Chapter 5

Aqueous Two-Phase Systems

Jorge F.B. Pereira*, João A.P. Coutinho†

**Department of Bioprocesses and Biotechnology, School of Pharmaceutical Sciences, São Paulo State University—UNESP, Araraquara, Brazil, †Department of Chemistry, CICECO, University of Aveiro, Aveiro, Portugal*

Chapter Outline

5.1 Introduction 158
5.2 Thermodynamic Fundamentals and Properties 158
5.2.1 Phase Diagrams and Tie-Lines 159
5.2.2 Physicochemical Properties of the Phases and Kinetics of Separation 161
5.3 Types of Aqueous Two-Phase Systems 162
5.3.1 Polymer/Polymer ATPS 163
5.3.2 Polymer/Salt ATPS 164
5.3.3 Salt/Salt ATPS 166
5.3.4 Other Types of ATPS 169
5.3.5 Effect of Temperature and pH 170
5.4 Applications of Aqueous Two-Phase Systems 171
5.4.1 Partition of Molecules and Particulates 171
5.4.2 Extraction and Purification of Products 172
5.4.3 Analytical Applications 173
5.4.4 Emerging and Non-Conventional Applications 174
5.5 Scaling-Up and Continuous Processing 174
5.6 Final Remarks and Future Perspectives 175
Acknowledgments 176
References 176

Liquid-Phase Extraction. https://doi.org/10.1016/B978-0-12-816911-7.00005-0

5.1 Introduction

As shown in this book, various unit operations are used in downstream processing to extract/separate solutes from a liquid phase. Among these, aqueous two-phase systems (ATPS) were proposed as alternatives liquid-liquid extraction (LLE) processes for the separation of biological products. ATPS were discovered by Beijerinck in 1896, who observed that a mixture of gelatin, agar, and water can create a biphasic system [1]. However, there were no further developments until 1956, when Albertsson proposed ATPS composed of polyethylene glycol (PEG) and dextran for the separation of proteins, peptides, nucleic acids, viral, and LLE particles [1]. Besides polymer/polymer, Albertson also formed ATPS by mixing a polymer and an inorganic salt [1]. Subsequently, a significant number of works were reported characterizing and understanding different types of ATPS or evaluating their uses for the separation, extraction, and purification of biomolecules and particles [1–13].

ATPS are biphasic systems that can be used in LLE processes, where two water-rich phases are formed by mixing, above given concentrations, at least two different water-soluble components, such as polymers, salts, sugars, alcohols, or surfactants. Although both components are water-soluble, they separate into two phases, each richer in one of the two components. Because of the hydrophilic nature of the immiscible water-rich phases and the low interfacial tension, their use in biotechnological processes has been paramount. ATPS are frequently associated with simple, biocompatible, amenable, and easily scalable separation platforms [1–3]. Depending on the type of ATPS, a range of downstream processing scenarios can be attained, for example, achieving selective extractions, concentrating diluted solutes, or removing significant amounts of contaminants and denaturing compounds. Despite these advantages, their application to biotechnological downstream processing is predominantly confined to laboratory and academic (basic and applied) studies [2, 4–6] without significant industrial use. In recent years a large number of reviews [3, 5–26] have appeared compiling most of the advances, concepts, and applications of ATPS-based platforms.

5.2 Thermodynamic Fundamentals and Properties

The most common ATPS are generated by mixing a pair of hydrophilic polymers (polymer/polymer), a polymer with a salt (polymer/salt), or two different salts (salt/salt). Many of these combinations can generate a biphasic regime within a certain concentration range, where the phase separation is controlled by water solvation of the phase-forming agents. This section provides an overview of the thermodynamic fundamentals and key properties of ATPS.

5.2.1 Phase diagrams and tie-lines

Phase diagrams represent the potential working region for an ATPS, and any attempt at using ATPS should start with the determination of the phase diagram. As shown in Fig. 5.1, this is based on the compositions (weight or molar) of two phase-forming agents (components 1 and 2), providing a set of useful data about the system in equilibrium: (*i*) concentration of the components 1 and 2 necessary to create a biphasic system, (*ii*) the concentration of phase components in the top (light) and bottom (heavy) phases, and (*iii*) ratio of the phase volumes. Although ATPS are ternary systems, composed of two phase-forming agents and water, these are in general depicted in an orthogonal representation in which the water concentration is omitted (pure water corresponds to the origin).

The ternary phase diagram, depicted in Fig. 5.1, is constituted by two phase-forming agents (components 1 and 2) and water. A solubility curve (binodal) divides the two-phase region (above the curve) from the single-phase region (below the curve). The composition of the phases in equilibrium are related by the tie-lines (TLs) that connect two points on the binodal curve, which correspond to the

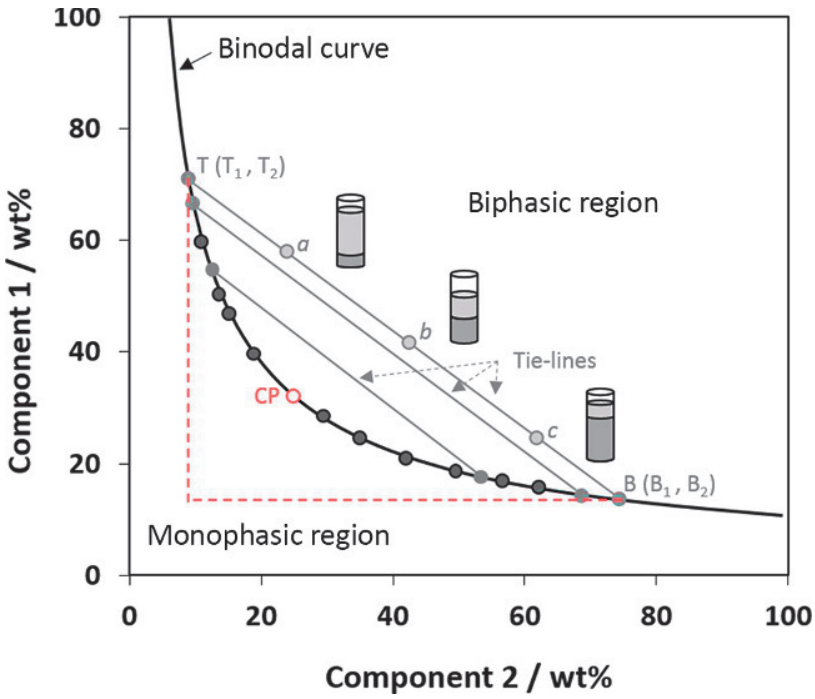

Fig. 5.1 Scheme of an orthogonal ternary phase diagram composed of component 1, component 2, and water (in weight fraction, wt%) and the respective binodal curve (—), tie-lines (—), and critical point (CP, ○). Top phase (component 1–rich phase) is plotted on the *y*-axis, and bottom phase (component 2 rich-phase) is plotted on the *x*-axis. *a*, *b*, and *c* (●) represent total compositions of three mixtures. The final composition of the top and bottom phase is represented by the nodes (●) *T* and *B*, respectively.

concentration of components 1 and 2 in the top and bottom phases. For example, a, b, and c correspond to three ATPS that have the same composition in equilibrium (T_1, T_2 and B_1, B_2, respectively, for the top and bottom phases). Thus, moving along the same tie-line (TL), the concentration of the phases remains the same, differing only in the total compositions and phase volume ratios—an interesting feature when ATPS are used for concentration of analytes since partition is maintained, while the volumes are reduced.

TLs are approximately parallel, and parameters such as tie-line length (TLL) and slope of the tie-line length (SLT) can be calculated, contributing to the understanding of the phase diagram and helping to draw other TLs. TLL has the same units as the concentration, and it is used to express the influence of the system composition on the partition of solutes. TLL and SLT can be related to the equilibrium phase composition according to Eqs. (5.1) and (5.2):

$$TLL = \sqrt{[T_2 - B_2]^2 + [T_1 - B_1]^2} \tag{5.1}$$

$$STL = \frac{[T_1 - B_1]}{[T_2 - B_2]} = \frac{\Delta\text{component 1}}{\Delta\text{component 2}} \tag{5.2}$$

For example, the TL of the system a is the segment TB, and the ratio of the length of the segments aB and aT corresponds to the top and bottom phase ratio (in wt%), as shown in Eq. (5.3):

$$\frac{m_T}{m_B} = \frac{V_T \rho_T}{V_B \rho_B} = \frac{aB}{aT} \tag{5.3}$$

where m, V, and ρ are the masses, volumes, and densities of the top (T) and bottom (B) phases. If the phase densities are known, the volume ratio of the phases can be easily determined.

As detailed by Hatti-Kaul [27], the binodal curves are mainly determined by three different methods, (*i*) turbidimetric titration, (*ii*) the cloud-point titration, and (*iii*) the analytic determination of the nodes (end points) of the systems. All these methods can be used for the determination of the binodal curve, but the most common are titration (turbidimetric and cloud-point) methods. The choice of a specific method should consider the type and nature of the phase-forming agents. For example, the determination of the solubility curves for a salt/salt ATPS can be easily carried out by cloud-point titration [3], but if some polydisperse polymers are used as phase-forming agents, in polymer/polymer or polymer/salt ATPS, a gradual decrease/increase in turbidity may occur, affecting the accuracy of the titration-based methods [27]. Although these methods are still the most applied, they are tedious and lengthy, and consume large amounts of reagents [13]. High-throughput screening alternatives have been proposed, using microfluidic devices [28] or 96-well microplate titration-based approaches [29].

The most common approach used for the description of the binodal curves is to fit them using empirical equations, particularly, the three-parameter equation (Eq. 5.4) proposed by Merchuk et al. [30]:

$$Y = A\exp\left(BX^{0.5} - CX^3\right) \tag{5.4}$$

where Y and X are the weight fractions (wt%) of components 1 and 2, respectively, and A, B, and C are adjusted parameters obtained by least-squares regression.

