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Aqueous Two-Phase Systems for Bioprocess Development for the Recovery of Biological Products

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Preface

Biotechnology has become one of the most fast-paced growing disciplines, having impact on society due to its presence in a wide range of industrial sectors. This includes the food, cosmetics, detergents, personal care, nutrimental supplements and pharmaceutical markets, among others. In this regards, the design and implementation of bioprocesses, oriented to the generation of such products and services, is of great relevance. Such processes should be effective so the product can be produced, isolated and purified in a time-effective and cost-effective manner. Furthermore, bioprocesses should be able to be implemented at large scale, so they can satisfy the markets demands. For more than 45 years the development in the bioprocessing area has mainly focused on the upstream and production stages. As a result, production-oriented strategies have become exceedingly efficient in time, particularly due to the use of genetic engineering and metabolic engineering approaches. This has generated highly concentrated process streams, complex in nature, that need to be further handled using downstream strategies in order to extract, isolate and purify the bioactives of interest. In this context, the development of robust and efficient primary recovery and purification technologies is of great relevance in the bioprocessing research area. Over the years, the use of aqueous two-phase systems (ATPS) based technology has proven to be effective for processing highly concentrated and complex biological samples.

Aqueous two-phase systems (ATPS) are liquid-liquid biphasic systems that form when two hydrophilic constituents are mixed over certain concentration threshold. This results in two independent phases with different characteristics in polarity, charge and excluded volume, allowing the fractionation of complex biological samples. But, what differentiates ATPS from other fractionation techniques currently available? Since the main solvent in both phases is water, ATPS are regarded as highly biocompatible, since most biological compounds and particles come from aqueous environments. Most constituents (polymers, salts, ionic liquids, low molecular weight alcohols, etc.) used for ATPS formation are relatively cheap, and some of them can be easily reused in the process, reducing the cost of the stage. Furthermore, process integration and intensification can be attained using ATPS due to their robustness while handling solid matter (cells, cell debris, organelles, bionanoparticles, etc.). Finally, ATPS can be readily scaled up, being able to be used from lab to industrial scale. Although for a long time (1960–1990) the use of ATPS focused on the recovery and purification of proteins, nowadays they are also used

for a wide variety of bioactives, including low molecular weight compounds, cells, organelles, virus and virus-like particles, among others. Altogether, ATPS represent an attractive alternative to more traditional downstream fractionation strategies.

This book gives an overview of the basic theory and the state of the art of aqueous two-phase systems technology for the recovery and purification of biological products. Chapters 1 and 2 present basic concepts related to the formation and handling of ATPS. Furthermore, they address traditional and novel analytical techniques used for the characterization of the physical, physicochemical and biochemical properties of ATPS. Chapters 3, 4, 5, 6 and 7 present current applications of traditional and novel ATPS strategies for the fractionation and recovery of biologicals, covering from low molecular weight compounds to bioparticles. For instance, Chap. 6 addresses the use of affinity ATPS for the selective fractionation of biomolecules. Chapter 8 focuses on the fractionation of biomolecules using ATPS in continuous operation mode. Most (> 99%) ATPS fractionation studies consider batch mode operation, and therefore, giving insights of the most relevant aspects to consider for the design of continuous operation ATPS stages represents a valuable element of this book. Chapter 9 addresses the evaluation of the potential of ATPS as a feasible alternative to traditional fractionation techniques from the process economics perspective. Finally, Chap. 10 presents the perspectives and future trends in ATPS-based technology, with particular emphasis in ATPS enhancement, alternative applications and economic analysis at large-scale implementation. We certainly believe that this book represents a valuable source of information for both, those who have already worked with ATPS and those taking their first steps in this field.

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General Concepts and Definitions of Aqueous Two-Phase Systems

1

Karla Mayolo-Deloisa, Jorge Benavides, and Marco Rito-Palomares

Abbreviations

$[C_4MIM]Br$	1-butyl-3-methylimidazolium bromide
[C6mim][C12SO3]	1-hexyl-3-methyl imidazole dodecyl sulfonate
$[N_{2,2,2,2}]Br$	Tetraethylammonium bromide
ATPS	Aqueous two-phase systems
BMIM Cl	1-butyl-3-methyl-imidazolium chloride
HIV-GFP VLP	Virus-like particles from human immunodeficiency virus with a covalent bond to a GFP tag
HTS	High-throughput screening
ILs	Ionic liquids
K_p	Partition coefficient
NaPA	Sodium polyacrylate
PEG	Polyethylene glycol
P_N	Polymer of N-isopropylacrylamide
PPG	Polypropylene glycol
PVBAm	Polymer of N-vinylcaprolactam, butyl methacrylate, and acrylamide
S	Selectivity

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TLL Tie-line length
UCON Ethylene oxide/propylene oxide copolymer
V_R Volume ratio
Y_T Recovery yield

1.1 Introduction

Aqueous two-phase systems (ATPS) are produced when combinations of hydrophilic solutes (polymers or polymer and certain salts) display incompatibility in aqueous solution above critical concentrations (Rito-Palomares 2004). When the polymers are mixed, large aggregates form, and the two polymers will tend to separate into two different phases due to steric exclusion. The most commonly used polymers are polyethylene glycol (PEG) and dextran. A similar exclusion phenomenon can be observed between a polymer and a high concentration of salt (e.g., PEG and phosphate, sulfate, citrate) since the salt will capture a large amount of the water present (Asenjo and Andrews 2011). As discovered by Albertsson (Albertsson 1986), these phases offer different physical and chemical environments which allow for the partition of different solutes. Nowadays, ATPS can be classified into five groups: polymer–polymer, polymer–salt, alcohol–salt, micellar, and ionic liquid based. Polymer–polymer and polymer–salt ATPS have advantages over conventional water–organic solvent systems for bioseparations. These systems are unique in that each of the phases typically contains over 80% water on a molal basis, and yet the phases are immiscible and differ in their solvent properties (Zaslavsky et al. 2016). The interfacial tension is extremely low, between 0.0001 and 0.1 dyne/cm compared with 1–20 dyne/cm for conventional water–organic solvent systems, creating a high interfacial contact area of the dispersed phases and thus an efficient mass transfer (Albertsson 1986). Among the main advantages of this technique include the partition equilibrium is reached very fast, low cost and has potential recycling of the materials used to form the systems, easy to scale up, processes integration capability and continuous operation (Reh et al. 2007; Benavides et al. 2011; Hardt and Hahn 2012).

Aqueous two-phase systems have been widely used for separation and recovery of biological products, specially proteins, such as lipase, laccase, bovine serum albumin, phospholipase, invertase, lactoferrin, protease, collagenase, β-phycoerythrin, and penicillin acylase (Benavides and Rito-Palomares 2004; Aguilar et al. 2006; Mayolo-Deloisa et al. 2009; Karkaş and Önal 2012; Lima et al. 2013; Moreira et al. 2013; Senphan and Benjakul 2014; Chow et al. 2015; Costa et al. 2015; da Silva et al. 2015; Souza et al. 2015), among others. Nowadays, ATPS are extensively used for valuable biopharmaceuticals such as monoclonal antibodies, DNA, cells, growth factors, and hormones. The high water content in each phase of the system ensures the high stability of biomolecules throughout the extraction process, which can result in higher yields (Pereira Alcântara et al. 2013).

The physicochemical events involved in the formation and separation of the ATPS are extremely complex; they may be simplified based on the hydration enthalpy and the entropy net balance. Although the two main compounds of the ATPS are primordially hydrophilic, the enthalpy of hydration between such compounds differs. As a result, two thermodynamic scenarios are possible. If the amount of energy in the system is high enough to overcome the net difference between entropy and hydration enthalpy, the two main chemical compounds may coexist at their present concentration in a single homogeneous phase. Otherwise, the separation of the two compounds is energetically favored, promoting the formation of two phases (Benavides et al. 2011). The initial characterization of ATPS is highly important regarding the determination of binodal curve and tie-lines which comprise the most important system properties such as phase composition and mass ratio of top and bottom phase (Amrhein et al. 2014).

In general, protein partitioning is driven by Van der Waals, hydrophobic, hydrogen bond, and ionic interactions between the biomolecules and the surrounding phase. Hence, the partition may be influenced by the concentrations and molecular weight of phase-forming polymer, type and concentration of phase-forming salt, and pH (Nagaraja and Iyyaswami 2015). The preference of a protein to migrate to a particular phase depends on several factors, among which are molecular weight, surface area, net electrochemical charge, isoelectric point, and content of hydrophobic residues (Sebastião et al. 1997). The parameters of ATPS play an important role during the partition of a molecule of interest toward a particular phase. To obtain a high purification factor and yield is necessary to do an appropriate selection of the type and molecular weight of polymer, type and salt concentration, tie-line length (TLL), volume ratio of phases (V_R), temperature, and pH of system.

Throughout this chapter, the basic concepts, the general classification of the systems, and the parameters that influence the partition will be described. It is important to remark the importance of this technique because despite the amount of research that is done in the area, there is still great potential to develop new systems and separate new molecules, hence the importance of addressing the bases that govern partitioning in ATPS.

1.2 Binodal Curve, Tie-Line Length, and Volume Ratio

ATPS is designed by a binodal curve which separates two-phase area from single-phase area (see Fig. 1.1). The phase diagram delineates the potential working area for a particular two-phase system, and it is a “fingerprint” unique to that system under set conditions of, for example, pH, temperature, and salt or polymer concentration (Kaul 2000).

