can be directly obtained from protein hydrolysates of fish processing by-products,[6] adding value to matrices that are secondary or residues from food industries. On the other hand, fermentation media are rich in a wide variety of nutrients, *e.g.* glucose or other sugars, as well as in other amino acids used as feedstocks or obtained as products.[7, 8] As an example, a mutant of the genus *Bacillus* is used for Trp production; yet, L-Phe, L-Leucine (L-Leu) and L-Methionine (L-Meth) must be added to the growth medium.[9] In the same line, when resorting to the modified pentose phosphate pathway by *Corynebacterium glutamicum*, amino acids such as Pro, Val, and Ala are the major by-products obtained (*ca.* 6% of the total amino acids).[10] Despite their high interest and value, amino acids obtained by proteins hydrolysis or fermentation lack in high purity standards.

The downstream processing of natural-derived amino acids comprises several stages, such as the cells removal by centrifugation and/or filtration[11], followed by chromatographic[12] and/or concentration/crystallisation[13] steps. However, these sequential methods are rather difficult to be transposed to a large-scale and require a high investment.[14] The recovery and purification costs of amino acids can reach up to 80% of the final product cost.[15] Therefore, there is a crucial demand on the development of cost-effective processes for their fractionation and selective separation aiming at obtaining amino acids with high purity levels.

The introduction of the "Green Chemistry" concept[16] triggered the research on more benign solvents and processes; it is in this context that ionic liquids (ILs) have been under the spotlight. Although some controversy still exists, and features such as biodegradability, toxicity and full life-cycle analyses need to be fulfilled[17], aprotic ILs are non-volatile solvents, and thus they do not contribute to atmospheric pollution. In addition to their

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†Electronic Supplementary Information (ESI) available: Identification of the systems able and not able to form ABS; materials and experimental procedure adopted; phase diagrams in molality units; detailed experimental weight fraction data; octanol-water partition coefficients and solubility in water of aliphatic amino acids; TLs, TLLs and pH of the coexisting phases; ternary phase diagrams comparison with literature; extraction efficiencies of L-Lys and L-Trp with the $[P_{4444}]$Br-based ABS at several pH values. See DOI: 10.1039/x0xx00000x

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non-volatile nature, one of their most important characteristics is related to their tailoring ability achieved by the large range of possible cation/anion combinations; as a result, effective ILs can be designed for a particular application. Along with their wide variety of applications, in the last decade, it was demonstrated that ILs form aqueous biphasic systems (ABS) when added to aqueous solutions of inorganic salts.[18]

Due to their water-rich media, ABS are adequate for liquid-liquid extraction processes, and have been used in the purification and concentration of cells, viruses, nucleic acids, proteins, antibiotics, among others.[19, 20] Albeit ABS composed of polymers have been largely investigated in the past six decades[181818181818], the use of ILs in these systems has led to enhanced extraction efficiencies and selectivity.[21] In addition to the most studied type of ABS comprising ILs and inorganic salts[21], in 2007, Zhang *et al.*[22] demonstrated that amino acids (Glycine (Gly), L-Ser, and L-Pro) are also able to form IL-based ABS. One of their main advantages comprises the possibility of using natural-derived compounds coupled to a medium of low ionic strength.

In spite of their advantages, only two works[22, 23] reported the use of amino acids to create IL-based ABS. This lacuna is a consequence of the low capacity of amino acids to induce the salting-out of ILs, and thus to create two-phase systems in aqueous media.[22, 23] Previous works[22, 23] demonstrated the possibility of ABS formation only with [C_4mim][DCA], [C_4mim][CF_3SO_3] and [C_4mim][BF_4] (the definition of these ILs is provided as a footnote‡). These imidazolium-based ILs are of a low benign character, relatively expensive due their fluorinated anions, and some of them are non-water-stable.[24] To the best of our knowledge, there are no reports in the literature regarding the use of ILs with a higher biodegradability, lower toxicity and lower cost, such as ammonium- and phosphonium-based[25, 26], to form ABS with amino acids. Furthermore, as it is only possible to form ABS with ILs of low hydrogen-bond basicity and more hydrophilic aliphatic amino acids (aromatic amino acids do not form ABS with ILs)[22, 23], these systems can be foreseen as adequate platforms to selectively separate mixtures of aliphatic and aromatic amino acids, a possibility that has never been attempted and that is explored herein.