Merchuk's equation, initially proposed to describe polymer/salt ATPS, was successfully applied to polymer/polymer [31], salt/salt [32], and ionic liquid/carbohydrate-based ATPS [33]. Other alternative empirical equations have been proposed [23, 24], but as recently assessed by Alvarez et al. [34], Merchuck's equation still remains the best equation to fit the binodal curve.

Regarding the determination of TLs, the gravimetric method (also proposed by Merchuk et al. [30]) coupling the fitted binodal data by Eq. (5.4) and a mass balance relationship is typically used. The compositions of the coexisting phases (top and bottom) are determined mathematically by the solution of the following system of Eqs. (5.5)–(5.8):

$$Y_T = A\exp\left(BX^{0.5} - CX_T{}^3\right) \tag{5.5}$$

$$Y_B = A\exp\left(BX^{0.5} - CX_B{}^3\right) \tag{5.6}$$

$$Y_T = \frac{Y_M}{\alpha} - \frac{1-\alpha}{\alpha}Y_B \tag{5.7}$$

$$X_T = \frac{X_M}{\alpha} - \frac{1-\alpha}{\alpha}X_B \tag{5.8}$$

where Y and X are the weight fractions (wt%) of components 1 and 2, respectively, and A, B, and C are adjusted parameters obtained by least-squares regression; subscripts T, B, and M correspond to top phase, bottom phase, and the mixture, respectively; α is the ratio between the mass of the top phase and the total mass of the mixture. Despite the popularity of this approach to determine the TLs of different types of ATPS, the analytic method is still the most accurate to determine the exact composition of each component of the coexisting phases.

5.2.2 Physicochemical Properties of the Phases and Kinetics of Separation

The molecular mechanisms behind the phase separation and partition of solutes are governed by the thermodynamic equilibrium of the system, according to the type and chemical nature of the phase-forming components and characteristics of the target solute. An overview of these mechanisms is provided in the succeeding text. Nevertheless, before considering thermodynamics, it is important to review the kinetics of the phase separation and their relationship to the physicochemical properties of the

coexisting phases. Asenjo and coworkers [18, 35] stated that three main forces (gravitational, flotation, and frictional) are acting on a drop during coalescence, with the drop movement a result of their balance. Interestingly, each force depends on specific properties of the phases in equilibrium, namely, (*i*) gravitational force, dependent on the density of the drops, and (*ii*) flotation or frictional forces—dependent on the rheological properties of the phases. Therefore, the balance of these forces, along with the interfacial tension, will control the drop coalescence. Considering that in most ATPS the phase densities are similar, the behavior of the drops is mainly controlled by the rheological differences of the coexisting phases [35]. The viscosity plays a key role in the phase separation process because it determines the settling time of the phases after the mixing [35], as well as the fluid dynamics in continuous processing [18, 35] or the formation of micropatterned droplets [36]. For a fast separation, salt/salt ATPS should be used instead of polymer-based systems. However, by decreasing the polymer molecular weight or by increasing the temperature, the settling time and phase dynamics can be adjusted. Similarly, density differences can also affect the rate of sedimentation and, consequently, the kinetics of phase separation [35]. It is important to note that by changing the phase densities, it is possible to reverse the top (light) and bottom (heavy) layers. Therefore, the modulation of the individual phases, by changing the concentration of polymers or surfactants or simply by adding additives (such as salts, ionic liquids, and cosolutes) [13], can be a good approach to optimize the phase separation kinetics and consequently to tailor an ATPS to a specific application. ATPS have a very low interfacial tension, when compared with traditional LLE systems, with the smallest interfacial tension values obtained for formulations close to the CP. Like the other two properties, interfacial tension can also be adjusted [13]. The choice of ATPS should thus consider the fluid viscosity, density, and interfacial tension. Depending on the application, other properties such as osmolarity and hydrophobicity should also be considered prior to implementation [13].

5.3 Types of Aqueous Two-Phase Systems

The most common ATPS are generated by mixing a pair of hydrophilic polymers (polymer/polymer) or a polymer and a salt (polymer/salt). They have been explored since the 1980s [1, 8, 11]. A significant advance occurred in 2003, when Rogers and collaborators [37] generated an ATPS by the codissolution of two salts in water (salt/salt-based ATPS), one of the salts having one or both ions of high charge density (water-ion interactions stronger than water-water interactions), while the other salts are based on low-charge density ions (water-ion interactions weaker than water-water interactions). The delocalization of charge in the salt ions induces a lower melting temperature of the salt itself, these being categorized as ionic liquids (ILs) [37]. Therefore, in the last two decades, many studies have focused on the

development of new ILs-based ATPS, combining ILs with inorganic and organic salts, sugars, carbohydrates, polymers, alcohol, or other compounds. In this section, considering the large number of possible ATPS, only three types of ATPS are discussed, namely, *5.3.1 Polymer/Polymer*, *5.3.2 Polymer/Salt*, and *5.3.3 Salt/Salt*. Other ATPS types are briefly highlighted in the Section 5.3.4. In the Section 5.3.5, the influences of temperature and pH on the phase behavior of traditional ATPS are compared.

5.3.1 Polymer/polymer ATPS

Polymer/polymer ATPS [1] are formed when pairs of water-soluble polymers are mixed above a critical concentration inducing the formation of two phases. Since the late 1950s, several polymer/polymer systems have been characterized for different purposes [1, 8, 21]. During approximately 60 years, several combinations of hydrophilic polymers were successfully employed for the formation of two-phase systems, obtained from the mixing of: (*a*) two nonionic polymers, such as the well-known PEG/dextran-based ATPS; (*b*) one nonionic and an ionic polymer, for example, PEG/poly(acrylic acid) (PAA) and PEG/dextran-based ATPS; and (*c*) two charged polyelectrolytes, namely, sodium dextran sulfate/polystyrene sulfonate (PSS). Fig. 5.2 summarizes representative examples of polymer/polymer ATPS reported to date [1, 26, 27, 38–43].

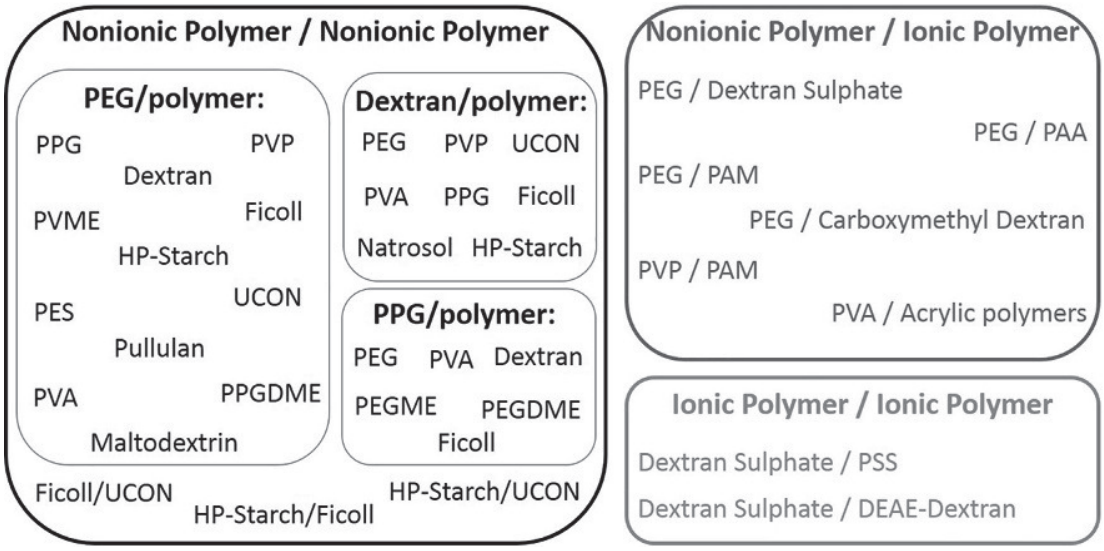

Fig. 5.2 Representative examples of polymer/polymer ATPS. (*Acronyms*: PEG, polyethylene glycol; PEGME, polyethylene glycol methyl ether; PEGDME, polyethylene glycol dimethyl ether; PPG, polypropylene glycol; PPGDME, polypropylene glycol dimethyl ether; PVP, polyvinyl pyrrolidone; PVA, polyvinyl alcohol; PES, polyether sulfones; HP, starch, hydroxypropyl starch; PAA, polyacrylic acid; PAM, polyacrylamide; PSS, polystyrene sulfonate).

Phase separation in solutions containing polymer mixtures is a common phenomenon, that is, most hydrophilic polymer pairs are "incompatible" in aqueous solutions [8]. Thermodynamically, polymer/polymer ATPS phase separation is described according to two points of view: the theory of polymer mixtures described by Flory-Huggins [44] on the basis of the energetically unfavorable segment interactions of polymers overcoming the entropy increase involved in phase separation [45, 46]; or the structure of water as a key factor of phase separation, that is, ordered polymeric water structures, supported by Zaslavsky [47]. To this day the phase formation in polymer/polymer ATPS is incompletely understood [40], and as recently highlighted by Sadeghi and Maali [43], only a few experimental and theoretical attempts have been made to fully understand the polymer/polymer phase separation mechanism.

These phase-forming mechanisms apply to nonionic-based ATPS, which, as shown in Table 5.1, are the most representative class of polymer/polymer ATPS. However, other systems can also be formed by mixing one polyelectrolyte and a nonionic polymer [39, 41, 59], two polyelectrolytes [60], or even using electrolytes as adjuvants [41, 46, 61], but their phase-forming mechanisms are far more complex. Herein, we do not detail these systems, but a comprehensive view of their phase separation mechanisms can be found in Picullel [45], Pfennig [61], Gupta [39], and Johansson [41, 62]. It is important to note that the phase behavior of polymer/polymer ATPS is strongly influenced by the presence of salts (or electrolytes). The addition of small concentrations of salt can significantly enhance the biphasic region or may allow the partitioning mechanisms of target solutes to be adjusted.