The binodal curve gives the exact composition of top and bottom phases. The compositions represented by the points below the binodal curve are homogeneous. Two phases are formed only by the compositions above the binodal curve (Sinha et al. 2000). Traditionally, binodal curves are constructed following the cloud point method. Polymer- or salt (or polymer–polymer, alcohol–salt solutions; it depends on

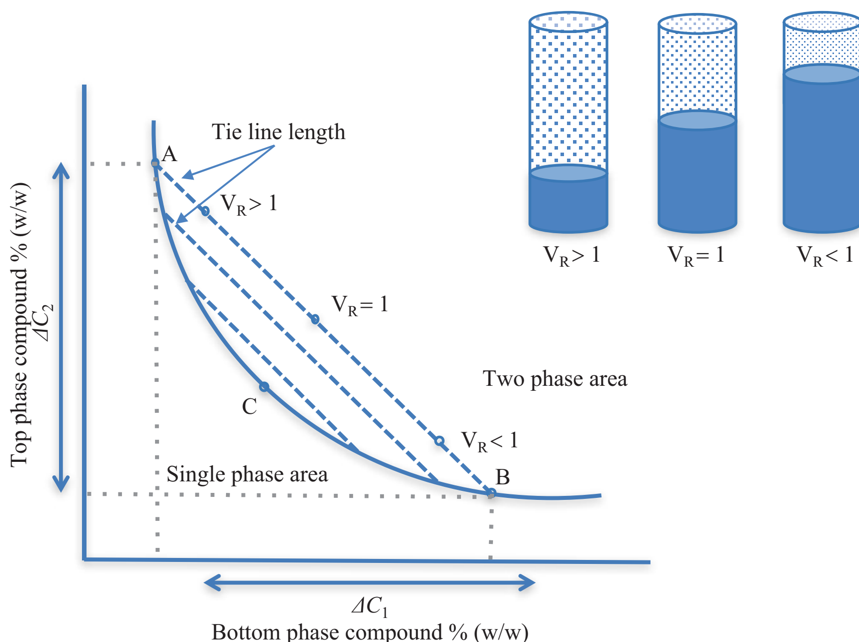

Fig. 1.1 Binodal curve or phase diagram. Bottom phase compound (polymer–salt) is plotted on the abscissa, and top phase compound (polymer) is plotted on the ordinate. *A*–*B*: Tie-line length; *A*: composition of top phase; *B*: composition of bottom phase; *C*: critical point; V_R: volume ratios

the case)-concentrated stock solutions are added dropwise to each other until the visual detection of a cloudy solution; at this critical point, the cloud point, the mixture turbid is indicative of two-phase formation. After that, the mixture composition can be calculated by weight quantification of all components added. The whole procedure must be carried out under constant magnetic stirred using a thermostatic water bath for temperature control (Yan and Cao 2014; de Araujo Sampaio et al. 2016). Lately, techniques as microfluidic and high-throughput screening (HTS) are used for to avoid consuming the time and use lower volumes of reagents and analytes (Amrhein et al. 2014; Silva et al. 2014). Silva et al. (2014) described the procedure for determination of a binodal curve using microfluidic device. Two solutions containing separate ATPS solution precursors were loaded into the side inlets of a three-inlet microfluidic channel, while milli-Q water was loaded into the middle inlet. By varying the flow rates of the three solutions, a wide range of concentrations inside the microchannel could be rapidly tested using limited volumes. By means of optical microscopy, depending on the concentrations inside the microchannel, three different states can be observed at the end of the microchannel: (1) the presence of an interface, (2) no presence of an interface, or (3) the presence of an unstable interface. The binodal curve was calculated using the points corresponding to unstable interfaces (Silva et al. 2014). There are many binodal curves reported that can be used to construct different systems under specific conditions. However, new binodal curves also continue to be reported due to the appearance of new phase-forming chemicals.

The tie-line length, which represents the final concentration of phase components in the top and bottom phases (Fig. 1.1, line A–B), is often used to express the effect of system composition on partitioned material (Kaul 2000). The TLL is calculated as:

$$TLL = \sqrt{\Delta C_1^2 + \Delta C_2^2} \quad (1.1)$$

where ΔC_1 and ΔC_2 are the absolute difference in the concentration of the phase-forming constituents, in the top and the bottom phases.

TLL are commonly parallel and influence the partitioning phenomenon. Moving along the TLL, coordinates are denoted systems with differing total compositions. All points on one tie-line length will have identical compositions in the top and bottom phases (Fig. 1.1) (Asenjo and Andrews 2011). As TLL decrease, they ultimately approach a critical point (C) on the binodal curve, where the TLL = 0. At this point, the composition and volume of both phases are almost equal (Zaslavsky 1995). In the practice, the TLL determination can be realized using gravimetric methods, refractive index, or polarimetry for quantitation of polymer concentration. Some thermo-responsive polymers may also be quantified using high-performance liquid chromatography (HPLC) (Xu et al. 2016). In the case of salts, the concentration can be obtained by conductimetry (Rengifo et al. 2015). However, the distribution of phase-forming components may change during a phase change operation (Sinha et al. 2000).

Although all ATPS within the same TLL have identical top and bottom phase compositions, their volume ratio (V_R) changes according to the global composition of the system (Benavides et al. 2011). The V_R is defined as the relation between the volume of the top phase (V_{TP}) and the volume of the bottom phase (V_{TP}). V_R is calculated as:

$$V_R = \frac{V_{TP}}{V_{BP}} \quad (1.2)$$

In the practice, determining the volume ratio is simplified if volumetrically graduated bottles are used to carry out the systems. The appropriate handling of V_R allows to concentrate the sample at the same time that the partition is being carried out. Many of the mechanisms that influence the partition behavior of solutes and bionanoparticles in ATPS are directly related to the TLL and the V_R. Therefore, the proper characterization of these two system parameters is desirable in order to quantify their effect on the fractionation of biological compounds (Benavides et al. 2011).

1.3 General Classification of ATPS

Traditionally, ATPS had been classified in two main groups: polymer–polymer and polymer–salt systems. So currently the family of ATPS could be categorized in five main groups that include ATPS formed by ethanol, micelles, and ionic liquids. In Fig. 1.2, general classification and principal characteristics of each type of system are presented. Table 1.1 shows examples of the composition of each type of ATPS

Polymer -polymer
- Systems based on polymer/electrolytes , or polymer/low molecular weight solutes
- Fractionation and recovery of proteins, nucleic acids, cells and organells
- Environment suitable for proteins and biomolecules sensitive to the ionic strength
- Some polymers are expensive

Polymer - salt
- Systems formed on addition of salts solutions of certain polymers
- Fractionation and recovery of proteins, nucleic acids, nanoparticles and low molecular weight compounds
- Low cost systems
- High concentrations of salt, high ionic strength

Alcohol - salt
- Systems based on aliphatic alcohols and aqueous salt solutions
- Fractionation and recovery of low molecular weight compounds
- Low viscosity, low cost and easy solvent recovery
- Many proteins are not compatible with alcohol rich phase

Micellar
- Systems formed by some surfactants at certain conditions
- Fractionation of products sensitive to ionic strength
- The components can be recycled
- Can provide a gentle and friendly environment for biological materials

Ionic liquid based
- Systems composed of hydrophilic ILs and kosmotropic salts
- Fractionation of macromolecules sensitive to ionic strength
- ILs bring beneficial properties compared to organic solvents like non-flammability and non-volatility
- Some ILs are very toxic

Fig. 1.2 General classification of ATPS and principal characteristics

Table 1.1 Types and composition of some aqueous two-phase systems

Type of ATPS	Composition	Target	Reference
Polymer–polymer	PEG 4000 – dextran P_{VBAm} – P_N UCON – dextran 75,000 PEG 1000 – NaPA 8000	L-glutaminase ε-Polylysine $CD133^+$ stem cells Natural red colorants	Singh and Banik (2012)) Xu et al. (2016) González-González et al. (2016) Santos-Ebinuma et al. (2015)
Polymer–salt	PEG 1500 – PO_4 PEG 20000 – sodium citrate PEG 400 – Na_2SO_4 PEG 1500 – $(NH_4)_2SO_4$ UCON – $(NH_4)_2SO_4$	Lipase Clavulanic acid Caffeine HIV-GFP VLP Laccase	Carvalho et al. (2017) Carneiro-da-Cunha et al. (2014) de Araujo Sampaio et al. (2016) Jacinto et al. (2015) Bertrand et al. (2016)
Alcohol–salt	Ethanol – $(NH_4)_2SO_4$ 2-propanol – $(NH_4)_2SO_4$ 1-propanol – $(NH_4)_2SO_4$ Ethanol – PO_4 Methanol – PO_4	Lignans Interferon alpha-2b Anthraquinones Fucoxanthin Proteins	Cheng et al. (2016) Lin et al. (2013) Tan et al. (2013) Gómez-Loredo et al. (2014) Phong et al. (2016)
Micellar	Triton X-100 – sodium citrate PEG4000/Triton X-100 Triton X-100 – sorbitol	Aromatic amino acids Lysozyme Pectinase	Salabat et al. (2011) Liu et al. (2012) Amid et al. (2015)
Ionic liquid based	[C6mim][C12SO3] – PEG 6000 BMIM Cl – PO_4 $[C_4MIM]Br$ – PO_4 $[N_{2,2,2,2}]Br$- potassium citrate $[Bmim]BF_4$ – PO_4	Au (III) Quinine Food colorants Natural red colorants Esterase	Zheng et al. (2015) Flieger and Czajkowska-Żelazko (2015) Sha et al. (2015) Ventura et al. (2013) Jiang et al. (2015)

PVBAm polymer of N-vinylcaprolactam, butyl methacrylate, and acrylamide, P_N polymer of N-isopropylacrylamide, *UCON* ethylene oxide/propylene oxide copolymer, *NaPA* sodium polyacrylate, *HIV-GFP VLP* virus-like particles from human immunodeficiency virus with a covalent bond to a GFP tag, *[C6mim][C12SO3]* 1-hexyl-3-methyl imidazole dodecyl sulfonate, *BMIM Cl* 1-butyl-3-methyl-imidazolium chloride, $[C_4MIM]Br$ 1-butyl-3-methylimidazolium bromide, $[N_{2,2,2,2}]Br$ tetraethylammonium bromide

and the molecules that have been separated using different ATPS types. As can be observed, the ATPS are versatile, the components of each system are varied, and their properties help to separate a broad spectrum of molecules as proteins, virus-like particles, cells, colorants, bioactive low weight molecular compounds, metals, alkaloids, etc. The selection of the more suitable ATPS depends on the characteristics of the molecule of interest, the source, and the application. In addition new components capable of forming ATPS are continuously being characterized.

1.3.1 Polymer–Polymer Systems

Generally, aqueous two-phase polymeric systems are based on polymer/electrolytes or polymer/low molecular weight solutes. High molecular weight dextran and PEG are two polymers that have been used so far because of their well-known physical and chemical structure. Both polymers are cheap, require only moderate concentrations, separate rapidly, have moderately low viscosities, and can be easily buffered and rendered isotonic (Schindler and Nothwang 2006). Systems containing dextran usually present a bottom phase mainly composed of dextran, while the top phase is composed of the other polymer (Mazzola et al. 2008). However, it is important to remember that both phases will contain a certain amount of each component of the system, due to the phase equilibrium attained. Given that the ionic strength of the polymer–polymer systems is extremely low, they are used preferentially for the separation of solutes extremely sensitive to ionic environments as well as viable cells and organelles susceptible to osmotic shock (Benavides et al. 2011). Frequently, these systems have a high viscosity due to the nature of the polymers. Another disadvantage is the high cost of some polymers that limit their usage for large scale, thus, to replace the dextran by less expensive polymers like crude dextran, starches, maltodextrins, or pullulans as bottom phase component can greatly reduce the process cost (Hatti-Kaul 2001). Or alternatively, these systems can be used to recover high-value molecules such as cells and antibodies.