As a first step, a wide range of amino acids as phase-forming components of ABS was investigated, namely L-Proline (L-Pro), L-Lysine (L-Lys), L-Lysine HCl (L-Lys HCl), L-Arginine (L-Arg), DL-Aspartic Acid (DL-Asp), L-Valine (L-Val), L-Isoleucine (L-Ile), L-Cysteine (L-Cys), L-Alanine (L-Ala) and L-Asparagine (L-Asn). These were combined with aqueous solutions of several tetraalkylphosphonium- and tetraalkylammonium-based ILs, *viz.* [P_{4442}][Et_2PO_4], [P_{4444}]Br, [P_{4444}]Cl, [$P_{i(444)1}$][TOS], [P_{4441}][$MeSO_4$], [$P_{444(14)}$]Cl, [N_{3333}]Br, [N_{4444}]Br and [N_{4444}]Cl. The definition of the ILs acronyms is provided as a footnote‡. Not all amino acids and ILs combinations tested are able to form ABS - a detailed list of the systems able (or not) to form ABS is given in the ESI†. For the combinations in which it was possible to create ABS, the respective ternary liquid–liquid equilibrium phase diagrams were determined at 25ºC to ascertain the mixture compositions which allow these systems to be used as extraction/purification processes. Then, the ability of these systems to selectively separate aliphatic and aromatic amino acids was evaluated by the determination of the extraction efficiency of each amino acid to a given phase. Fig. 1 depicts a schematic overview of the proposed process, as well as the chemical structures of the amino acids investigated. Further

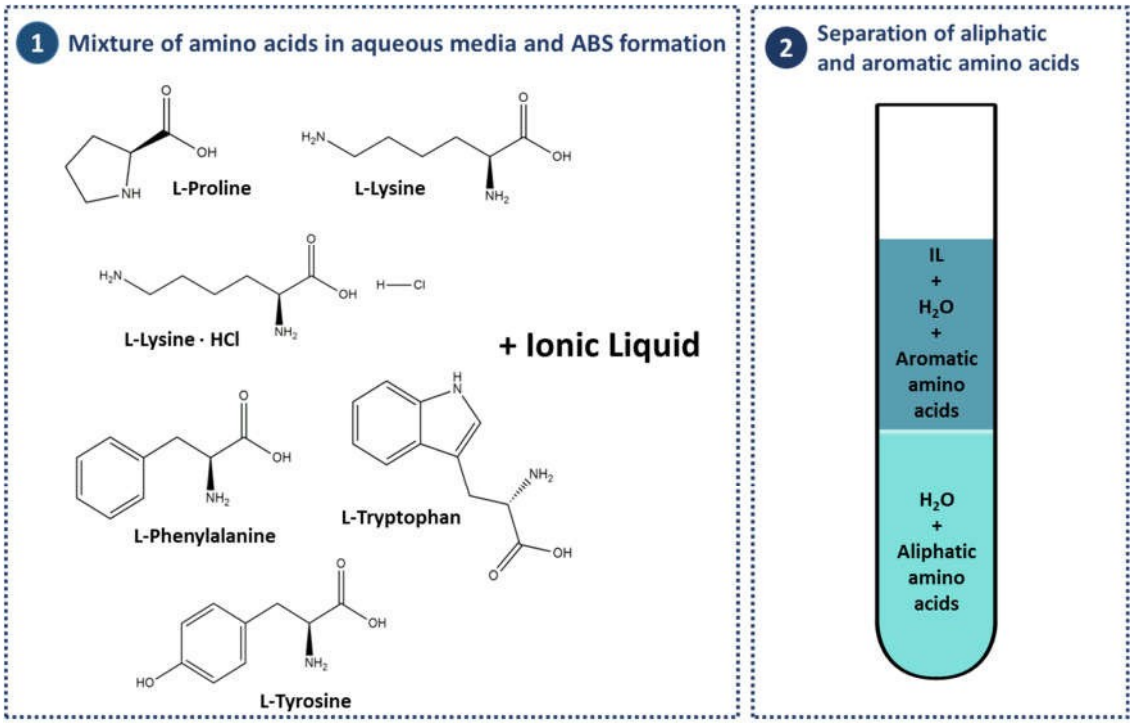

Figure 1. Chemical structures of the investigated amino acids and schematic view of the proposed process for the selective separation of aliphatic and aromatic amino acids mixtures.