The next point to discuss is the effects that can influence the phase diagram, such as polymer concentration, molecular weight, the presence of additives, temperature, and pH. Since the last two factors also influence other types of ATPS, these will be discussed together in Section 5.3.5. Regarding the effect of polymer concentration, as highlighted in the description of the phase diagram, at low concentrations, both polymers are fully miscible in water, and no phase separation occurs. With increasing polymer concentration, a phase demixing will occur [1, 11]. It should be highlighted that depending on the nature of the polymer or its molecular weight, the effect on phase separation can be intensified or reduced affecting the "phase diagram symmetry." For example, in PEG/dextran ATPS, the increase of polymer molecular weight enhances their ATPS phase-forming ability, that is, low polymer concentrations are required for the formation of a two-phase region, as well as increasing the asymmetry between the phases [1, 11].

5.3.2 Polymer/salt ATPS

Polymer/salt ATPS, the second type of ATPS discovered in the mid-1950s [1], are formed by the dissolution of a water-soluble polymer and inorganic (or organic) salt

above critical concentrations [1, 27], promoting a salt-rich, polymer-poor bottom phase and a polymer-rich, salt-poor top phase [1, 27]. As reviewed by Hatti-Kaul [8], Grilo et al. [7], and Ruiz-Ruiz et al. [10], in the last decades, polymer/salt systems have been widely studied, carefully characterized, and applied to many separations. A wide array of polymers and salts can be combined for the formation of ATPS. As shown in Fig. 5.3, PEG and PPG polymers are the most used polymeric phase-forming agents [1, 38, 63, 64], while the inorganic phosphate, sulfate-based salts are the most common ionic components [1, 38, 63, 64]. However, ATPS composed of polymer and hydroxide [63, 64], nitrate [65], and carbonate salts [63, 64] can be produced; chloride salts [66] can also form ATPS if combined with a more hydrophobic PPG polymer. Noteworthy, more recently, a large number of ATPS used organic salts as phase-forming components, for example, citrate, tartrate, acetate, and formate salts [67–69], as more eco-friendly alternatives. Alternatively, it is possible to find less common polymer/salt systems, using polymers such polyethylene glycol dimethyl ether (PEGDME) [70], polyalkylene glycols (UCON) [71], or polyoxyethylene (20) cetyl ether (POELE) [72].

Regarding the demixing mechanisms of polymer/salt ATPS, in general, the effectiveness of different salts in promoting phase separation follows the Hofmeister series [73], where salt ions (preferentially the anion contribution) are ranked according to their salting-out ability [74, 75]. This sequence is the empirical ordering of salts according to the minimum concentration required for protein precipitation from an aqueous solution, which is, in general, the following:

$$\textit{Anions} \text{ —} \; CO_3^{2-} > SO_4^{2-} > S_2O_3^{2-} > H_2PO_4^{-} > F^{-} > Cl^{-} > Br^{-} \approx NO_3^{-} > I^{-} > ClO_4^{-} > SCN^{-}.$$

$$\textit{Cations} \text{ —} \; (CH_3)_4N^{+} > Cs^{+} > Rb^{+} > NH_4^{+} > K^{+} > Na^{+} > Li^{+} > Mg^{2+} > Ca^{2+}.$$

Multivalent anions and highly charged ions (i.e., with large negative Gibbs free energies of hydration), such as HPO_4^{2-} and SO_4^{2-}, are highly effective at forming

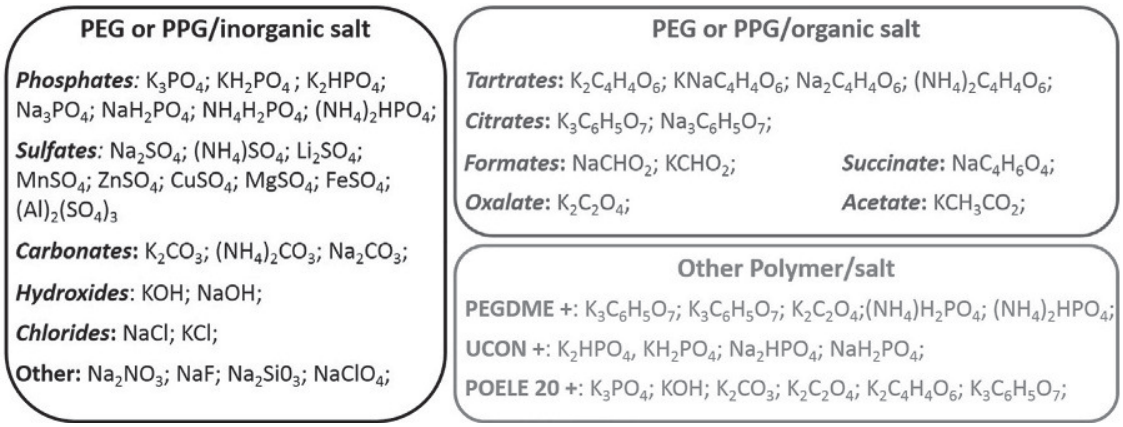

Fig. 5.3 Representative examples of polymer/salt ATPS.

polymer/salt ATPS, while, for example, chloride and bromide univalent salts are not as effective [73].

Although some details of the mechanisms are not fully understood, it seems that the phase separation results from the competition for hydration between the polymer and the salt [40]. For example, triply charged anions are strongly hydrated, being more effective in polymer salting out than the doubly charged sulfate and the monovalent hydroxide anions, that is, they are better ATPS-forming agents [73]. In the case of cations, the effect is more complex and, in some cases, results from a competition between two opposite effects, hydration and cation-polymer specific interaction (e.g., interaction between lithium and EO groups of PEO polymers) [73]. Anyway, Ananthapadmanabhan and Goddard [73] demonstrated that in most cases the fundamental forces behind the formation of polymer/salt ATPS are the salting-out aptitude of each salt and the partial dehydration of the polymer [73]. Further studies validated these earlier speculations for a wide range of salts and polymers [63, 65, 68, 69, 71, 72], even correlating the ions' salting-out aptitude with their Gibbs free energies of hydration (ΔG_{hyd}) [69, 71, 72], that is, the more negative ions' ΔG_{hyd} is, the stronger is its salting-out aptitude [76].

As discussed in the previous paragraphs, the phase separation of polymer/salt is both dependent on the type and molecular weight of the polymer and salting-out ability of the salt (i.e., position in the Hofmeister series ranking), but as for other types of ATPS, they are strongly influenced by temperature, pH (as a result of ion speciation), or even the presence of additives (as discussed later in Section 5.3.5).

5.3.3 Salt/salt ATPS

As highlighted in the previous sections, polymer/polymer and polymer/salt ATPS were for long the most studied and applied. However, due to the limited ability to manipulate the difference in polarities between the coexisting phases, their application was limited, often exhibiting narrow extraction and purification capabilities. ATPS composed of two polymers have similar phase polarities, while polymer/salt ATPS display distinctly different characteristics between the coexisting phases [3, 4]. To overcome these limitations, several alternatives were proposed, such as polymer derivatization or the use of additives [9, 10, 61].

A disruptive innovation on the ATPS field occurred in 2003, when Rogers and coworkers [37] demonstrated that a hydrophilic ionic liquid can be salted-out and concentrated from aqueous solution by inorganic salts forming an ATPS. The formation of this type of salt/salt ATPS, generally known as ionic liquid/salt ATPS, results from the codissolution of two salts, one with one or two highly charged ions (water-ion interactions stronger than water-water interactions) and other salt with low-charge density ions (water-ion interactions stronger than water-water interactions)

[3, 77]. The first salt can be any organic or inorganic salt (with mainly high-charge density ions dominated by coulomb interactions) with "salting-out" nature, while the second, due to low-symmetry and charge-delocalized ions, fits within a "particular" category defined as ionic liquids (ILs). By definition, ILs are "salts with melting temperatures below 100°C" [78]. However, the definition of ILs based on their melting temperature restricts the window for salts that can fit within ILs category [79]. Therefore, considering that salts with melting temperatures above 100°C also form salt/salt ATPS [80] and to avoid a distinction between IL/salt and salt/salt ATPS, this categorization was adopted in this chapter.

Due to the tunability of ILs (covering the whole hydrophilicity-hydrophobicity range [3]), the study of salt/salt ATPS has grown exponentially. In particular, after 2009 a large number of phase diagrams were characterized and extensively applied as potential separation platforms for a wide range of compounds [3, 4]. A detailed description of ILs-based ATPS can be found in the book by Freire [4]. In the next paragraphs, the major concepts behind the formation of the salt/salt ATPS with representative examples (Fig. 5.4), are discussed.

Imidazolium-based ILs (with halogens, sulfates, sulfonates, alkanoates, tetrafluoroborate, and triflate anions) are the most studied as the first phase-forming component, while high-charge density inorganic salts, such as phosphates, sulfates, and carbonates, are used as the second component of polymer/polymer ATPS [3, 4]. However, due to environmental concerns, some ILs-based ATPS have been proposed, in which inorganic salts have been replaced by more benign organic salts (citrates, tartrates, etc.) or even by other species such as amino acids, carbohydrates, and polymers (alternative ATPS discussed in the next subsection). Independently of the salt/salt ATPS, the use of two salts as phase-forming agents with low phase viscosities allows a quick phase splitting, overcoming an important drawback of the polymer-based ATPS [3].