1.3.2 Polymer–Salt Systems

ATPS may also form in addition of salt solutions in combination with polymeric solutions. These systems have particular relevance in biotechnological applications because salt is cheap relative to second polymers such as dextran (Huddleston et al. 1991). They have been extensively used for protein purification from crude feedstocks. The system has marked hydrophobic difference between the phases. The relative effectiveness of various salts in promoting phase separation is seen to follow the Hofmeister series (a classification of ions based upon salting-out ability) (Huddleston et al. 1991). The contribution of the anion is more important than that of the cation in determining the effectiveness of a particular salt. The multivalent anions like HPO_4^{2-} and SO_4^{2-} are most effective in inducing phase separation with PEG (Huddleston et al. 1991, Hatti-Kaul 2001). But this leads to high phosphate

and sulfate concentration in the effluent streams and hence an environmental concern. In the polymer–salt systems, PEG–phosphate ATPS are commonly used, due to several process advantage, including the range of pH (from six to nine) under which the ATPS are stable (Rito-Palomares 2004), low viscosity, and short separation time. Currently, use of citrate salts as a phase-forming component with PEG is highly used since citrate salts are biodegradable and nontoxic.

1.3.3 Alcohol–Salt Systems

Water-miscible methanol, ethanol, 1-propanol, and 2-propanol were investigated as additives to ATPS (Wang et al. 2010). However, under appropriate concentrations, these aliphatic alcohols and aqueous salt solutions form ATPS with an alcohol-rich top phase and salt-rich bottom phase. By removing the alcohol using evaporation, the target product can be easily extracted and recovered (Greve and Kula 1991). Specific advantages of these systems include the use of inexpensive constituents (particularly compared with some expensive polymers and copolymers), easy constituent recovery and reutilization, reduced settling times, high polarity, low viscosity, and low toxicity to environment (Ooi et al. 2009). The major disadvantage of alcohol–salt ATPS is that even though they have been used for the separation of some enzymes, many proteins are incompatible with organic solvent phase, which can inactivate or denature the biomolecules (Ooi et al. 2009). Therefore, a conformational shift leading to agglomeration, precipitation, and even denaturation is a risk for macromolecules partitioned on this type of system (Benavides et al. 2011).

1.3.4 Micellar Systems

Aqueous surfactant solutions (mainly nonionic mixtures) split into two phases if the temperature is increased beyond the cloud point temperature. One of the phases is a concentrated surfactant solution, the surfactant-rich phase, and the other one is the surfactant lean phase (or aqueous phase), where surfactant concentration is close to its critical micelle concentration (Tani et al. 1998; Safonova et al. 2014). The micelles are aggregates made of surfactant molecules which are self-assembled with hydrophilic head groups facing the aqueous solution and hydrophobic chains pointing inwards, so the micelles form the hydrophobic microstructure in their nonpolar cores. The existing micelle structures bring the obvious excluded volume interactions with the hydrophilic biomolecules, which could impel the hydrophilic biomolecules partitioning to the polymer-rich phase according to biomolecular size (Liu et al. 2012). The size and shape of the micelles can be controlled by varying parameters such as the surfactant concentration, temperature, pH, and ionic strength. Consequently, salts and some low molecular weight solvents can be added in order to further control the partition selectivity (Benavides et al. 2011). Aqueous two-phase micellar systems offer both hydrophobic and hydrophilic environments, providing selectivity to the molecule partitioning according to its hydrophobicity.

They can provide a gentle and friendly environment for biologic materials. So, these systems have been evaluated for the extraction and purification of proteins, viruses, enzymes, antibiotics, DNA, and nucleic acids (Lopes et al. 2011).

1.3.5 Ionic Liquid-Based Systems

Ionic liquids are salts with unique physical properties, such as a negligible vapor pressure, low viscosity, and high thermal stability, depending on their structure (Oppermann et al. 2011). Some hydrophilic ionic liquids are able to form ionic liquid ATPS when mixed with aqueous solutions of inorganic salts (Novak et al. 2012). The partition behavior of solutes in the system can be manipulated based on the chemical nature of the ionic liquid (the presence of aliphatic chains, cyclic groups, and electrochemical charge) as well as its concentration (Benavides et al. 2011). Ionic liquid-based ATPS were firstly proposed by Huddleston and coworkers (1998) and have since been successfully studied by a growing number of researchers for the separation, concentration, and purification of proteins, antioxidants, metal ions, alkaloids, and antibiotics (Ventura et al. 2013). Among the various ionic liquid families, the cholinium based have been the focus of attention as ATPS phase formers, since they can form biphasic systems with both salts and polymers. Cholinium chloride (2-hydroxyethyltrimethylammonium chloride) is one of the most recent families of ionic liquids being utilized in the extraction and purification of biomolecules due to it is a cheap raw material, widely used as food additive, and thus a safer and more environmentally friendly salt when compared with some of most common ionic liquid cations (Santos et al. 2016). Nonetheless, the green aspect of the ionic liquids has often been picked out as a central benefit compared to organic solvents. Due to the negligible vapor pressure, they are less air-polluting than organic solvents. On the other hand, there are various kinds of organic solvents used during the ionic liquid production or recycling process, which reduces the eco-friendliness of their application in biotechnological processes (Oppermann et al. 2011).

1.4 Definitions of Parameters of ATPS: Partition Coefficient, Selectivity, Recovery Yield, and Purity

The number of experiments needed to determine the process conditions of an ATPS extraction can be reduced by using a practical approach which exploits the known effect of system parameters such as tie-line length, phase volume ratio, system pH, and molecular mass of the polymer on the protein partition (Rito-Palomares 2004). It is well known that changes in TLL affect the free volume available for a defined solute to accommodate in the phase, as a consequence, the partition behavior (Grossman and Gainer 1988). Alike, the effect of increasing molecular mass of the polymer (specially PEG) upon solute partitioning behavior has been explained on the basis of protein hydrophobicity and phase excluded volume (Huddleston et al. 1991). So, it is important to evaluate the effectiveness of the partition through the analysis of the following parameters: partition coefficient, selectivity, recovery yield, and purity.

1.4.1 Partition Coefficient

In general, the fractionation behavior of biomolecules is described using the partition coefficient (K_p) defined as:

$$K_p = \frac{C_T}{C_B} \quad (1.3)$$

where C_T and C_B represent the equilibrium concentrations of the partitioned protein in the top and bottom phases, respectively (Asenjo and Andrews 2011). K_p is often used to evaluate the extension of biomolecule separation in the aqueous two-phase systems. K_p is a function of the properties of the phases and the substance and also temperature; it is independent of the solute concentration and volume ratio of the phases (Chu and Chen 2000). When K_p is significantly distinct for the target biomolecule and for other biomolecules present in the system, the extraction is better. In other words, K_p values greater than unity indicate the effectiveness of partitioning in the aqueous two-phase system (Mazzola et al. 2008). Some authors calculate ln (K_p) for to remark the negative and positive values. A positive value of ln (K_p) indicates the preference of the molecules for the top phase, while a negative value indicates the preference of the molecule for the bottom phase (González-Valdez et al. 2011).

1.4.2 Selectivity

The selection of conditions favoring differential partition of the molecules of interest versus contaminants is achieved by adjusting the biphasic environment to each particular situation (Soares et al. 2015). Some researchers use selectivity to relate the partition coefficient of a specific molecule and the partitioning of the rest of the contaminants. In other words, if it is working with a protein, the partition coefficient of that protein is related to the partition coefficient of the total protein (contaminants). Then, the selectivity is defined as:

$$S = \frac{K_{\text{p target}}}{K_{\text{p contaminants}}} \quad (1.4)$$

where $K_{\text{p target}}$ is the partition coefficient of the target or molecule of interest and $K_{\text{p contaminants}}$ is the partition coefficient of the rest of molecules or contaminants (Ventura et al. 2013).

1.4.3 Recovery Yield

Recovery yield (Y_T) is another important parameter for to evaluate the efficiency of the ATPS. Y_T is calculated at the phase where the target is partitioned preferentially,

and it is expressed relative to the initial amount of the molecule of interest in the sample loaded to the ATPS according to the next equation:

$$Y_{\mathrm{T}} = \frac{C_{\mathrm{t}} V_{\mathrm{t}}}{C_{\mathrm{i}} V_{\mathrm{i}}} \times 100\% \quad (1.5)$$

where C_t is the concentration of the target in the partitioned phase, V_t is the volume of the phase where the target is partitioned, and $C_i$and V_i are the concentration and volume of the stock solution added to the system.

1.4.4 Purity

Although ATPS is more recognized as a primary recovery technique, it is also capable of purifying different biomolecules. So one of the important parameters that can be calculated is purity. Purity (%) is the fraction of the main component present in a given sample, in other words, the mass percentage of a specific compound in the total solid content or extract. It is calculated using the next expression:

$$\text{Purity}\left(\%\right) = \frac{C_{\mathrm{o}} V_{\mathrm{o}}}{M_{\mathrm{e}}} \times 100 \quad (1.6)$$

where C_{o} and V_{o} are the measured concentration and volume of the specific phase (top or bottom phase), respectively, and M_e is the mass of extract powder (Cheng et al. 2016).

1.5 Factors that Affect Partitioning in ATPS: Molecular Weight, Electrochemical Interactions and pH, Hydrophobicity, and Affinity

The mechanisms that influence the partition behavior can be exploited separately or altogether in order to achieve an effective product migration toward one phase while contaminants fractionate to the opposite phase. The molecular properties that govern partition behavior in ATPS have been categorized in four groups: (a) molecular weight or size, (b) electrochemical interactions and pH, (c) hydrophobicity, and (d) affinity.

1.5.1 Molecular Weight or Size

Since the solutes to be partitioned in the system have a defined size, they are subjected to the steric effects imposed by the constituents of the system. These steric effects are typically related to the available volume for the solutes to be fractionated toward a particular phase and are generally known as the free volume effect. Polymer–polymer and polymer–salt ATPS are well known to promoting

size-dependent partition on the solutes. As these systems are constructed using polymers, with most of them of considerable length and molecular weight, the free volume available in polymer-rich phases is limited. This effect is enlarged as the TLL of the systems increases, since the concentration of the polymer is also increased (Benavides et al. 2011). The molecular weight of the polymer used also influences the partitioning. As polymer concentration increases, differences in density, refractive index, and viscosity between the phases increase (de Oliveira et al. 2008). In PEG–salt systems, the partitioning is governed by volume exclusion effect (polymer-rich) and salting-out effect (salt-rich). The systems with high concentration or high molecular weight polymer and high salt concentration will result in partitioning of biomolecules at the interphase due to the influence of both volume exclusion and salting-out effect (Sharma et al. 2008).

1.5.2 Electrochemical Interactions and pH

Considering that protein and biomolecule solubility are mostly related to superficial charge interaction with the ions in solution, the charge distribution is a very important parameter in ATPS (Mazzola et al. 2008). Some ATPS constituents, such as salts and ionic liquids, are ionizable species. Furthermore, although some other constituents such as polymers (PEG and dextran) do not ionize in solution, they present weak dipole moments due to the presence of functional groups with strong electronegativity. The influence of pH on the electrochemical interactions is fundamental. The use of pH values above the isoelectric point (pI) of proteins may induce an additional affinity toward the PEG-rich phase (Benavides et al. 2011) and increase the partition coefficient. It may be because of the electrostatic interactions between the molecules and PEG units (Schmidt et al. 1994). A pH value above 7 is suitable for the PEG–phosphate system, and a pH below 6.5 is compatible with the PEG–sulfate system. Most of the biomolecules, especially proteins and enzymes, are stable at neutral pH that is a favorable condition to conduct the partitioning (Goja et al. 2013).