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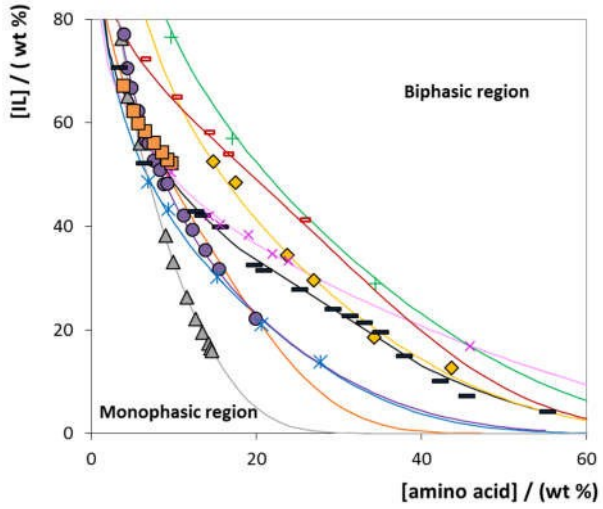

Figure 2. Phase diagrams of ABS composed of ILs + amino acids + H_2O at 25ºC: $[P_{444(14)}]Cl$ + L-Lys HCl (▲); $[P_{4444}]Br$ + L-Lys HCl (●); $[N_{4444}]Br$ + L-Lys HCl (■); $[P_{4444}]Br$ + L-Pro (♦); $[P_{444(14)}]Cl$ + L-Pro (−); $[P_{4441}][MeSO_4]$ + L-Pro (+); $[P_{444(14)}]Cl$ + L-Lys (—); $[P_{4444}]Br$ + L-Lys (✳); $[P_{4441}][MeSO_4]$ + L-Lys (×).

details on the materials and experimental procedure adopted are given in the ESI†.

A wide range of aliphatic amino acids and ILs were evaluated as phase-forming components of ABS. It was found that only combinations of ILs with a more hydrophobic character and amino acids with a more hydrophilic nature or higher affinity for water are able to form ABS, meaning that amino acids act as salting-out species.

The ternary phase diagrams of the IL-amino-acid mixtures that form ABS are depicted in Fig. 2 (the respective phase diagrams in molality units are reported in the ESI†). For mixture compositions above each solubility curve there is the formation of a two-phase system, while mixture compositions below the same curve result in the formation of a homogeneous solution with no phase-separation. We have found nine IL-amino-acid pairs able to create ABS, namely those formed by L-Pro + $[P_{4444}]Br$, L-Pro + $[P_{4441}][MeSO_4]$, L-Pro + $[P_{444(14)}]Cl$, L-Lys + $[P_{4444}]Br$, L-Lys + $[P_{4441}][MeSO_4]$, L-Lys + $[P_{444(14)}]Cl$, L-Lys HCl + $[P_{4444}]Br$, L-Lys HCl + $[P_{444(14)}]Cl$ and L-Lys HCl + $[N_{4444}]Br$. These results confirm that appropriate ammonium- and phosphonium-based ILs can form ABS with amino acids, overcoming therefore the need of using less benign fluorinated imidazolium-based fluids.

For a given IL, the ability of amino acids to create ABS follows the order: L-Lys·HCl > L-Lys > L-Pro (Fig. 2). This trend is in accordance with their octanol-water partition coefficients and solubility in water - *cf.* the ESI†, confirming therefore their salting-out aptitude over ILs in aqueous media. On the other hand, the ILs ability to form ABS in presence of a given amino acid is as follows: $[P_{444(14)}]Cl$ > $[P_{4444}]Br$ > $[N_{4444}]Br$ > $[P_{4441}][MeSO_4]$. Although composed of an anion with a high hydrogen-bond basicity[27], $[P_{444(14)}]Cl$ is amongst the best ILs to form ABS with amino acids, a result of the high hydrophobic nature of the cation derived from the long tetradecyl alkyl chain. Contrarily, $[P_{4441}][MeSO_4]$ is constituted by shorter alkyl side chains at the cation coupled to an anion with high hydrogen-bond basicity[27], and thus displays a higher affinity for water, further reflected in the need of higher amounts of amino acid to create two-phase systems. The same phenomenon is behind the ABS formation ability of $[N_{4444}]Br$ *versus* $[N_{4444}]Cl$ (the former being not able to form ABS as described in the ESI†). In general, the higher the IL hydrophilic nature or affinity for water, the lower it is its ability to form ABS with amino acids - following the same trend observed in IL-salt ABS.[21]