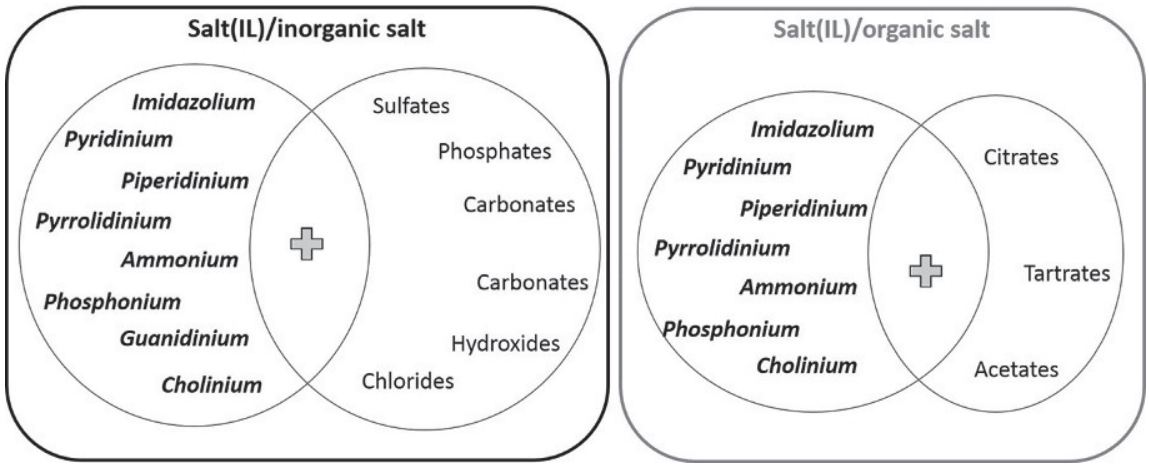

Fig. 5.4 Representative examples of salt/salt ATPS.

In these salt/salt systems, since both phase-forming components are ionic, a distribution of the constituent ions between the coexisting phases occurs but is always restricted by the electroneutrality of the overall system and individual phases [81]. Bridges et al. [81] have shown that, besides maintaining electroneutrality, the most chaotropic ion is preferentially concentrated into the top phase, while the most salting-out ion is partitioned to the bottom phase, with negligible deviation in the speciation of the ions along any given tie-line; thus each salt/salt phase diagram can be determined and interpreted on whole salt concentrations rather than ion concentration yields [81].

Regarding the phase separation mechanisms, the formation of salt/salt ATPS and its efficiency of separation are associated with the Gibbs' free energy of hydration (ΔG_{hyd}) of the most "hydrophobic" salt, namely, K_3PO_4, which due to its very negative ΔG_{hyd} is preferentially solvated [37], "salting out" the ILs from aqueous solution. The ΔG_{hyd} trend was demonstrated by the efficiency of separation in ATPS composed of 1-butyl-3-methylimidazolium chloride ([C_4mim]Cl) with a series of inorganic salts (KOH, K_2CO_3, Na_2HPO_4, and $Na_2S_2O_3$) [37]. Similarly to the previous polymer/salt ATPS, the ability of the low ΔG_{hyd} salts to salt out different hydrophilic salts ([C_4mim]Cl, [C_4mmim]Cl, [C_4py]Cl, [N_{4444}]Cl, and [P_{4444}]Cl) can also be ranked according the Hofmeister series [81]. These first insights were further confirmed by Coutinho [32, 82–84] and Zafarani-Moattar [53, 85] research groups, which demonstrated that the cornerstone of the salt/salt ATPS formation is the salting-out effect [3], due to the creation of water-ion complexes, in opposition to the dominant ion-ion interactions in the salting-in inducing ions [77]. Therefore, as stated by Freire et al. [3], the addition of high-charge density salts (salting-out inducing salts) to aqueous solutions of salts containing low-symmetry charge-delocalized ions (ILs) leads to the preferential hydration of the first over the second and consequently to the salting out of the IL to the opposite (upper) phase. Shariari et al. [32] performed an extensive study of the molecular mechanisms for the effect of salt ions in the formation of salt/salt ATPS. Different ATPS containing 1-butyl-3-methylimidazolium triflate ([C_4mim][CF_3SO_3]) and aqueous solutions of conventional salts were evaluated, illustrating the influence of both the cation and anion of the salt on phase demixing [32]. The large set of data showed that both the cations' and anions' ability to induce the salting-out phenomenon follows the well-known Hofmeister series. Furthermore, they also found a close correlation between the IL molality required for ATPS formation and the ions' molar entropy of hydration, concluding that the creation of ion-water complexes plays a key role in the formation of salt/salt ATPS [32].

In addition to the nature of the salts, both temperature and pH significantly influence phase demixing for salt/salt ATPS. In Section 5.3.5 the general effects of both parameters are summarized. A more detailed discussion can be found in Refs. [3, 4].

5.3.4 Other types of ATPS

In addition to the well-studied polymer/polymer, polymer/salt, and salt/salt ATPS, several other ATPS formed through the mixture of a wide range of compounds have been studied, for example: (*i*) aqueous micellar two-phase systems (AMTPS), micellar and reverse micellar, using surfactants (anionic, cationic, zwitterionic, or nonionic) [14, 16, 20, 86–88]; (*ii*) IL-based ATPS with polymers [89–92], carbohydrates [33, 93, 94], amino acids [95], and others [3, 4]; (*iii*) carbohydrate-based ATPS [33, 93, 94, 96, 97]; (*iv*) copolymer-based ATPS [98–100]; (*v*) ATPS composed of deep eutectic solvents [101–103]; and (*vi*) ATPS composed of hydrophilic organic solvents, mainly short-chain alcohols [104–106]. While interesting, these systems are also more complex.

Among these "alternative" systems the most studied are AMTPS, which were introduced by Watanabe and Tanaka in 1978 for the extraction of zinc (II) [88]. These systems are formed by mixing surfactants (anionic, cationic, zwitterionic, or nonionic), that is, amphiphilic molecules with a hydrophilic and a hydrophobic core, and water, which can form aggregates known as micelles above a certain concentration (critical micellar concentration, CMC) [87]. A homogeneous micellar isotropic surfactant aqueous solution, when subjected to certain conditions, particularly temperature, can form spontaneously a micelle-rich (concentrated) phase and a micelle-poor (diluted) phase. Micelles start to interact preferentially with each other, resulting in a phase separation at a specific temperature (known as cloud-point temperature) [86]. The formation of AMTPS depends mainly on surfactant structure, charge, and concentration. In the presence of additives (e.g., inorganic and organic salts, biopolymers, alcohols, and ionic liquids), the phase demixing aptitude can be enhanced or reduced [86, 87].

A new type of ATPS was created by mixing polymers with ILs [89, 90]. While initially it was presumed that this polymer/IL systems would be simply another series of polymer/salt ATPS [90], it was soon realized that the replacement of high-charge density salts by more amenable ILs increased the complexity and changed the nature of interactions at the molecular level [89, 91, 92], enlarging the phases' polarity range, increasing the solute specific interactions, and reducing the crystallization problem [4]. Freire et al. [90] formed an ATPS composed of PEG polymers (different molecular weight) and a series of ILs (from imidazolium, piperidinium, pyridinium, pyrrolidinium, and phosphonium families). Thence, many ATPS combining ILs and different PEG- and PPG-based polymers have been used for several purposes [3, 4, 90, 92]. The formation of these polymer/IL ATPS not only is dependent on the IL ions' ability to form hydration complexes or the formation of polymer-water structure complexes but also results from a delicate balance between competing interactions that occur in IL-polymer solute pairs, where the larger the immiscibility between IL and the polymer, the greater is the phase separation [92]. Therefore, depending on the polymer type and molecular weight, nature of the ILs (e.g., the

ion chain length), and temperature, opposite phase demixing trends can occur as a result of the balance between complex interactions occurring between all the phase-forming agents [91, 92].

5.3.5 Effect of temperature and pH

ATPS phase diagrams can be influenced by many parameters, including the type and concentration of phase-forming components, temperature, pH, and the presence of additives or contaminants. In previous sections, the influence of phase-forming components on the formation of ATPS was discussed. However, both pH and temperature exhibit a strong influence on the phase separation mechanisms. A comparison of the main ATPS and their response to temperature and pH changes is presented in Table 5.1.

As the temperature increases, the binodal curves of the polymer/polymer [11, 48, 49] and salt/salt ATPS [3, 53, 54] shift to higher concentrations of phase-forming agents (away from the origin) corresponding to a decrease in the biphasic region. This shift with temperature is characteristic of an upper critical solution temperature (UCST) behavior. On the other hand, polymer/salt ATPS resemble a lower critical solution temperature (LCST) behavior in which an increase in temperature leads to an enlargement of the biphasic region, that is, less polymer and/or salt is required for the phase separation [49, 50, 107].

TABLE 5.1 Influence of pH and Temperature in the Phase Behavior of the Main ATPS Types

Temperature effect			
Increase (↑) of the temperature	Polymer-polymer ATPS [11, 48, 49]	Polymer-salt ATPS [50–52]	Salt-salt ATPS [3, 53, 54]
Biphasic region (binodal curve)	Decrease (↓) (shift to higher polymer concentrations)	Increase (↑) (shift to smaller polymer/salt concentrations)	Decrease (↓) (shift to higher salt concentrations)
Type of critical solution temperature behavior	UCST	LCST	UCST
pH effect			
Increase (↑) of the pH	Polymer-polymer ATPS [55, 56]	Polymer-salt ATPS [50–52]	Salt-salt ATPS [4, 57, 58]
Biphasic region (binodal curve)	Decrease (↓) (shift to higher polymer concentrations)	Increase (↑) (shift to lower polymer/salt concentrations)	Increase (↑) (shift to lower salt concentrations)

Several reports detail the effect of pH on phase separation of polymer/salt [101–103] and salt/salt [4, 57, 58] ATPS, but the effect of pH on the phase diagrams for polymer/polymer ATPS has been rarely studied. An exception is the study of Planas et al. [55], where different phase diagrams for systems containing EOPO-PEI titrated with lactic acid and DEX at different pH values (from 2.0 to 6.0) are described. The biphasic region increases with a decrease in pH, but the polymer concentrations in the two coexisting phases are not affected (constant slope of the TLs over the whole pH range) [55]. Yan and Cao also demonstrated that the binodal curve for two pH-responsive polymers move close to the origin (a high biphasic region) with a decrease of pH [56]. On the other hand, for both polymer/salt [50–52] and salt/salt [4, 57, 58], phase diagrams at increasing pH resulted in enlargement of the two-phase region, that is, less phase-forming components (polymers or salts) are required for the formation of the ATPS.