1.5.3 Hydrophobicity

Hydrophobicity is one of the main factors that determine the partitioning of proteins in ATPS. Two well-known effects are involved in the hydrophobic interactions: the phase hydrophobicity effect and the salting-out effect. Although both phases are rather hydrophilic, the top phase (PEG-rich or other) is usually more hydrophobic. This favors the partition of amphipathic and less hydrophilic solutes and particles toward that particular phase. In polymer–salt systems, the phase hydrophobicity may be manipulated by varying the TLL and the polymer molecular weight and by the addition of a salt such as NaCl (at a concentration of up to 10%). When the TLL increases, an intrinsic reduction of the water content is achieved. Therefore, the system becomes more hydrophobic as less water is available. Regarding polymer

molecular weight, as this parameter increases, an induced hydrophobicity is generated due to the presence of extensive hydrophobic areas. This is particularly evident for polymers such as PEG, which only has hydrophilic functional (hydroxyl) groups at its extremes, whereas the rest of the chain is primordially hydrophobic. Therefore, as the molecular weight of the polymer increases, the ratio of hydrophilic groups to hydrophobic area decreases, reflecting a rise in hydrophobicity (Benavides et al. 2011).

Regarding the salting-out effect, this is also related to the hydrophobic-dependent partitioning in ATPS. This effect is observed in systems with at least one highly ionic phase (polymer–salt ATPS, ionic liquid-based ATPS, etc.). In these cases, since the amount of water needed to dissolve the salts in the system is high, the solutes to be partitioned are only partially hydrated. Consequently, partitioning toward the less hydrophilic phase is favored under such circumstances (Benavides et al. 2011).

Salt additives are among the factors which are known to strongly influence the solute partitioning in ATPS (Zaslavsky 1995). In general, addition of neutral salts to the ATPS results in an increase in the hydrophobic difference due to generation of an electrical potential difference between two phases (Goja et al. 2013). This increase in the hydrophobicity is related to the decrease of the amount of bound water in order to keep constant the final composition of the systems and also because of the ion solvation (Rosa et al. 2009). The most widely used salt additive in ATPS is NaCl. NaCl is generally used in the concentration range of up to 15 wt%, i.e., up to ca. 2.6 M (Ferreira et al. 2011). However, the addition of high concentration of neutral salts may cause denaturation of proteins; thus, low concentration range from 0.0 to 1.0 M is preferred (Goja et al. 2013). The effects of several other salt additives, such as perchlorate and different chlorides, on protein partitioning in PEG–salt ATPS were also reported (Ferreira et al. 2011).

1.5.4 Affinity

To increase the selectivity of the partitioning of molecule of interest to make extraction predictable as well as to achieve sufficient purification during this step is one of the main objectives of scientists (Hatti-Kaul 2000). In that sense, specific affinity partitioning exploits the attachment of a ligand to a phase-forming polymer to introduce a general or specific binding affinity of target solutes for the polymer-rich phase and to enhance the selectivity (Huddleston et al. 1991). In addition, affinity partitioning in liquid–liquid extraction systems can also be achieved by the addition of free ligands (biological or chemical molecules) in solution to a traditional ATPS in order to induce a partition shift of the desired target molecule without the chemical modification of the phase-forming component (Azevedo et al. 2009). The ligands are often polymers modified with hydrophobic or charge groups or protein ligands conjugated with polymers. During the past 40 years, polymer-coupled ligands have been studied for cell and protein partitioning (Soares et al. 2015). At preparative scale, affinity ATPS also present important advantages for the development of novel

downstream strategies such as high ligand concentration per volume of polymer solution, which translates to high concentration of bound target biomolecule in one of the system phases (process intensification), and fast equilibrium achieving (Ruiz-Ruiz et al. 2012).

1.6 Concluding Remarks

ATPS is a versatile technique for the primary recovery and partial purification of a wide selection of biomolecules. Up to now, the binodal curves of many pairs of phase-forming chemicals and their characteristics are known. However, publications with reports of new components and therefore new binodal curves appear continuously. For many researchers, the typical application of the ATPS has been exhausted; however, the number of publications in the area shows otherwise. An example of this is the number of publications where PEG–phosphate systems are used, especially for the separation of proteins. The general classification of the ATPS includes now alcohol–salt systems. Although this is a system that is not exactly formed by two aqueous phases, the mild characteristics of alcohol allow to separate other types of molecules. The mechanisms that governed the partition are still complex but permit to exploit the technique with the objective to enhance the partition coefficient, selectivity, and recovery.

References

Aguilar O, Albiter V, Serrano-Carreon L, Rito-Palomares M. Direct comparison between ion-exchange chromatography and aqueous two-phase processes for the partial purification of penicillin acylase produced by E. coli. J Chromatogr B. 2006;835(1–2):77–83. doi:10.1016/j.jchromb.2006.03.016.

Albertsson P-A. Partition of cell particles and macromolecules. 3rd ed. New York: Wiley; 1986.

Amid M, Murshid FS, Manap MY, Hussin M. A novel aqueous micellar two-phase system composed of surfactant and sorbitol for purification of pectinase enzyme from psidium guajava and recycling phase components. Biomed Res Int. 2015;2015:815413. doi:10.1155/2015/815413.

Amrhein S, Schwab ML, Hoffmann M, Hubbuch J. Characterization of aqueous two phase systems by combining lab-on-a-chip technology with robotic liquid handling stations. J Chromatogr A. 2014;1367:68–77. doi:10.1016/j.chroma.2014.09.042.

Asenjo JA, Andrews BA. Aqueous two-phase systems for protein separation: a perspective. J Chromatogr A. 2011;1218(49):8826–35. doi:10.1016/j.chroma.2011.06.051.

Azevedo AM, Rosa PAJ, Ferreira IF, Pisco AMMO, de Vries J, Korporaal R, Visser TJ, Aires-Barros MR. Affinity-enhanced purification of human antibodies by aqueous two-phase extraction. Sep Purif Technol. 2009;65(1):31–9. doi:10.1016/j.seppur.2008.03.006.

Benavides J, Rito-Palomares M. Bioprocess intensification: a potential aqueous two-phase process for the primary recovery of B-phycoerythrin from Porphyridium cruentum. J Chromatogr B. 2004;807(1):33–8. doi:10.1016/j.jchromb.2004.01.028.

Benavides J, Rito-Palomares M, Asenjo JA. 2.49 – Aqueous two-phase systems. In: Moo-Young M, editor. Comprehensive biotechnology. 2nd ed. Burlington: Academic; 2011. p. 697–713.

Bertrand B, Mayolo-Deloisa K, González-González M, Tinoco-Valencia R, Serrano-Carreón L, Martínez-Morales F, Trejo-Hernández MR, Rito-Palomares M. Pleurotus ostreatus laccase recovery from residual compost using aqueous two-phase systems. J Chem Technol Biotechnol. 2016;91(8):2235–42. doi:10.1002/jctb.4995.

Carneiro-da-Cunha MN, Souza KPS, Mota AMO, Teixeira JA, Porto CS, Porto TS, Porto ALF. Stability of clavulanic acid in PEG/citrate and liquid–liquid extraction in aqueous two-phase system. Fluid Phase Equilibr. 2014;375:104–9. doi:10.1016/j.fluid.2014.04.036.

Carvalho T, Finotelli PV, Bonomo RCF, Franco M, Amaral PFF. Evaluating aqueous two-phase systems for Yarrowia lipolytica extracellular lipase purification. Process Biochem. 2017;53:259–66. doi:10.1016/j.procbio.2016.11.019.

Cheng Z, Cheng L, Song H, Yu L, Zhong F, Shen Q, Hu H. Aqueous two-phase system for preliminary purification of lignans from fruits of Schisandra Chinensis Baill. Sep Purif Technol. 2016;166:16–25. doi:10.1016/j.seppur.2016.04.013.

Chow YH, Yap YJ, Tan CP, Anuar MS, Tejo BA, Show PL, Ariff AB, Ng E-P, Ling TC. Characterization of bovine serum albumin partitioning behaviors in polymer-salt aqueous two-phase systems. J Biosci Bioeng. 2015;120(1):85–90. doi: http://dx.doi.org/10.1016/j.jbiosc.2014.11.021

Chu IM, Chen W-Y. Partition of amino acids and peptides in aqueous two-phase systems. In: Hatti-Kaul R, editor. Aqueous two-phase systems: methods and protocols: methods and protocols. Totowa: Humana Press; 2000. p. 95–105.

Costa ARD, Coimbra JSDR, Ferreira LA, Marcos JC, Santos IJB, Saldaña MDA, Teixeira JAC. Partitioning of bovine lactoferrin in aqueous two-phase system containing poly(ethylene glycol) and sodium citrate. Food Bioprod Process. 2015;95:118–24. doi: http://dx.doi.org/10.1016/j.fbp.2015.04.002

da Silva RMM, JSDR C, da Silva CA, da Costa AR, da Rocha RA, ARM G, IJB S. Green extraction by aqueous two-phase systems of porcine pancreatic and snake venom phospholipase A2. Sep Purif Technol. 2015;141:25–30. doi:10.1016/j.seppur.2014.11.029.

de Araujo Sampaio D, Mafra LI, Yamamoto CI, de Andrade EF, de Souza MO, Mafra MR, de Castilhos F. Aqueous two-phase (polyethylene glycol + sodium sulfate) system for caffeine extraction: equilibrium diagrams and partitioning study. J Chem Thermodyn. 2016;98:86–94. doi:10.1016/j.jct.2016.03.004.

de Oliveira RM, JSDR C, Minim LA, da Silva LHM, Ferreira Fontes MP. Liquid–liquid equilibria of biphasic systems composed of sodium citrate + polyethylene(glycol) 1500 or 4000 at different temperatures. J Chem Eng Data. 2008;53(4):895–9. doi:10.1021/je7004209.

Ferreira LA, Teixeira JA, Mikheeva LM, Chait A, Zaslavsky BY. Effect of salt additives on partition of nonionic solutes in aqueous PEG–sodium sulfate two-phase system. J Chromatogr A. 2011;1218(31):5031–9. doi:10.1016/j.chroma.2011.05.068.

Flieger J, Czajkowska-Żelazko A. Aqueous two phase system based on ionic liquid for isolation of quinine from human plasma sample. Food Chem. 2015;166:150–7. doi:10.1016/j.foodchem.2014.06.037.