Given the capability of aliphatic amino acids to form ABS with phosphonium- and ammonium-based ILs, against the non-ability of aromatic ones, these liquid-liquid platforms were further evaluated in what concerns their performance to separate mixtures of amino acids. Fig. 3 depicts the extraction efficiencies of the investigated IL-based ABS for aromatic and aliphatic amino acids. For comparison purposes, imidazolium-based ABS reported in the literature[22, 23] were also tested. Percentage extraction efficiencies of amino acids (EE_{aa}%) correspond to the percentage ratio between the amount of each amino acid in a given phase and that in the total mixture. All extractions were carried out at a common tie-line length (≈ 80) to avoid discrepancies in the extraction efficiencies which could arise from differences between the compositions of the coexisting phases. Detailed values and respective uncertainties, are given in the ESI†.

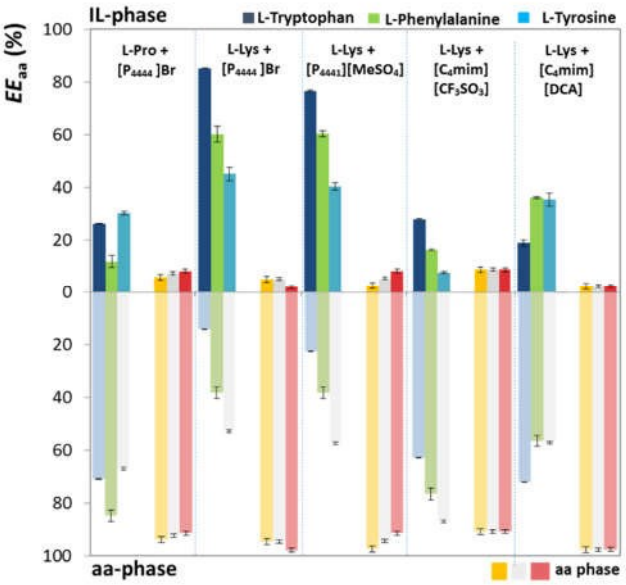

Figure 3. Extraction efficiencies of amino acids (EE_{aa}%) in the studied systems at 25ºC. ■ ■ represents the EE_{aa}% of aliphatic amino acids to the IL-rich opposite phase.

Remarkably, and with the exception of the L-Pro + $[P_{4444}]Br$ ABS, amongst the phoshonium-based ABS there is the preferential migration of aromatic amino acids to the IL-rich phase while aliphatic amino acids are enriched in the opposite layer, hence allowing their effective separation. Within the best systems

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identified, EE_{aa}% of the IL-rich phase for aromatic amino acids ranging between 40 and 85%, and EE_{aa}% of the opposite phase for aliphatic amino acids ranging between 91 and 98%, were obtained in a single-step. The higher the extraction efficiency values to opposite phases, the higher the selective separation of aromatic/aliphatic amino acids mixtures. In fact, phosphonium-based ABS appear as remarkable separation techniques when compared with ABS formed by imidazolium-based fluids. The selectivity values of IL-based ABS to separate aromatic amino acids from aliphatic ones are provided in the ESI†. The selectivity values for phosphonium-based ILs range between 1.5 and 121, corresponding to significantly higher values than those found with the imidazolium-based systems (from 0.01 to 0.07).

Amongst the ABS investigated, the [P_{4444}]Br + L-Lys system provides the best results in what concerns the selective separation of amino acids mixtures. With this system it is possible to recover L-Trp in one-step with an extraction efficiency higher than 85% to the IL-rich, whereas L-Lys is almost completely enriched in the opposite phase (EE_{aa}% higher than 90%). In addition to the high ability of the IL-rich phase to extract aromatic amino acids, it should be remarked the almost null cross-contamination of this phase with aliphatic amino acids (EE_{aa}% of the IL-rich phase lower than 9% for all aliphatic amino acids, in all systems investigated). The obtained results, in terms of separation performance, are comparable to those obtained with conventional techniques,[28, 29] and that usually are more complex processes and require the use of less benign compounds and more expensive equipment.