5.4 Applications of Aqueous Two-Phase Systems

ATPS have been utilized for the extraction, separation, and purification of a plethora of solutes. While some studies are focused on the understanding of the partition of molecules and particulates in these systems, most address their application to the extraction, concentration, and purification of products, both as downstream processing platforms or as an analytic tool. Over the last decade, approximately 100 ATPS application-based articles were published each year [7]. These were mostly focused on the separation/purification of enzymes, followed by purification of DNA, nucleic acids, monoclonal antibodies, and antibiotics, while several also addressed the extraction of metals [108]. The number of articles using ATPS as an analytic/characterization technique is also growing. In this section, some of these applications are summarized.

5.4.1 Partition of Molecules and Particulates

ATPS are regarded as useful tools for the extraction and purification of biocompounds [1, 11, 21]. Extraction by ATPS involves the transfer of a target solute from one aqueous phase to another with a partition coefficient (K), defined as the distribution ratio of the solute between the coexisting phases ($K = C_T/C_B$). Albertsson [1] proposed a model to correlate the K for a specific biomolecule, based on the different driving forces: (*i*) size-dependent (solutes are separated according to their size and surface area); (*ii*) electrochemical (solutes are separated according to their charge and the electric potential of the coexisting phases); (*iii*) hydrophobicity (solutes are separated according to hydrophobic interactions between molecules and the relative hydrophobicity of the phases); (*iv*) biospecific affinity (one of the phase-forming polymers has

specific binding sites for the target molecule); and (*v*) conformation-dependent (the conformation of the solute is the key factor for partitioning). This model, initially proposed for protein partitioning [1], can be expressed as Eq. (5.5):

$$\ln(K) = \ln(K_0) + \ln(K_{size}) + \ln(K_{elec}) + \ln\left(K_{hfob}\right) + \ln\left(K_{biosp}\right) + \ln\left(K_{conf}\right)$$

where the subscripts *size*, *elec*, *hfob*, *biosp*, and *conf* refer to the electrochemical, hydrophobic, size, biospecific, and conformational contributions to K from both protein structural properties and the surrounding environmental conditions of the system. K_0 includes all other factors. As discussed in Refs. [1, 7, 11, 21], the K of a biomolecule is the result of the protein structure-related factors (such as charge, hydrophobicity, and/or surface properties) and the surrounding environmental conditions, as: (1) salt type and concentration; (2) pH; (3) phase-forming polymer type, molecular weight, and concentration; (4) the presence of polymer derivatives (charged, hydrophobic, or affinity types); (5) temperature; and (6) salt additives.

Although several theoretical and experimental studies provide some support for the key factors for biomolecule partition [109–111], these are still too complex and poorly understood. As discussed by Grilo et al. [7], depending of the type of biomolecule and ATPS, different theories are used to explain the experimental results. While some authors suggest that hydrophobicity controls the partition of biomolecules [109, 110], others suggest that electrostatic interactions play the major role [111]. Grilo et al. [7] suggested that the partitioning "should be regarded as a synergetic effect of all these different mechanisms." Thus, depending on the characteristics of the biomolecule and properties of ATPS, each mechanism will have a higher or lower influence on the partition. Since most ATPS partition mechanisms are quite complex and unpredictable, most experimental work optimized the partition according to the product properties and operation conditions, among others. In fact, the factors influencing the partition behavior of solutes in ATPS are (*i*) the polymer molecular weight and concentration, (*ii*) salt (or ionic liquid) type and concentration, (*iii*) relative hydrophobicity, (*iv*) pH and charges, (*v*) temperature, (*vi*) density and viscosity, (*vii*) interfacial tension, (*viii*) settling time, and (*iv*) solute size and concentration. The influence of each factor on partitioning for different ATPS is discussed in earlier reviews [3, 5–22, 26] and books [1, 2, 4, 27, 38].

5.4.2 Extraction and purification of products

In spite of the ATPS partition mechanisms not being fully understood, these systems have been widely used as effective downstream platforms for the recovery and purification of products ranging from biocompounds to metals. They are mainly used to replace traditional organic solvent liquid-liquid extraction procedures [5, 7, 21, 22],

but due to their low interfacial tension, they can be applied as well to stabilize fragile biological structures [1, 13]. A number of reviews provide additional details [3, 5–22, 25]. Some representative applications are presented in the succeeding text.

As expected, most ATPS are used for the extraction of bioproducts, ranging from small and simple biomolecules, such as amino acids, antibiotics, peptides, alkaloids, carotenoids, or biocolorants, to complex compounds such as proteins, enzymes, monoclonal antibodies, virus, virus-like particles (VLPs), cells and organelles, and DNA and nucleic acids [3, 5–22]. Among these, protein-related applications are the most widely studied, with several reviews focused on this topic [9, 16, 18, 21]. In particular, ATPS have been used extensively for the recovery of protein-based biopharmaceuticals (e.g., monoclonal antibodies, growth factors, and therapeutic enzymes) from complex cell cultures (like microbial, animal, and vegetal) [5, 7, 8, 12, 22, 25]. However, as suggested by Gonzalez-Valdez et al. [12], ATPS have a great potential for the selective fractionation, recovery, and purification of low-molecular weight solutes, other types of macromolecules, organelles, and even whole cells. In certain cases, due to the target-bioproduct degradation or cell culture inhibition, in situ approaches are used, where the extraction and production steps are integrated in a single unit, recovering the product during the bioconversion in a concept known as *extractive fermentation* or *extractive bioconversion* [5, 8, 11]. Alternatively, ATPS have been realized as a powerful tool for the selective extraction of metal ions [108].

5.4.3 Analytical applications

ATPS are becoming more widely used for analytic applications [4, 5, 7, 10, 13], for example, the use of ATPS for the concentration of residual drugs and pollutants in water [112–114] and food [113, 114] in which the ATPS allowed high concentration factors enhancing detection limits. The analytic aptitude of polymer/salt ATPS was also extended to label-free cell technologies, being used to differentiate promyelocytic cell line HL-60 through a high-throughput cell partitioning analysis [115]. Benavides et al. [10] highlighted the possibility of using ATPS for the molecular characterization of proteins. Since the protein partition is a surface-dependent phenomenon, where the exposed residues interact with the phase-forming agents, it can be used to discriminate between similar proteins based on their molecular properties, such as molecular size or relative hydrophobicity. Zaslavsky et al. [26] reviewed advances in analytic application of solute partitioning in ATPS, emphasizing systems that explore protein structural changes and protein-partner interaction (in vitro and in vivo). This solvent interaction analysis method allows the analysis and characterization of individual proteins in solution, detecting small changes, such as single-point mutations, chemical modifications, posttranslational modifications, aggregation, and protein misfolding, among other conformational and interactions

changes. Considering that clinical proteomics and disease biomarkers are often protein-related, ATPS seems to be a convenient technique for automated discovery and monitoring of structure-based protein biomarkers in biological fluids and subsequent use in clinical disease diagnostics.

5.4.4 Emerging and non-conventional applications

Recent advances in the biotechnology and materials science fields have enlarged their use to alternative applications, such as micropatterning and bioprinting, high-throughput 3-D tissue assembly, microcapsule production, synthetic biology, and microscale biomolecular assay development. Teixeira et al. [13] provide an overview of emerging and nonconventional biotechnology applications of ATPS, anticipating five major areas for future growth, namely, (1) application-focused ATPS polymer design, (2) *stimuli*-responsive systems, (3) scaling-industrial separation reactions, (4) therapeutic microencapsulation and drug delivery, and (5) artificial cells and synthetic biology. We also believe that these new applications will change the focus of ATPS and its communities, spreading their use beyond current applications.

5.5 Scaling-Up and Continuous Processing

The use of ATPS as a large-scale separation process or as a bioengineering tool is yet to become a widespread "reality." Albeit new emerging biotechnological uses can be performed on small scale using microdevices, the downstream processing of biological products at industrial scale is fully dependent on a proper scale-up or integration in continuous processing platforms. From the beginning the attractiveness of ATPS-based separations to industry was associated with the simplicity of scaling, equipment, and facility demands [11, 25]. Despite these advantages, the large-scale applications of ATPS are limited in number [6, 8]. Considering the use of ATPS for large-scale downstream processing, it may be divided into two categories: (*i*) batch or continuous mode by using single-stage or multistage mixing/settling units; and (*ii*) continuous mode by using countercurrent distribution, liquid-liquid partition chromatography, or continuous countercurrent chromatography (CCC). The first category has been more extensively studied, mainly in the early stages of purification (low resolution); the second category, originally designed for aqueous-organic- and organic-organic two-phase systems (as discussed in another chapter in this book), is less common for ATPS-based processes but exhibits a strong potential for use as a high-resolution bioproducts' purification platform. Rosa et al. [25] have reviewed some case studies of batch scale-up of ATPS for the manufacture of

biopharmaceuticals (viz., interleukin, human growth hormones, and monoclonal antibodies), highlighting their advantages and drawbacks. Particularly, the authors concluded that three main questions still need to be addressed to introduce these platforms in biopharmaceutical production processes: their maximum capacity, limited predictive design, and economic and environmental sustainability (compared with established chromatographic platforms) [25]. It is important to note that recycling, back extraction, and multistage procedures can reduce some of the process costs and increase the sustainable character of ATPS [6].