Goja A, Yang H, Cui M, Li C. Aqueous two-phase extraction advances for bioseparation. J Bioproces Biotechniq. 2013;4(1):140–8. doi:10.4172/2155-9821.1000140.

González-González M, Willson RC, Rito-Palomares M. Elimination of contaminants from cell preparations using aqueous two-phase partitioning. Sep Purif Technol. 2016;158:103–7. doi:10.1016/j.seppur.2015.12.006.

González-Valdez J, Cueto LF, Benavides J, Rito-Palomares M. Potential application of aqueous two-phase systems for the fractionation of RNase A and α-Lactalbumin from their PEGylated conjugates. J Chem Technol Biotechnol. 2011;86(1):26–33. doi:10.1002/jctb.2507.

Greve A, Kula MR. Cost structure and estimation for the recycling of salt in a protein extraction process. Bioprocess Eng. 1991;6(4):173–7. doi:10.1007/BF00369255.

Grossman PD, Gainer JL. Correlation of aqueous two-phase partitioning of proteins with changes in free volume. Biotechnol Prog. 1988;4(1):6–11. doi:10.1002/btpr.5420040103.

Gómez-Loredo A, Benavides J, Rito-Palomares M. Partition behavior of fucoxanthin in ethanol-potassium phosphate two-phase systems. J Chem Technol Biotechnol. 2014;89(11):1637–45. doi:10.1002/jctb.4514.

Hardt S, Hahn T. Microfluidics with aqueous two-phase systems. Lab Chip. 2012;12(3):434–42. doi:10.1039/C1LC20569B.

Hatti-Kaul R. Aqueous two-phase systems. In: Hatti-Kaul R, editor. Aqueous two-phase systems: methods and protocols: methods and protocols. Totowa: Humana Press; 2000. p. 1–10.

Hatti-Kaul R. Aqueous two-phase systems. Mol Biotechnol. 2001;19(3):269–77. doi:10.1385/MB:19:3:269.

Huddleston JG, Ottomar KW, Ngonyani DM, Lyddiatt A. Influence of system and molecular parameters upon fractionation of intracellular proteins from saccharomyces by aqueous two-phase partition. Enzyme Microb Technol. 1991;13(1):24–32. doi:10.1016/0141-0229(91)90184-C.

Huddleston JG, Willauer HD, Swatloski RP, Visser AE, Rogers RD. Room temperature ionic liquids as novel media for 'clean' liquid-liquid extraction. Chemical Commun. 1998;16:1765–6. doi:10.1039/A803999B.

Jacinto MJ, Soares RRG, Azevedo AM, Chu V, Tover A, Conde JP, Aires-Barros MR. Optimization and miniaturization of aqueous two phase systems for the purification of recombinant human immunodeficiency virus-like particles from a CHO cell supernatant. Sep Purif Technol. 2015;154:27–35. doi:10.1016/j.seppur.2015.09.006.

Jiang B, Feng Z, Liu C, Xu Y, Li D, Ji G. Extraction and purification of wheat-esterase using aqueous two-phase systems of ionic liquid and salt. J Food Sci Tech Mys. 2015;52(5):2878–85. doi:10.1007/s13197-014-1319-5.

Karkaş T, Önal S. Characteristics of invertase partitioned in poly(ethylene glycol)/magnesium sulfate aqueous two-phase system. Biochemical Eng J. 2012;60(0):142–50. doi:10.1016/j.bej.2011.11.005.

Kaul A. The phase diagram. In: Hatti-Kaul R, editor. Aqueous two-phase systems. Hardcover: Human Press; 2000.

Lima CA, Júnior ACVF, Filho JLL, Converti A, Marques DAV, Carneiro-da-Cunha MG, Porto ALF. Two-phase partitioning and partial characterization of a collagenase from Penicillium aurantiogriseum URM4622: application to collagen hydrolysis. Biochem Eng J. 2013;75:64–71. doi:10.1016/j.bej.2013.03.012.

Lin YK, Ooi CW, Tan JS, Show PL, Ariff A, Ling TC. Recovery of human interferon alpha-2b from recombinant *Escherichia coli* using alcohol/salt-based aqueous two-phase systems. Sep Purif Technol. 2013;120:362–6. doi:10.1016/j.seppur.2013.09.038.

Liu Y, Wu Z, Dai J. Phase equilibrium and protein partitioning in aqueous micellar two-phase system composed of surfactant and polymer. Fluid Phase Equilibr. 2012;320:60–4. doi:10.1016/j.fluid.2012.02.002.

Lopes AM, Silva DP, Vicente AA, Pessoa-Jr A, Teixeira JA. Aqueous two-phase micellar systems in an oscillatory flow micro-reactor: study of perspectives and experimental performance. J Chem Technol Biotechnol. 2011;86(9):1159–65. doi:10.1002/jctb.2642.

Mayolo-Deloisa K, Trejo-Hernandez MD, Rito-Palomares M. Recovery of laccase from the residual compost of Agaricus bisporus in aqueous two-phase systems. Process Biochem. 2009;44(4):435–9. doi:10.1016/j.procbio.2008.12.010.

Mazzola PG, Lopes AM, Hasmann FA, Jozala AF, Penna TCV, Magalhaes PO, Rangel-Yagui CO, Pessoa A Jr. Liquid–liquid extraction of biomolecules: an overview and update of the main techniques. J Chem Technol Biotechnol. 2008;83(2):143–57. doi:10.1002/jctb.1794.

Moreira S, Silvério SC, Macedo EA, Milagres AMF, Teixeira JA, Mussatto SI. Recovery of Peniophora cinerea laccase using aqueous two-phase systems composed by ethylene oxide/propylene oxide copolymer and potassium phosphate salts. J Chromatogr A. 2013;1321(0):14–20. doi:10.1016/j.chroma.2013.10.056.

Nagaraja VH, Iyyaswami R. Aqueous two phase partitioning of fish proteins: partitioning studies and ATPS evaluation. J Food Sci Tech Mys. 2015;52(6):3539–48. doi:10.1007/s13197-014-1425-4.

Novak U, Pohar A, Plazl I, Žnidaršič-Plazl P. Ionic liquid-based aqueous two-phase extraction within a microchannel system. Sep Purif Technol. 2012;97(0):172–8. doi:10.1016/j.seppur.2012.01.033.

Ooi CW, Tey BT, Hii SL, Kamal SMM, Lan JCW, Ariff A, Ling TC. Purification of lipase derived from Burkholderia pseudomallei with alcohol/salt-based aqueous two-phase systems. Process Biochem. 2009;44(10):1083–7. doi:10.1016/j.procbio.2009.05.008.

Oppermann S, Stein F, Kragl U. Ionic liquids for two-phase systems and their application for purification, extraction and biocatalysis. Appl Microbiol Biot. 2011;89(3):493–9. doi:10.1007/s00253-010-2933-4.

Pereira Alcântara LA, do Nascimento KS, Mourão CA, VPR M, Minim LA. Aqueous two-phase poly(ethylene glycol)–sodium polyacrylate system for amyloglucosidase purification: equilibrium diagrams and partitioning studies. Sep Purif Technol. 2013;118:888–94. doi:10.1016/j.seppur.2013.08.039.

Phong WN, Le CF, Show PL, Chang J-S, Ling TC. Extractive disruption process integration using ultrasonication and an aqueous two-phase system for protein recovery from Chlorella sorokiniana. Eng Life Sci. 2016;17:357–69. doi:10.1002/elsc.201600133.

Reh G, Spelzini D, Tubio G, Pico G, Farruggia B. Partition features and renaturation enhancement of chymosin in aqueous two-phase systems. J Chromatogr B. 2007;860(1):98–105. doi:10.1016/j.jchromb.2007.10.012.

Rengifo AFC, Ferreira GMD, Ferreira GMD. Hespanhol da Silva MC, Mendes da Silva LH. Phase diagrams, densities and refractive indexes of poly(ethylene oxide) + organic salts + water aqueous two-phase systems: effect of temperature, anion and molar mass. Fluid Phase Equilibr. 2015;406:70–6. doi:10.1016/j.fluid.2015.08.002.

Rito-Palomares M. Practical application of aqueous two-phase partition to process development for the recovery of biological products. J Chromatogr B. 2004;807(1):3–11. doi:10.1016/j.jchromb.2004.01.008.

Rosa PAJ, Azevedo AM, Sommerfeld S, Mutter M, Aires-Barros MR, Bäcker W. Application of aqueous two-phase systems to antibody purification: a multi-stage approach. J Biotechnol. 2009;139(4):306–13. doi:10.1016/j.jbiotec.2009.01.001.

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. doi:10.1016/j.chroma.2012.04.077.

Safonova EA, Mehling T, Storm S, Ritter E, Smirnova IV. Partitioning equilibria in multicomponent surfactant systems for design of surfactant-based extraction processes. Chem Eng Res Des. 2014;92(12):2840–50. doi:10.1016/j.cherd.2014.04.005.

Salabat A, Far MR, Moghadam ST. Partitioning of amino acids in surfactant based aqueous two-phase systems containing the nonionic surfactant (Triton X-100) and salts. J Solut Chem. 2011;40(1):61–6. doi:10.1007/s10953-010-9628-4.

Santos PL, Santos LNS, Ventura SPM, de Souza RL, Coutinho JAP, Soares CMF, Lima ÁS. Recovery of capsaicin from Capsicum frutescens by applying aqueous two-phase systems based on acetonitrile and cholinium-based ionic liquids. Chem Eng Res Des. 2016;112:103–12. doi:10.1016/j.cherd.2016.02.031.

Santos-Ebinuma VC, Lopes AM, Pessoa A, Teixeira MFS. Extraction of natural red colorants from the fermented broth of Penicillium purpurogenum using aqueous two-phase polymer systems. Biotechnol Prog. 2015;31(5):1295–304. doi:10.1002/btpr.2127.

Schindler J, Nothwang HG. Aqueous polymer two-phase systems: effective tools for plasma membrane proteomics. Proteomics. 2006;6(20):5409–17. doi:10.1002/pmic.200600243.

Schmidt AS, Ventom AM, Asenjo JA. Partitioning and purification of α-amylase in aqueous two-phase systems. Enzyme Microb Technol. 1994;16(2):131–42. doi:10.1016/0141-0229(94)90076-0.

Sebastião MJ, Martel P, Baptista A, Petersen SB, Cabral JMS, Aires-Barros MR. Predicting the partition coefficients of a recombinant cutinase in polyethylene glycol/phosphate aqueous two-phase systems. Biotechnol Bioeng. 1997;56(3):248–57. doi:10.1002/(sici)1097-0290(19971105)56:3<248::aid-bit2>3.0.co;2-j.

Senphan T, Benjakul S. Use of the combined phase partitioning systems for recovery of proteases from hepatopancreas of Pacific white shrimp. Sep Purif Technol. 2014;129:57–63. doi:10.1016/j.seppur.2014.03.025.