According to the results depicted in Figure 3, the IL nature has a relevant influence through the partition behaviour of aromatic amino acids and a more negligible effect on the partition trend of aliphatic ones. While almost all systems are able to effectively separate mixtures of aliphatic and aromatic amino acids, the trend obtained for the extraction efficiencies is also related to the salting-out aptitude of the aliphatic amino acid. For instance, the system constituted by the strongest salting-out amino acid (L-Lys, according to Fig. 2) is the one that displays the best performance in terms of selective separation. Furthermore, electrostatic interactions established between charged amino acids and ILs can also play a role on the amino acids partition and cannot be neglected. The pH of the coexisting phases of the L-Pro + [P_{4444}]Br system is ≈ 5, corresponding to the system with the lowest selectivity (Fig. 3). On the other hand, both [P_{4444}]Br + L-Lys and [P_{4441}][$MeSO_4$] + L-Lys systems, have aqueous phases with pH values ranging from 10 to 11, and both systems provide better extraction efficiencies for aromatic amino acids. The pH of the coexisting phases of all ABS investigated is presented in the ESI†. The isoelectric points (pI) of the aromatic amino acids investigated are 5.89 for L-Trp, 5.48 for L-Phe, and 5.66 for L-Tyr,[30] indicating that electrostatic interactions between amino acids and ILs decrease at pH values *ca.* 5. To address the relevance of electrostatic interactions, we carried out the separation of L-Lys and L-Trp with the ABS composed of [P_{4444}]Br at several pH values, and explored the ability of the ABS formed by [P_{4444}]Br and L-Lys.HCl, that will be more acidic in nature, for the separation of aromatic and aliphatic amino acids. The results obtained for both sets of experiments are shown in the ESI†. In general, an increase in the pH leads to an increase of the L-Trp partitioning to the IL-rich phase, and therefore confirms the relevance of electrostatic interactions in the performance of the systems investigated. In fact, EE_{aa}% of the IL-rich phase for L-Trp up to 87%, and EE_{aa}% of the opposite phase for L-Lys up to 95%, were obtained in a single-step at pH 12. In the same line, a lower separation performance was observed with the L-Lys.HCl-based system, as a result of its more acidic character (the pH of the coexisting phases is *ca.* 3). This dependence on the pH can be additionally used to further improve the selective separation ability of the investigated IL-based ABS.

Most of the works on IL-based ABS have mainly focused on the evaluation of their extraction performance;[21] yet, and although scarcely investigated, the products recovery from the IL-rich phase is a crucial task aiming at proving the "real" utility of these separation systems. In this context, we further evaluated the possibility of separating the aromatic amino acids from the ILs that constitute the ABS IL-rich-phase. To this end we used a solid-phase extraction approach, by means of a cation exchange column[31], able to retain the IL cation. The elution of the IL-rich phase was conducted at a high pH value (*ca.* 12) to avoid the amino acids adsorption. Experimental details are given in the ESI†. At the optimized conditions we successfully eluted the aromatic amino acid and retained the IL by adsorption onto the column, allowing thus its removal from the original aqueous phase. With the [P_{4444}]Br + L-Lys ABS enriched in L-Trp, we were able to recover 93% of the aromatic amino acid and to remove 79% of the IL present in the original IL-rich phase. Even though these are already very promising results, we believe that by further optimizing the mobile phase composition, for instance the pH and ionic strength, and other operation variables, such as bed volume, an even better chromatographic performance will be reached. In addition to the amino acid/IL separation, this strategy also allows the IL recovery and reuse towards the development of cost-effective and more sustainable technologies.

Wang *et al.*[32] demonstrated the selective separation of L-Trp with imidazolium-based ILs, finding that this amino acid could be effectively separated from a fermentation broth, although requiring multiple steps. However, most studies reported in the literature on similar topics address the extraction of only one aromatic amino acid (*i.e.*, the selectivity toward other amino acids were not evaluated) while using less "benign" ILs.[32-38] Herein, we demonstrate the remarkable ability of IL-based ABS for the separation of two classes of amino acids usually present in proteins hydrolysates or fermentation broths, and that more benign and non-fluorinated ILs can be efficiently used for such purpose.