Albeit that the number of studies focused on continuous ATPS are scarce, these appear as the most promising for industrial purification processes. Depending on the target product, continuous processing has clear advantages in comparison with the batch mode, namely, low processing times, high productivity and purification yields, and lower cost. Espitia-Saloma et al. [19] presented a comparative analysis of different ATPS-based techniques in continuous processing, from the most common approaches using conventional column contactors to novel mixer-settler processing units. In summary, although column contactors are more studied, due to their lack of versatility and limitations related to mass transfer and separation performance, the interest in mixer-settler devices is increasing [19]. Anyway, similar to batch-mode ATPS, an effective industrial implementation of continuous systems is yet dependent on the definition of parameters such as phase recycling, feasible predictive models, practical guidelines for design and scale-up, control, and automation [19].

5.6 Final Remarks and Future Perspectives

This chapter addresses the theoretical and practical aspects of the use of ATPS. These systems were proposed by the middle of the 20th century and have since been regarded as powerful alternatives to conventional liquid-liquid extraction systems for the isolation of bioproducts. They share a series of key advantages, such as versatility, biocompatibility, low cost, and outstanding purification performances for a range of molecules and products. However, as herein summarized, they still lack a significant application at an industrial scale.

The most studied classes of ATPS are formed by mixing a large range of polymers and salts, under different process conditions (pH, temperature, and additives). Nevertheless, other types of systems were also proposed and characterized, by using ILs, carbohydrates, amino acids, or surfactants. These combinations, using benign, renewable, and biodegradable phase-forming components (such as sugars, short-alkyl chain alcohols, ionic liquids, and amino acids), have been raising the "greenness" of ATPS, but regarding a future and sustainable application at industrial scale, it is crucial to develop novel and economical approaches for the full recovery and reuse of these components.

Several theoretical and experimental studies have tried to reveal and develop models and thermodynamic relationships to characterize the phase separation mechanisms and solute partitioning. However, their widespread use has yet been limited because of their complexity and the number of possible combinations. Furthermore, additional efforts are required to fully reveal the nature of these systems and thus to allow the development of phase separation predictive models. Similarly, considering the plethora of solutes that can be partitioned/separated with these platforms and envisaging the implementation at large scale, establishing reliable models to predict solute partitioning is of upmost importance.

Finally, considering the excellent extractive performances, integrability, biocompatibility, and sustainable characteristics of many ATPS, we believe that these can be commercially applied for the purification of several products, particularly, complex bio-based materials (such as VLPs, membrane proteins, and DNA fragments) and metal-based products (recycling and urban-mining procedures). Nevertheless, their alternative uses as (bio)analytic and nonconventional biotechnological approaches are nowadays very promising and may lead to alternative industrial applications.

Acknowledgments

This work was developed within the scope of the projects: FAPESP (São Paulo Research Foundation, Brazil) 2014/19793-3, cofunded by FAPESP and FCT (Portuguese Foundation for Science and Technology, Portugal); FAPESP 2014/16424-7; CICECO-Aveiro Institute of Materials, POCI-01-0145-FEDER-007679 (FCT Ref. UID/CTM/50011/2013); and MultiBiorefinery (POCI-01- 0145-FEDER-016403), financed by national funds through the FCT/MEC and when appropriate cofinanced by FEDER under the PT2020 Partnership Agreement.

References

[1] Albertsson P-A. Partition of cell particles and macromolecules. 3rd ed. New York: Wiley; 1986.
[2] Benavides J, Rito-Palomares M, Asenjo JA. Aqueous two-phase systems. In: Comprehensive biotechnology. 2nd ed. Elsevier; 2011. p. 697–713.
[3] Freire MG, Cláudio AFM, Araújo JMM, Coutinho JAP, Marrucho IM, Lopes JNC, et al. Aqueous biphasic systems: a boost brought about by using ionic liquids. Chem Soc Rev 2012;41:4966–95.
[4] Freire MG. Ionic-liquid-based aqueous biphasic systems: fundamentals and applications. Berlin Heidelberg: Springer; 2016.
[5] Iqbal M, Tao Y, Xie S, Zhu Y, Chen D, Wang X, et al. Aqueous two-phase system (ATPS): an overview and advances in its applications. Biol Proced Online 2016;18:1–18.
[6] Soares RRG, Azevedo AM, Van Alstine JM, Aires-Barros MR. Partitioning in aqueous two-phase systems: analysis of strengths, weaknesses, opportunities and threats. Biotechnol J 2015;10 (8):1158–69.
[7] Grilo AL, Aires-Barros MR, Azevedo AM. Partitioning in aqueous two-phase systems: fundamentals, applications and trends. Sep Purif Rev 2016;45(1):68–80.

[8] Hatti-Kaul R. Aqueous two-phase systems: a general overview. Appl Biochem Biotechnol 2001; 19:269–77.
[9] Johansson G. Affinity partitioning in aqueous two-phase systems. J Sci Food Agric 2000;90 (9):1385–92.
[10] Ruiz-Ruiz F, Benavides J, Aguilar O, Rito-Palomares M. Aqueous two-phase affinity partitioning systems: current applications and trends. J Chromatogr A 2012;1244:1–13.
[11] Diamond AD, Hsu JT. Aqueous two-phase systems for biomolecule separation. In: Tsao GT, editor. Bioseparation. Berlin, Heidelberg: Springer; 1992. p. 89–135.
[12] González-Valdez J, Mayolo-Deloisa K, Rito-Palomares M. Novel aspects and future trends in the use of aqueous two-phase systems as a bioengineering tool. J Chem Technol Biotechnol 2018;93: 1836–44.
[13] Teixeira AG, Agarwal R, Ko KR, Grant-Burt J, Leung BM, Frampton JP. Emerging biotechnology applications of aqueous two-phase systems. Adv Healthc Mater 2018;7(6):1–19.
[14] Liu CL, Nikas YJ, Blankschtein D. Novel bioseparations using two-phase aqueous micellar systems. Biotechnol Bioeng 1996;52(2):185–92.
[15] Rogers RD, Bond AH, Bauer CB. Metal ion separations in polyethylene glycol-based aqueous biphasic systems. Sep Sci Technol 1993;28(5):1091–126.
[16] Liu C, Kamei DT, King JA, Wang DIC, Blankschtein D. Separation of proteins and viruses using two-phase aqueous micellar systems. J Chromatogr B Biomed Sci Appl 1998;711(1):127–38.
[17] Benavides J, Aguilar O, Lapizco-Encinas BH, Rito-Palomares M. Extraction and purification of bioproducts and nanoparticles using aqueous two-phase systems strategies. Chem Eng Technol 2008;31:838–45.
[18] Asenjo JA, Andrews BA. Aqueous two-phase systems for protein separation: phase separation and applications. J Chromatogr A 2012;1238:1–10.
[19] Espitia-Saloma E, Vázquez-Villegas P, Aguilar O, Rito-Palomares M. Continuous aqueous two-phase systems devices for the recovery of biological products. Food Bioprod Process 2014;92 (2):101–12.
[20] Álvarez MS, Rivas M, Deive FJ, Sanromán MA, Rodríguez A. Ionic liquids and non-ionic surfactants: a new marriage for aqueous segregation. RSC Adv 2014;4(62):32698–700.
[21] Asenjo JA, Andrews BA. Aqueous two-phase systems for protein separation: a perspective. J Chromatogr A 2011;1218(49):8826–35.
[22] Dos Santos NV, de Carvalho Santos-Ebinuma V, Pessoa Junior A, Pereira JFB. Liquid–liquid extraction of biopharmaceuticals from fermented broth: trends and future prospects. J Chem Technol Biotechnol 2018;93(7):1845–63.
[23] Cabezas H. Theory of phase formation in aqueous two-phase systems. J Chromatogr B Biomed Sci Appl 1996;680(1-2):3–30.
[24] Chakraborty A, Sen K. Impact of pH and temperature on phase diagrams of different aqueous biphasic systems. J Chromatogr A 2016;1433:41–55.
[25] Rosa PAJ, Ferreira IF, Azevedo AM, Aires-Barros MR. Aqueous two-phase systems: a viable platform in the manufacturing of biopharmaceuticals. J Chromatogr A 2010;1217(15):2296–305.
[26] Zaslavsky BY, Uversky VN, Chait A. Analytical applications of partitioning in aqueous two-phase systems: exploring protein structural changes and protein-partner interactions in vitro and in vivo by solvent interaction analysis method. Biochim Biophys Acta, Proteins Proteomics 2016;1864 (5):622–44.
[27] Hatti-Kaul R. Aqueous two-phase systems: methods and protocols. New Jersey: Humana Press; 2000.
[28] Silva DFC, Azevedo AM, Fernandes P, Chu V, Conde JP, Aires-Barros MR. Determination of aqueous two phase system binodal curves using a microfluidic device. J Chromatogr A 2014;1370: 115–20.