Sha O, Zhu X, Feng Y, Ma W. Aqueous two-phase based on ionic liquid liquid–liquid microextraction for simultaneous determination of five synthetic food colourants in different food samples by high-performance liquid chromatography. Food Chem. 2015;174:380–6. doi:10.1016/j.foodchem.2014.11.068.

Sharma K, Babu PVC, Sasidhar P, Srinivas VK, Mohan VK, Krishna E. Recombinant human epidermal growth factor inclusion body solubilization and refolding at large scale using expanded-bed adsorption chromatography from Escherichia coli. Protein Expres Purif. 2008;60(1):7–14. doi:10.1016/j.pep.2008.02.020.

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. doi:10.1016/j.chroma.2014.10.035.

Singh P, Banik RM. Partitioning studies of l-glutaminase production by Bacillus Cereus MTCC 1305 in different PEG–salt/dextran. Bioresour Technol. 2012;114:730–4. doi:10.1016/j.biortech.2012.03.046.

Sinha J, Dey PK, Panda T. Aqueous two-phase: the system of choice for extractive fermentation. Appl Microbiol Biot. 2000;54(4):476–86. doi:10.1007/s002530000342.

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. doi:10.1002/biot.201400532.

Souza RL, Lima RA, Coutinho JAP, Soares CMF, Lima ÁS. Novel aqueous two-phase systems based on tetrahydrofuran and potassium phosphate buffer for purification of lipase. Process Biochem. 2015;50(9):1459–67. doi:10.1016/j.procbio.2015.05.015.

Tan Z-j, Li FF, Xu XL. Extraction and purification of anthraquinones derivatives from Aloe vera L. using alcohol/salt aqueous two-phase system. Bioprocess Biosyst Eng. 2013;36(8):1105–13. doi:10.1007/s00449-012-0864-4.

Tani H, Kamidate T, Watanabe H. Aqueous micellar two-phase systems for protein separation. Anal Sci. 1998;14(5):875–88. doi:10.2116/analsci.14.875.

Ventura SPM, Santos-Ebinuma VC, Pereira JFB, Teixeira MFS, Pessoa A, Coutinho JAP. Isolation of natural red colorants from fermented broth using ionic liquid-based aqueous two-phase systems. J Ind Microbiol Biot. 2013;40(5):507–16. doi:10.1007/s10295-013-1237-y.

Wang Y, Han J, Xu X, Hu S, Yan Y. Partition behavior and partition mechanism of antibiotics in ethanol/2-propanol–ammonium sulfate aqueous two-phase systems. Sep Purif Technol. 2010;75(3):352–7. doi:10.1016/j.seppur.2010.09.004.

Xu C, Dong W, Wan J, Cao X. Synthesis of thermo-responsive polymers recycling aqueous two-phase systems and phase formation mechanism with partition of ε-polylysine. J Chromatogr A. 2016;1472:44–54. doi:10.1016/j.chroma.2016.10.016.

Yan B, Cao X. Phase diagram of novel recycling aqueous two-phase systems composed of two pH-response polymers: experiment and modeling. Fluid Phase Equilibr. 2014;364:42–7. doi:10.1016/j.fluid.2013.11.037.

Zaslavsky B. Aqueous two-phase partitioning: physical chemistry and bioanalytical applications. New York: Marcel Dekker Inc.; 1995.

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. BBA-Proteins Proteom. 2016;1864(5):622–44. doi:10.1016/j.bbapap.2016.02.017.

Zheng Y, Tong Y, Wang S, Zhang H, Yang Y. Mechanism of gold (III) extraction using a novel ionic liquid-based aqueous two phase system without additional extractants. Sep Purif Technol. 2015;154:123–7. doi:10.1016/j.seppur.2015.09.014.

2 Characterization of Aqueous Two-Phase Systems and Their Potential New Applications

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and Marco Rito-Palomares

Abbreviations

ADSA	Axisymmetric drop shape analysis
ATPS	Aqueous two-phase system
DARA	Distribution analysis of radiolabeled analytes
HPLC	High-performance liquid chromatography
HTS	High throughput screening
K_P	Partition coefficient
LHS	Liquid-handling stations
PEG	Polyethylene glycol
TLL	Tie-line length
V_R	Volume ratio

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2.1 Introduction

Successful implementation of aqueous two-phase system (ATPS) strategies requires a complete understanding of the different physical and chemical phenomena happening within a particular system. This said, the design of ATPS involves the correct selection of parameters such as phase-forming chemicals (i.e., polymers, salts, ionic liquids, alcohols, etc.), pH, tie-line length (TLL), and phase volume ratio (V_R) for the particular product or products being recovered (Rito-Palomares 2004). The mixed interactions of these parameters upon the physicochemical characteristics of the products being fractionated will cause the molecules of interest to partition to either of the phases either by concentration or by separation from the rest of the contaminants in the mixtures (González-Valdez et al. 2013).

In most cases, the appropriate selection of system design parameters requires a thorough experimentation of all these variables since, to date, models fail to predict the behavior of solutes (especially in mixtures) in ATPS (Mistry et al. 1996). This is particularly important because, being a primary recovery operation, ATPS are usually involved in the separation of a particular set of products from streams carrying mixtures of contaminants that must be eliminated (Rito-Palomares 2004). More importantly, because of its characteristics, a well-designed ATPS operation is usually regarded as a viable option for substituting other more complicated or time-consuming procedures including chromatography (Mayolo-Deloisa et al. 2011). With this in mind, the engineering design of a biphasic separation should be optimal, and in doing so, ATPS should be completely characterized and understood from an intrinsic (i.e., the physicochemical interactions affecting it) and operational point of view. This chapter aims to present in a logical manner the different procedures to characterize the physicochemical properties of an ATPS and the solutes that are partitioned within them and to present novel and potential new uses where ATPS strategies could be successfully implemented.

2.2 Aqueous Two-Phase System Characterization

The first step in implementing an ATPS operation involves the selection of the phase-forming components to be used in the system. These chemicals can be broadly categorized into six main groups: polymers, salts, alcohols, ionic liquids, micelle-forming agents, and low molecular weight solvents (Benavides et al. 2011). The selection and use of at least one of the chemicals in a group with at least one of another group (or the same group in polymer-polymer and ionic liquid systems) above a critical concentration generate a biphasic system with two immiscible phases that may be used for extraction and fractionation of different products (González-Valdez et al. 2013). Each one of these types of ATPS formed (i.e., polymer-polymer, polymer-salt, alcohol-polymer, alcohol-salt, and ionic liquid-based and micellar systems) is mainly used for the recovery and fractionation of specific kinds of molecules depending on the properties they possess as it has been extensively described in literature (Diamond and Hsu 1992; Rosa et al. 2010).

The most common ATPS are generated with the use of two incompatible polymers or one polymer and a salt. For many of these cases, there is published equilibrium data (i.e., binodal curves) that might be used to then choose the concentration of the phase-forming chemicals in each of the phases (Zaslavsky 1995). However, this data is only available for commonly used systems and should be generated for particular operations which is becoming a common practice since the use of novel phase-forming chemicals such as "smart" polymers, new ionic liquids, or affinity ligands for product fractionation is gaining momentum (Leong et al. 2016; Montalvo-Hernández et al. 2012). Furthermore, published equilibrium data should only be used as a reference since in most cases, experimental errors, variations in the molecular weights of the species, poor temperature control, and uncontrolled or unreported addition of additives are rarely reported, and all of these aspects might cause variations in the position of the binodal curve (Forciniti 2000).

Therefore, the generation of binodal curves and phase diagrams is one of the first steps to be made toward the characterization of ATPS. On their part, phase diagrams are unique to each combination of phase-forming chemicals and additives under specific pH and temperature conditions. These diagrams are delineated by their corresponding binodal curve, under which no phase formation can be achieved and above which all the different operation conditions are found. Coordinates for all of these possible systems will then give the information needed for the total composition of the system and will lie on a specific tie-line length (TLL) that denotes the composition of the phase-forming chemicals in both the top and bottom phases (Kaul 2000). Even more, the specific "working coordinates" for that particular system will also indicate according to its position along the tie-line and the volume ratio (V_R) of the top and bottom phases of the system which has also a particular influence in product partition (Diamond and Hsu 1989).

Generation of binodal curves can be achieved by different experimental approaches. For instance, in turbidimetric titration, a series of mixed stock solutions of phase-forming chemicals in known concentrations is prepared and then diluted with an appropriate solvent. If, when agitated, these mixtures become visually turbid, a clear indication of being "above" the binodal curve is given. Then, solvent addition is made in a dropwise manner until the mixture becomes clear indicating that the mixture has reached the binodal curve. The final concentrations of the phase-forming chemicals at that point will then indicate one of the coordinates of the binodal curve that can then be completed with the other points obtained from the series of mixed stock solutions (de Oliveira et al. 2008). In a similar manner, in the cloud point method, a concentrated stock solution of one of the ATPS components is added dropwise to a known amount of a concentrated solution of the other component. At a certain point, the mixture will become turbid indicating the chemical compositions that lay on the binodal curve which again can be extrapolated with a series of similar experiments varying the known amounts of the second component (Kaul 2000). Within this context, a novel approach involving the use of microdevices for the characterization of binodal curves is starting to show promising results since the appearance of two distinct phases can be controlled by tuning the volumetric flow of the phase components. By knowing the concentrations, densities, and

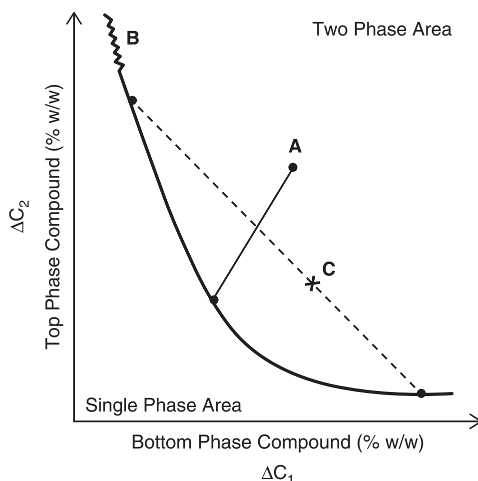

Fig. 2.1 Schematic representation of the different methodologies available for binodal curve characterization. (*A*) Shows the procedure of turbidimetric titration where a series of known two-phase points are titrated until one single phase is observed indicating the point where the curve lies. (*B*) Presents the cloud point method procedure (indicated by the *zigzag line*) where stocks of either system components are mixed and taking the solution above and below the cloud point locating the place where the cure lies. (*C*) Illustrates the determination of nodes, where the composition of each of the phase-forming components is calculated for each of the phases and graphed to determine the binodal curve. This also helps with determining the tie-line length composition of that particular system

flows of the said stock phase-forming solution, the visual formation of two distinct phases in the microdevice can serve as an indicator to calculate the concentrations laying in the binodal curve (Vazquez-Villegas et al. 2016). Finally, in another more traditional method, calculation of the binodal curve can also be achieved by determining the nodes in a series of systems by analysis of the amounts of phase-forming chemicals in each of the generated phases providing the different points in the binodal. In fact, this is also helpful in calculating the different TLL values in the diagram where $TLL = (\Delta X^2 + \Delta Y^2)^{1/2}$. In this case, ΔX and ΔY represent the difference between the compositions (in % w/w) between the top and bottom phases of each of the phase-forming chemicals. Other TLL values can then be calculated by extrapolating the slopes of these lines since in most cases tie-lines are parallel to each other (Kaul 2000). Figure 2.1 presents a schematic representation of the use of the different methodologies available to generate binodal curves.