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Notes and references

‡ethyl(tributyl)phosphonium diethylphosphate ($[P_{4442}][Et_2PO_4]$), tetrabutylphosphonium bromide ($[P_{4444}]Br$), tetrabutylphosphonium chloride ($[P_{4444}]Cl$), triisobutyl(methyl)phosphonium tosylate ($[P_{(4441)}][TOS]$), tributyl(methyl)phosphonium methylsulfate ($[P_{4441}][MeSO_4]$), tributyl(tetradecyl)phosphonium chloride ($[P_{444(14)}]Cl$, tetrapropylammonium bromide ($[N_{4444}]Br$), tetrabutlyammonium bromide ($[N_{4444}]Br$), tetrabutlyammonium chloride ($[N_{4444}]Cl$), 1-butyl-3-methylimidazolium dicyanamide ($[C_4mim][DCA]$, 1-butyl-3-methylimidazolium trifluoromethanesulfonate ($[C_4mim][CF_3SO_3]$) and 1-butyl-3-methylimidazolium tetrafluoroborate ($[C_4mim][BF_4]$).

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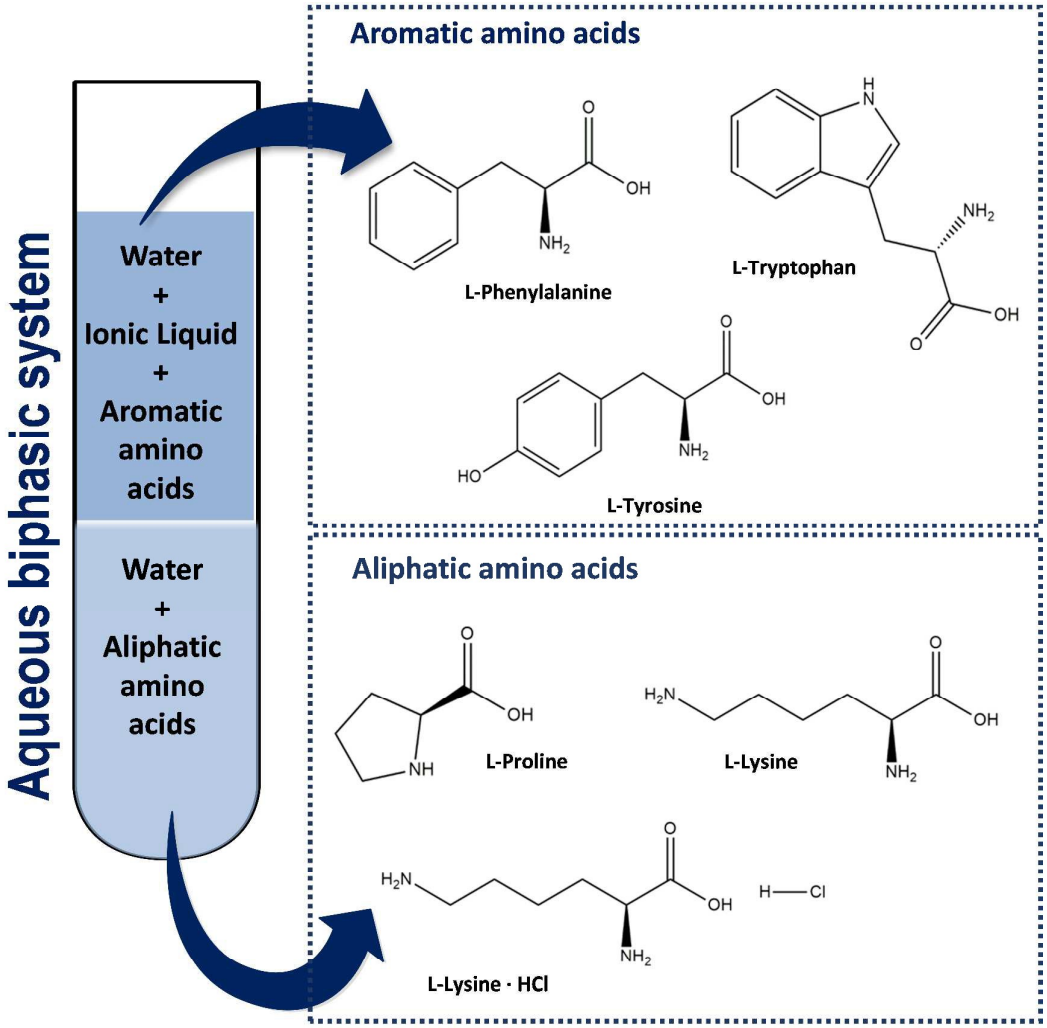

Ionic-liquid-based aqueous biphasic systems allow an efficient and selective separation of aliphatic and aromatic amino acids mixtures usually present in protein hydrolysates or fermentation broths