[29] Ruthven M, Ko KR, Agarwal R, Frampton JP. Microscopic evaluation of aqueous two-phase system emulsion characteristics enables rapid determination of critical polymer concentrations for solution micropatterning. Analyst 2017;142(11):1938–45.
[30] Merchuk JC, Andrews BA, Asenjo JA. Aqueous two-phase systems for protein separation: studies on phase inversion. J Chromatogr B 1998;711(1-2):285–93.
[31] Moody ML, Willauer HD, Griffin ST, Huddleston JG, Rogers RD. Solvent property characterization of polyethylene glycoD/dextran aqueous biphasic systems using the free energy of transfer of a methylene group and a linear solvation energy relationship. Ind Eng Chem Res 2005;44 (10):3749–60.
[32] Shahriari S, Neves CMSS, Freire MG, Coutinho JAP. Role of the Hofmeister series in the formation of ionic-liquid-based aqueous biphasic systems. J Phys Chem B 2012;116(24):7252–8.
[33] Freire MG, Louros CLS, Rebelo LPN, Coutinho JAP. Aqueous biphasic systems composed of a water-stable ionic liquid + carbohydrates and their applications. Green Chem 2011;13(6):1536–45.
[34] Alvarez-Guerra E, Ventura SPM, Alvarez-Guerra M, Coutinho JAP, Irabien A. Modeling of the binodal curve of ionic liquid/salt aqueous systems. Fluid Phase Equilib 2016;426:10–6.
[35] Asenjo JA, Mistry SL, Andrews BA, Merchuk JC. Phase separation rates of aqueous two-phase systems: correlation with system properties. Biotechnol Bioeng 2002;79(2):217–23.
[36] Lesher-Perez SC, Frampton JP, Takayama S. Microfluidic systems: a new toolbox for pluripotent stem cells. Biotechnol J 2012;8(2):180–91.
[37] Gutowski KE, Broker GA, Willauer HD, Huddleston JG, Swatloski RP, Holbrey JD, et al. Controlling the aqueous miscibility of ionic liquids: aqueous biphasic systems of water-miscible ionic liquids and water-structuring salts for recycle, metathesis, and separations. J Am Chem Soc 2003;125 (22):6632–3.
[38] Zaslavsky BY. Aqueous two-phase partitioning—physical chemistry and bioanalytical applications. New York, Basel, Oxford: Marcel Dekker, Inc.; 2018
[39] Gupta V, Nath S, Chand S. Role of water structure on phase separation in polyelectrolyte-polyethyleneglycol based aqueous two-phase systems. Polymer 2002;43(11):3387–90.
[40] Huddleston JG, Willauer HD, Griffin ST, Rogers RD. Aqueous polymeric solutions as environmentally benign liquid/liquid extraction media. Ind Eng Chem Res 1999;38(7):2523–39.
[41] Johansson H-O, Feitosa E, Junior AP. Phase diagrams of the aqueous two-phase systems of poly(ethylene glycol)/sodium polyacrylate/salts. Polymers 2011;3(4):587–601.
[42] Kang CH, Sandler SI. Phase behavior of aqueous two-polymer systems. Fluid Phase Equilib 1987;38(3):245–72.
[43] Sadeghi R, Maali M. Toward an understanding of aqueous biphasic formation in polymer-polymer aqueous systems. Polymer 2016;83:1–11.
[44] Flory PJ. Principles of polymer chemistry. Cornell University Press; 1953.
[45] Piculell L, Lindman B. Association and segregation in aqueous polymer/polymer, polymer/surfactant, and surfactant/surfactant mixtures: similarities and differences. Adv Colloid Interf Sci 1992;41:149–78.
[46] Gustafsson Å, Wennerström H, Tjerneld F. The nature of phase separation in aqueous two-polymer systems. Polymer 1986;27(11):1768–70.
[47] Zaslavsky BY, Bagirov TO, Borovskaya AA, Gulaeva ND, Miheeva LH, Mahmudov AU, et al. Structure of water as a key factor of phase separation in aqueous mixtures of two nonionic polymers. Polymer 1989;30(11):2104–11.
[48] Forciniti D, Hall CK, Kula MR. Influence of polymer molecular weight and temperature on phase composition in aqueous two-phase systems. Fluid Phase Equilib 1991;61(3):243–62.
[49] Meghna D, Griffin ST, Spear SK, Rodríguez H, Rijksen C, Rogers RD. Comparison of temperature effects on the salting out of poly(ethylene glycol) *versus* poly(ethylene oxide)-poly(propylene oxide) random copolymer. Ind Eng Chem Res 2010;49(5):2371–9.

[50] Barani A, Pirdashti M, Heidari Z, Dragoi EN. Influence of the molecular weight of polymer, temperature and pH on phase diagrams of poly (ethylene glycol) + di-potassium tartrate aqueous two-phase systems. Fluid Phase Equilib 2018;459:1–9.
[51] Glyk A, Scheper T, Beutel S. Influence of different phase-forming parameters on the phase diagram of several PEG–salt aqueous two-phase systems. J Chem Eng Data 2014;59(3):850–9.
[52] Tubío G, Pellegrini L, Nerli BB, Picó GA. Liquid – liquid equilibria of aqueous two-phase systems containing poly(ethylene glycols) of different molecular weight and sodium citrate. J Chem Eng Data 2006;51(1):209–12.
[53] Zafarani-Moattar MT, Hamzehzadeh S. Salting-out effect, preferential exclusion, and phase separation in aqueous solutions of chaotropic water-miscible ionic liquids and kosmotropic salts: effects of temperature, anions, and cations. J Chem Eng Data 2010;55(4):1598–610.
[54] Dilip M, Bridges NJ, Rodríguez H, Pereira JFB, Rogers RD. Effect of temperature on salt-salt aqueous biphasic systems: manifestations of upper critical solution temperature. J Solut Chem 2015; 44(3-4):454–68.
[55] Planas J, Kozlowski A, Harris JM, Tjerneld F, Hahn-Hägerdal B. Novel polymer-polymer conjugates for recovery of lactic acid by aqueous two-phase extraction. Biotechnol Bioeng 1999; 66(4):211–8.
[56] Yan B, Cao X. Phase diagram of novel recycling aqueous two-phase systems composed of two pH-response polymers: experiment and modeling. Fluid Phase Equilib 2014;364:42–7.
[57] Mourão T, Cláudio AFM, Boal-Palheiros I, Freire MG, Coutinho JAP. Evaluation of the impact of phosphate salts on the formation of ionic-liquid-based aqueous biphasic systems. J Chem Thermodyn 2012;54:398–405.
[58] Sintra TE, Cruz R, Ventura SPM, Coutinho JAP. Phase diagrams of ionic liquids-based aqueous biphasic systems as a platform for extraction processes. J Chem Thermodyn 2014;77:206–13.
[59] Hughes P, Lowe CR. Purification of proteins by aqueous two-phase partition in novel acrylic co-polymer systems. Enzym Microb Technol 1988;10(2):115–22.
[60] Nakajima A, Sato H. Phase relationships of an equivalent mixture of sulfated polyvinyl alcohol and aminoacetalyzed polyvinyl alcohol in microsalt aqueous solution. Biopolymers 2018;11 (7):1345–55.
[61] Pfennig A, Schwerin A, Gaube J. Consistent view of electrolytes in aqueous two-phase systems. J Chromatogr B Biomed Appl 1998;711(1-2):45–52.
[62] Johansson H-O, Karlström G, Tjerneld F, Haynes CA. Driving forces for phase separation and partitioning in aqueous two-phase systems. J Chromatogr B Biomed Sci Appl 1998;711(1):3–17.
[63] Rogers RD, Bauer CB. Partitioning behavior of group 1 and 2 cations in poly(ethylene glycol)-based aqueous biphasic systems. J Chromatogr B Biomed Sci Appl 1996;680(1):237–41.
[64] Huddleston JG, Willauer HD, Rogers RD. Phase diagram data for several PEG + salt aqueous biphasic systems at 25 °C. J Chem Eng Data 2003;48(5):1230–6.
[65] Zafarani-Moattar MT, Sadeghi R. Phase diagram data for several PPG + salt aqueous biphasic systems at 25 °C. J Chem Eng Data 2005;50(3):947–50.
[66] Cheluget EL, Gelinas S, Vera JH, Weber ME. Liquid-liquid equilibrium of aqueous mixtures of poly(propylene glycol) with sodium chloride. J Chem Eng Data 1994;39(1):127–30.
[67] Zafarani-Moattar MT, Hamzehzadeh S. Liquid–liquid equilibria of aqueous two-phase systems containing polyethylene glycol and sodium succinate or sodium formate. Calphad 2005;29(1):1–6.
[68] Xie X, Yan Y, Han J, Wang Y, Yin G, Guan W. Liquid – liquid equilibrium of aqueous two-phase systems of PPG400 and biodegradable salts at temperatures of (298.15, 308.15, and 318.15) K. J Chem Eng Data 2010;55(8):2857–61.
[69] Silvério SC, Gracia J, Teixeira JA, Macedo EA. Polyethylene glycol 8000+ citrate salts aqueous two-phase systems: relative hydrophobicity of the equilibrium phases. Fluid Phase Equilib 2016; 407:298–303.