Understanding the physicochemical parameters that will affect product partition and being able to measure them provide the means of developing better ATPS strategies and have a better quality control in industrial processes. As noted in Fig. 2.2, several of these parameters may have an important influence in solute partition behavior. For instance, interfacial tension provides a significantly more sensitive measurement of the effective concentration of a polymer solution than does the determination of TLL or the phase diagram which is important in repetitive batch

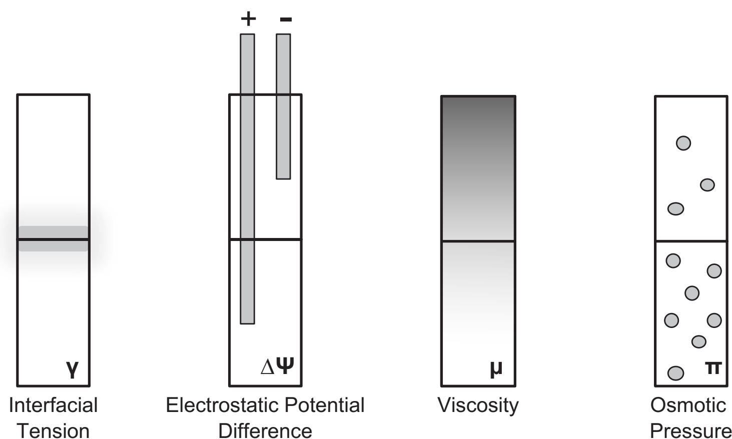

Fig. 2.2 Important parameters for characterization of the intrinsic properties of aqueous two-phase systems. Interfacial tension (γ) can be estimated by the rotating drop method or the sessile drop method. Electrostatic potential difference ($\Delta\Psi$) is acquired with the use of reversible electrodes connected to salt bridges. Viscosity (μ), on its part, is simply estimated with the use of viscometers and/or pycnometers. Finally, osmotic pressure (π) can be obtained with the use of a vapor pressure or membrane osmometer

operations where polymer batches are changed frequently (Brooks and Jones 2000). To calculate interfacial tension in the typical low tension values observed in ATPS, two methods are recommended. First is the rotating drop method that considers that when a fluid drop (top phase) is placed in a liquid of higher density (bottom phase) contained in a rotating horizontal tube, its elongation along the axis will continue until the deformation forces due to the centrifugal field are balanced by the interfacial tension. Using the proper equipment and a series of equations, the interfacial tension can then be calculated (Princen et al. 1967; Walter 1994). In the other recommended method (i.e., the sessile drop method), the contact angle between the phase interface and the solid support can also be calculated besides interfacial tension. This is important because this contact angle might be closely related to the partition behavior of small suspended solids (Brooks and Jones 2000). In this method, a sufficiently large droplet of a phase solution is deposited over a surface and becomes deformed due to the gravitation force action. The surface area of the sphere is proportional to its squared radius, and the gravitational deformation depends on its volume which is proportional to the radius raised to the third power. If the effects of surface gravitational forces are comparable, then the interfacial tension (or in this particular case surface tension) of the phase can be calculated from the droplet shape (Staicopolus 1962). Modern computer program analyses of the droplet shapes have proven successful in the calculation of this parameter. In fact, recent developments in this area have allowed the use of entropic edge detector

schemes to enable edge detection of drops of ternary liquid systems with ultralow interfacial tensions. For instance, axisymmetric drop shape analysis (ADSA), a well-established methodology for interfacial tension measurements in conventional fluid-liquid systems, has given comparable results within an uncertainty of 0.001 mJ/m^2 with the sessile drop technique (Atefi et al. 2014).

The presence of some salts makes ATPS behave as if there was an electrostatic potential difference ($\Delta\Psi$) between them. This potential is driven by the unequal partition of some ion species because of the partial exclusion from the polymer-rich phase and is supported by the partitioning behavior of charged macromolecules in ATPS (Walter 1994). In this sense, potentials in biphasic systems are only a few millivolts at the most, but since most interaction energies in these systems are small, these electrostatic potentials can be able to dominate all partitioning behavior. Hence, measurements of this parameter can be crucial in the understanding of partition mechanisms. However, this is not a crucial task since potential measurements may lay on the detection limits of the equipment being used and do not provide thermodynamic potential difference between the phases due probably to the presence of liquid junction potentials at the tips of the salt bridges used for this purpose (Brooks and Jones 2000). In electrostatic potential measurements, reversible electrodes are connected by salt bridges to the two phases of the systems, one electrode is then slowly moved through the interface into the bottom phase, and the change in voltage is noted in time. The differences between the readings from the time when both bridges are immersed in one phase while one of the capillaries is slowly moved into the other phase provide an estimate for $\Delta\Psi$ (Walter 1994). It is important to mention, however, that this procedure needs to be repeated at least ten times for reliable estimations. Furthermore, if the change of potential across the interface is of interest, the measurement of electrophoretic mobilities of drops of one phase to another could provide this information. Nonetheless, the interpretation of these mobilities remains uncertain and requires further investigations, but it is believed that the potential reverses sign at the phase boundary implying the presence of a potential well on each side of the interface that could accumulate material with a net charge (Brooks and Jones 2000). This provides further information on the usual observation of product accumulation between both phases besides the effects of reduction of free volume and free energy that also contribute to this phenomenon.

All of the biomolecule transport processes in an ATPS and other aspects such as phase separation rate, mixture, centrifugation, and pumping energy (especially in large-scale operations) heavily depend on viscosity. Phase viscosity on its part depends on the phase-forming chemicals being used and the location of the system in the phase diagram. In this manner, concentration and polymer molecular weight are proportional to the viscosities obtained. In the particular case of polymer-polymer systems, viscosity measurements become complicated when a big difference in the molecular weight of the polymers being used exists. In these cases, the viscosity of the low molecular weight polymer phase remains practically constant, while the viscosity of the other phase (i.e., the high polymer molecular weight phase) strongly increases with TLL increments (Walter 1994). Despite this, viscosity measurements in ATPS are straightforward, inexpensive, and sensitive

procedures since they can be done with a simple capillary viscometer, a controlled temperature bath, an electronic timer, and a pycnometer (Brooks and Jones 2000).

Finally, when partitioning cells, organelles, or liposomes, the characterization of the osmotic pressure in the system is also recommended. In ATPS, buffer salts are the phase-forming chemicals that most contribute to this parameter, while the contribution of polymers is almost null (Walter 1994). Therefore, the low molecular weight components in the system should be isotonic with the products being partitioned. When necessary, osmotic pressure can be measured with the use of a vapor pressure or membrane osmometer. Membrane osmometers in the context of ATPS are used by placing a pre-equilibrated phase sample separated from pure water by a semipermeable membrane. The phase is not able to cross this membrane but water flow across it to dilute the phase. The pressure required to stop this water flow corresponds to the osmotic pressure. In the procedure involving vapor pressure osmometers, the concentration of osmotically active particles that reduce the vapor pressure of a particular ATPS phase is calculated, but this procedure is not regularly used.

As it can be seen, the successful implementation of an ATPS operation is heavily dependent on developing or adapting a phase diagram all the way to the measurement of the different properties of the system. None of these tasks are trivial, but their understanding and study can represent a great advantage in processing yields and reproducibility. These aspects are of great importance particularly in large-scale operations where numerous runs have to be made with a robust and reliable operation which to some point has been one of the main drawbacks in finding a larger amount of adapted ATPS processes in the biotechnological industry.

2.3 Solute Partition Characterization

As it has been stated in the previous section, partition of the different biotechnological products has a dependence on both the system characteristics and the physicochemical properties of the molecules being recovered in a rather complex thermodynamic scenario. However, to study this behavior, a simple description of the partition coefficient of the molecule (K_P), defined as the relation of the concentration of a particular product between the top and bottom phases of the system, is regularly used (Rito-Palomares and Lyddiatt 2002). The molecules involved in an ATPS operation interact with the phase-forming chemicals with ionic and hydrophobic interactions, hydrogen bonding, van der Waals forces, and noncovalent interactions and can also interact with themselves when high solute concentrations are being used (Benavides et al. 2011). The net effect of these interactions will then cause a selective partition of the molecule to either of the phases depending on the most favorable energy state that can be reached in each of the cases.

The three most influential solute physicochemical properties that influence partition in ATPS are size, electrochemical charge, and hydrophobic character (Olivera-Nappa et al. 2004). In the ATPS context, phase-forming chemicals are usually larger than the solutes, subjecting these molecules to steric effects and changing the available volume for them to partition toward a particular phase in a phenomenon

generally known as free volume effect. For the cases where some of the system materials are ionizable, electrochemical partition effects play an important role in the operation. Therefore, pH control in the system becomes crucial to maximize these types of interactions between ionizable solutes like proteins and the phase-forming components by generating charges that may provoke a selective fractionation. Finally, the hydrophobic interactions that play a role in solute partitioning in ATPS can be categorized in two well-known effects: phase hydrophobicity and the salting-out effect. Both effects generate hydrophobic interactions that promote solutes with lower amphipathicity and hydrophobicity to partition toward the most hydrophobic phase (Andrews et al. 2005; Schmidt et al. 1996). Furthermore, it should also be mentioned that addition of affinity ligands to ATPS is another exploitable strategy to promote ad libitum solute partitioning. In this case, ligands whose partition behavior in ATPS is already known and that present a certain biological activity or affinity toward a specific molecule are included in the system to increase the selectivity of the biphasic system (Ruiz-Ruiz et al. 2012).

With these interactions in mind, once the systems under study have reached equilibrium, the measurement of the concentrations of the solutes in the top and bottom phases of the system and in many cases that of the accumulated mass in the interface is required. As mentioned, this measurement allows the quantification of the partition coefficients of the molecules and allows a final characterization of the systems. To do so, the selection of appropriate analytical techniques should be considered. Commonly, this can be done spectrophotometrically either with the use of a calibration curve prepared at the maximum absorbance of the solutes under study or with specific colorimetric assays for that particular solute. In the case of systems where the product of interest can be visually observed with the aid of a microscope, like cells, or nanoparticles, concentration can be estimated in this way. More advanced analytical techniques such as high-performance liquid chromatography (HPLC), infrared, or mass spectroscopy, to mention some examples, can also be used depending on the level of accuracy needed in each case. However, it should always be taken into consideration that the phase constituents may interact with the selected analytical technique and appropriate blanks or precautions should always be used in order to obtain a reliable measurement (González-Valdez et al. 2011). Also, it should be remembered that the partition behavior of molecules in ATPS is difficult to model and that empirical approaches are usually used (Benavides et al. 2011). This can sometimes become a tedious task mainly because of all of the parameters that need to be evaluated in each of the partition procedures and the increasing degrees of freedom that each one of the possibilities represents within the system. In this context, high throughput characterization of solute partitioning is becoming an important tool that aids in the acceleration of finding the best conditions to acquire a particular behavior of a specific set of molecules.