[70] Sadeghi R, Kahaki HB. Thermodynamics of aqueous solutions of poly ethylene glycol di-methyl ethers in the presence or absence of ammonium phosphate salts. Fluid Phase Equilib 2011;306 (2):219–28.
[71] Silvério SC, Rodríguez O, Teixeira JA, Macedo EA. Liquid – liquid equilibria of UCON + (sodium or potassium) phosphate salt aqueous two-phase systems at 23 °C. J Chem Eng Data 2010; 55(3):1285–8.
[72] Yang X, Lu Y, Sun Z, Cui K, Tan Z. Measurement and correlation of phase equilibria in aqueous two-phase systems containing polyoxyethylene cetyl ether and three organic salts at different temperatures. J Chem Eng Data 2018;63(3):625–34.
[73] Ananthapadmanabhan KP, Goddard ED. Aqueous biphase formation in polyethylene oxide-inorganic salt systems. Langmuir 1987;3(1):25–31.
[74] Hofmeister F. Zur Lehre von der Wirkung der Salze. Arch Exp Pathol Pharmakol 1888;24 (4):247–60.
[75] Kunz W, Henle J, Ninham BW. "Zur Lehre von der Wirkung der Salze" (about the science of the effect of salts): Franz Hofmeister's historical papers. Curr Opin Colloid Interface Sci 2004;9 (1):19–37.
[76] Marcus Y. Thermodynamics of solvation of ions. Part 5—Gibbs free energy of hydration at 298.15 K. J Chem Soc Faraday Trans 1991;87(18):2995–9.
[77] Freire MG, Neves CMSS, Silva AMS, Santos LMNBF, Marrucho IM, Rebelo LPN, et al. ^{1}H NMR and molecular dynamics evidence for an unexpected interaction on the origin of salting-in/salting-out phenomena. J Phys Chem B 2010;114(5):2004–14.
[78] Wilkes JS. A short history of ionic liquids—from molten salts to neoteric solvents. Green Chem 2002;40:73–80.
[79] E Silva FA, Pereira JFB, Kurnia KA, Ventura SPM, Silva AMS, Rogers RD, et al. Temperature dependency of aqueous biphasic systems: an alternative approach for exploring the differences between Coulombic-dominated salts and ionic liquids. Chem Commun 2017;53(53):7298–301.
[80] Shahriari S, Tomé LC, Araújo JMM, Rebelo LPN, Coutinho JAP, Marrucho IM, et al. Aqueous biphasic systems: a benign route using cholinium-based ionic liquids. RSC Adv 2013;3(6):1835–43.
[81] Bridges NJ, Gutowski KE, Rogers RD. Investigation of aqueous biphasic systems formed from solutions of chaotropic salts with kosmotropic salts (salt/salt ABS). Green Chem 2007;9(2):177–83.
[82] Neves CMSS, Ventura SPM, Freire MG, Marrucho IM, Coutinho JAP. Evaluation of cation influence on the formation and extraction capability of ionic-liquid-based aqueous biphasic systems. J Phys Chem B 2009;113(15):5194–9.
[83] Ventura SPM, Neves CMSS, Freire MG, Marrucho IM, Oliveira J, Coutinho JAP. Evaluation of anion influence on the formation and extraction capacity of ionic-liquid-based aqueous biphasic systems. J Phys Chem B 2009;113(27):9304–10.
[84] Cláudio AFM, Ferreira AM, Shahriari S, Freire MG, Coutinho JAP. Critical assessment of the formation of ionic-liquid-based aqueous two-phase systems in acidic media. J Phys Chem B 2011;115 (38):11145–53.
[85] Zafarani-Moattar MT, Hamzehzadeh S. Phase diagrams for the aqueous two-phase ternary system containing the ionic liquid 1-Butyl-3-methylimidazolium bromide and tri-potassium citrate at T = (278.15, 298.15, and 318.15) K. J Chem Eng Data 2009;54(3):833–41.
[86] Blankschtein D, Thurston GM, Benedek GB. Phenomenological theory of equilibrium thermodynamic properties and phase separation of micellar solutions. J Chem Phys 1986;85(12):7268–88.
[87] Santos-Ebinuma VC, Lopes AM, Converti A, Pessoa A, Rangel-Yagui CO. Behavior of Triton X-114 cloud point in the presence of inorganic electrolytes. Fluid Phase Equilib 2013;360:435–8.
[88] Watanabe H. Tanaka H. A non-ionic surfactant as a new solvent for liquid—liquid extraction of zinc(II) with 1-(2-pyridylazo)-2-naphthol. Talanta 1978;25(10):585–9.

[89] Pereira JFB, Kurnia KA, Cojocaru OA, Gurau G, Rebelo LPN, Rogers RD, et al. Molecular interactions in aqueous biphasic systems composed of polyethylene glycol and crystalline vs. liquid cholinium-based salts. Phys Chem Chem Phys 2014;16(12):5723–31.
[90] Freire MG, Pereira JFB, Francisco M, Rodríguez H, Rebelo LPN, Rogers RD, et al. Insight into the interactions that control the phase behaviour of new aqueous biphasic systems composed of polyethylene glycol polymers and ionic liquids. Chem Eur J 2012;18(6):1831–9.
[91] Neves CMSS, Shahriari S, Lemus J, Pereira JFB, Freire MG, Coutinho JAP. Aqueous biphasic systems composed of ionic liquids and polypropylene glycol: insights into their liquid-liquid demixing mechanisms. Phys Chem Chem Phys 2016;18(30):20571–82.
[92] Pereira JFB, Kurnia KA, Freire MG, Coutinho JA, Rogers RD. Controlling the formation of ionic-liquid-based aqueous biphasic systems by changing the hydrogen-bonding ability of Polyethylene glycol end groups. ChemPhysChem 2015;16(10):2219–25.
[93] Jamehbozorg B, Sadeghi R. Evaluation of the effect of carbohydrates as renewable, none-charged and non-toxic soluting-out agents on the ionic-liquid-based ABS implementation. J Mol Liq 2018;255:476–91.
[94] Quental MV, Pereira MM, Ferreira AM, Pedro SN, Shahriari S, Mohamadou A, et al. Enhanced separation performance of aqueous biphasic systems formed by carbohydrates and tetraalkylphosphonium- or tetraalkylammonium-based ionic liquids. Green Chem 2018;20 (13):2978–83.
[95] Capela EV, Quental MV, Domingues P, Coutinho JAP, Freire MG. Effective separation of aromatic and aliphatic amino acid mixtures using ionic-liquid-based aqueous biphasic systems. Green Chem 2017;19(8):1850–4.
[96] Da Silva LM, Meirelles AA. Phase equilibrium in polyethylene glycol/maltodextrin aqueous two-phase systems. Carbohydr Polym 2000;42(3):273–8.
[97] De Brito Cardoso G, Mourão T, Pereira FM, Freire MG, Fricks AT, Soares CMF, et al. Aqueous two-phase systems based on acetonitrile and carbohydrates and their application to the extraction of vanillin. Sep Purif Technol 2013;104:106–13.
[98] Johansson HO, Persson J, Tjerneld F. Thermoseparating water/polymer system: a novel one-polymer aqueous two-phase system for protein purification. Biotechnol Bioeng 1999;66(4):247–57.
[99] Chen J, Ding Z, Pan H, Cao X. Development of pH-responsive polymer and citrate aqueous two-phase system for extractive bioconversion of cefprozil. Talanta 2017;174:256–64.
[100] Wang W, Wan J, Ning B, Xia J, Cao X. Preparation of a novel light-sensitive copolymer and its application in recycling aqueous two-phase systems. J Chromatogr A 2008;1205(1):171–6.
[101] Passos H, Tavares DJP, Ferreira AM, Freire MG, Coutinho JAP. Are aqueous biphasic systems composed of deep eutectic solvents ternary or quaternary systems? ACS Sustain Chem Eng 2016;4(5):2881–6.
[102] Baghlani M, Sadeghi R. Thermodynamics investigation of phase behavior of deep eutectic solvents-polymer aqueous biphasic systems. Polymer 2018;143:115–28.
[103] Farias FO, Passos H, Sanglard MG, Igarashi-Mafra L, Coutinho JAP, Mafra MR. Designer solvent ability of alcohols in aqueous biphasic systems composed of deep eutectic solvents and potassium phosphate. Sep Purif Technol 2018;200:84–93.
[104] Liu D, Zou X, Gao M, Gu M, Xiao H. Hydrophilic organic/salt-containing aqueous two-phase solvent system for counter-current chromatography: a novel technique for separation of polar compounds. J Chromatogr A 2014;1356:157–62.
[105] Reis IAO, Santos SB, Santos LA, Oliveira N, Freire MG, Pereira JFB, et al. Increased significance of food wastes: selective recovery of added-value compounds. Food Chem 2012;135(4):2453–61.
[106] Guo W, Ma J, Wang Y, Han J, Li Y, Song S. Liquid–liquid equilibrium of aqueous two-phase systems composed of hydrophilic alcohols (ethanol/2-propanol/1-propanol) and $MgSO_4$/$ZnSO_4$ at (303.15 and 313.15)K and correlation. Thermochim Acta 2012;546:8–15.

[107] González-Amado M, Rodil E, Arce A, Soto A, Rodríguez O. The effect of temperature on polyethylene glycol (4000 or 8000)–(sodium or ammonium) sulfate aqueous two phase systems. Fluid Phase Equilib 2016;428:95–101.
[108] Karmakar R, Sen K. Aqueous biphasic extraction of metal ions: an alternative technology for metal regeneration. J Mol Liq 2019;273:231–47.
[109] Diamond AD, Hsu JT. Fundamental studies of biomolecule partitioning in aqueous two-phase systems. Biotechnol Bioeng 1989;34(7):1000–14.
[110] Hachem F, Andrews BA, Asenjo JA. Hydrophobic partitioning of proteins in aqueous two-phase systems. Enzym Microb Technol 1996;19(7):507–17.
[111] Schluck A, Maurer G, Kula M-R. Influence of electrostatic interactions on partitioning in aqueous polyethylene glycol/dextran biphasic systems: part I. Biotechnol Bioeng 1995;46(5):443–51.
[112] Dinis TBV, Passos H, Lima DLD, Sousa ACA, Coutinho JAP, Esteves VI, et al. Simultaneous extraction and concentration of water pollution tracers using ionic-liquid-based systems. J Chromatogr A 2018;1559:69–77.
[113] Shiri S, Khezeli T, Lotfi S, Shiri S. Aqueous two-phase systems: a new approach for the determination of brilliant blue FCF in water and food samples. J Chem 2013;236196:1–6.
[114] Han J, Wang Y, Yu CL, Yan YS, Xie XQ. Extraction and determination of chloramphenicol in feed water, milk, and honey samples using an ionic liquid/sodium citrate aqueous two-phase system coupled with high-performance liquid chromatography. Anal Bioanal Chem 2011;399 (3):1295–304.
[115] Zimmermann S, Gretzinger S, Schwab ML, Scheeder C, Zimmermann PK, Oelmeier SA, et al. High-throughput downstream process development for cell-based products using aqueous two-phase systems. J Chromatogr A 2016;1464:1–11.