The use of high throughput screening (HTS) platforms has gained momentum in the biotechnological industry during the last decade (Amrhein et al. 2014). This has allowed the development of highly automated and optimized processes with advantages that mainly include the reduction of operation materials, sample, and experimental efforts with more accurate analytics. These platforms are already being used in upstream operations such as the screening of strain mutants, substrates, and

microbial cultivation processes but are less used in downstream operations where most advances have been made in chromatographic operations. Nonetheless, this does not mean that ATPS have not been subject to optimization efforts using these kinds of technologies. In doing so, liquid handling stations (LHS) have provided the means to prepare, characterize, and optimize ATPS (Bensch et al. 2007). With this technology, binodal curves and tie-lines can be characterized without previous knowledge of the system behaviors. Binodal curves are obtained with dyes that are dissolved in the system components and by later correlating the top phase volume to the concentrating factor of the dye. Tie-lines are calculated by applying the lever arm rule on the phase volume, and the effect of density differences may be calculated with the use of lab-on-a-chip technologies (Amrhein et al. 2014). But furthermore, solute partition coefficients and yields can be quickly obtained with coupled sensors and measurement equipment compatible to robotic systems like turbidity measurements for the determination of cell debris and ELISA activity assays for the characterization of specific proteins (Bensch et al. 2007). The current capabilities of HTS in the context of ATPS allow the characterization of between 600 and 1000 phase systems per day. Problems and error sources such as liquid handling inaccuracies, top and bottom phase sampling, and meniscus forming because of the phase compositions that influence measurements are being addressed with the use of other integrated chip technologies within the HTS operations. It is important to mention that HTS procedures are in most manners compatible with traditional techniques used in ATPS solute characterization and can be also adapted to include more novel approaches such as distribution analysis of radiolabeled analytes (DARA) with rapid reversed phase chromatography analysis that aid with decision-making with regard to controlled conversion of monophasic systems into bi- or triphasic ones because of the addition of biomass and the addition of phase-forming chemicals to fine-tune a separation, variations between batches, and ATPS operation with phase-recycling (Lebreton et al. 2002). With this, the implementation of HTS methods in ATPS will certainly allow the coping with industrial relevant issues such as time to market demands, material consumption, cost efficiency, and process robustness according to quality-by-design requirements which are often some of the limitations that ATPS face before its industrial implementation. In this context, Fig. 2.3 presents the different considerations to have for the characterization of solute partitioning in ATPS.

2.4 Novel Operational Strategies and Potential New Applications for Aqueous Two-Phase Systems

Being a highly biocompatible operation, traditional ATPS strategies involve mainly the primary recovery and purification of proteins, nucleic acids, virus, viruslike and other bionanoparticles, cells, organelles, and low molecular weight compounds, and in this matter, extensive reviews and scientific publications can be found on this subject (Benavides and Rito-Palomares 2008). But furthermore, ATPS are starting to be implemented in novel strategies and in different manners that are extending their use while opening new application possibilities.

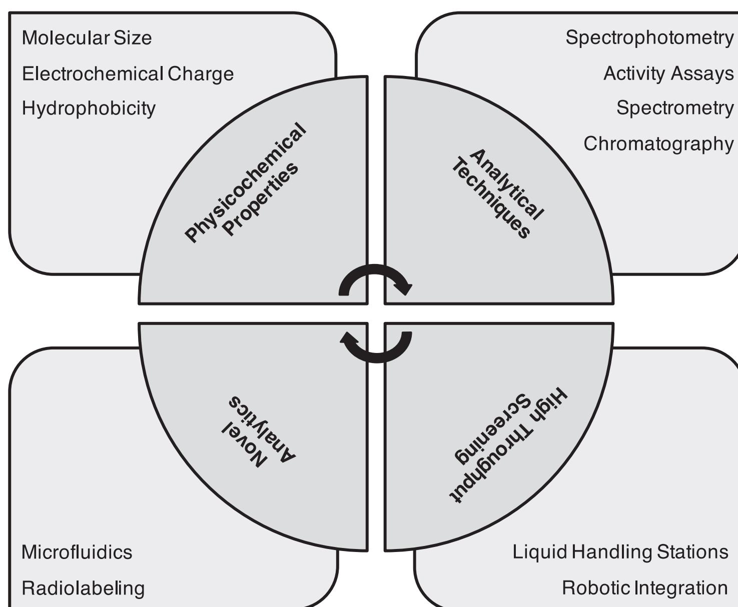

Fig. 2.3 Considerations for solute partition characterization. The diagram presents the different aspects to be taken into account to establish the partition behavior of the different solutes loaded in an aqueous two-phase system. These include the physicochemical properties of both the solutes and the phase-forming chemicals, the appropriate common and novel analytical techniques for concentration measurements, and the possibility of using high throughput screening techniques for this purpose

One of the new strategies being implemented with ATPS involves unit operation integration. In this manner, ATPS offer the possibility of serving as an extraction and primary recovery operation with the implementation of cell disruption within the system in a single operation. With this strategy, after culture and centrifugation, cells can be transferred to the system where cell disruption can be achieved by mechanical or chemical methods liberating the products and offering a partition environment where products will partition toward a specific phase while cell debris and other molecules preferably partition toward the other (Rito-Palomares and Lyddiatt 2002). Even more, going back a few stages, ATPS offer as well the possibility of serving as a reactor or fermenter and a recovery operation. In these approaches, ATPS can theoretically be designed to be formed by a “culture medium” phase and a polymeric one where, after inoculation, cells grow in one of the phases and liberate the products toward the other phase. This is achievable especially by those organisms that grow under saline conditions and where products may represent a growth inhibitor since once in the system products can be continuously released to the opposite phase. Enzymatic reactions can

also be accommodated in specific ATPS with an appropriate knowledge of the partition behaviors of the substrates, products, and enzymes. Furthermore, certain chemical reactions can also be achieved in biphasic systems in a similar fashion (Andersson and Hahn-Hägerdal 1990).

The use of "intelligent" or "smart" polymers capable of responding to external physical stimuli by changing some of their mechanical properties is a current matter of interest in ATPS development. The use of the said polymers is allowing the appearance of novel systems that besides presenting interesting partition and recovery yields for many products allow the simplification of the operations needed to later remove the phase-forming components from the processing streams. For example, thermosensitive polymers are being used in the generation of ATPS for the recovery of proteins. After phase separation, the proteins can be removed from the polymeric solution by allowing the precipitation of the polymer with the addition of heat. Besides interchanging a rather expensive operation like ultrafiltration for the removal of the polymers for a much simpler one where only heat is applied, this procedure allows the recovery of the polymer that was used that can later be recycled back into the ATPS operation (Lladosa et al. 2012).

Another novel approach in ATPS usage refers to the refolding of proteins. Usually, recombinant molecules expressed in certain microorganisms are obtained in insoluble forms or inclusion bodies. Recent studies have shown that ATPS possess an interesting potential in becoming a protein refolding operation, specifically because of their high-water content that favors the reduction of chaotropic agents used in the solubilization of these molecules and because polymers like polyethylene glycol (PEG) usually used in ATPS operations can function as chaperone molecules that promote the correct refolding of the proteins in solution. In the same line, refolding operations can be optimized with the addition of additives that depending on the protein can enormously enhance its correct refolding like is the case for the addition of metal ions to ATPS to refold denatured metalloenzymes (Sánchez-Trasviña et al. 2015). In fact, the advantages of ATPS usage in protein refolding have shown multiple advantages over more traditional refolding procedures involving the use of packed columns (Kuboi et al. 2000).

The use of microfluidic devices in ATPS operations is also a major trend in the area. The advantages presented by these devices like their operation in laminar flow, portability, and low solution usage are starting to become an interesting opportunity for product recovery especially in those systems where the product of interest is in very low concentrations (Munchow et al. 2007; Vázquez-Villegas et al. 2013, 2016). As mentioned, microfluidic devices can also be exploited for ATPS characterization purposes making this approach versatile in the sense in which it can be used as both a processing and an analytical technique. In fact, the use of microdevices in ATPS operations is allowing the characterization of ATPS operation in continuous mode in contrast to the traditional batch operation. In fact, ATPS operating in continuous mode are now operational up to a pilot scale with comparable results to those observed in batch mode operations (Vázquez-Villegas et al. 2015).

As an analytical tool, ATPS provide the means of obtaining and measuring some of the physicochemical properties of the molecules, particles, or cells being partitioned. The correlations between their partition coefficients and the known properties of the systems with the superficial physicochemical properties of the solutes allow the calculation of their superficial properties and hydrophobicity in a simple manner that can later be of utility in sorting and other separation procedures (Andrews et al. 2005; Trindade et al. 2005). In the same line, ATPS are being used as in three-dimensional proteomic analysis in combination with 2D electrophoresis where crude protein extracts can be characterized according to their molecular weight, isoelectric point, and partition coefficient that serves as an indicator of hydrophobicity (Aguilar et al. 2009).

As seen, the uses of ATPS go much further than their traditional application in the primary recovery of biotechnological products. Novel ATPS are constantly being developed for more and more particular applications with the use of novel components or techniques both for operational and analytical purposes. ATPS is a versatile and robust technique that can be used in a macro- or a microscale with different advantages for specific operations and available also for its use in batch and continuous processes. This technique that has been constantly used during more than half a century continues to be of importance in the research and industrial biotechnological fields. Figure 2.4 presents a graphical summary of the novel operational strategies and applications for ATPS presented in this section.

2.5 Concluding Remarks

The successful implementation of ATPS strategies requires a careful consideration of many designs and physicochemical aspects to meet the required purification and recovery standards needed in the operation. The characterization of ATPS starts with the appropriate selection of the phase-forming chemicals, the generation of the binodal curve, and all of the thermodynamic parameters like TLL and V_R for those specific components. The measurement of the intrinsic properties of each of the generated systems is as well a desirable procedure to better understand the observed partition behaviors of the solutes or to predict such behavior in an educated manner. Afterwards, the study of the partition of molecules in them should be performed extensively.

ATPS characterization is important because this liquid-liquid extraction strategy continues to be an important operation because of the great advantages it has in comparison to other more complicated, expensive, and time-consuming operations. Furthermore, ATPS is not only a purification strategy found in some biotechnological processes but is also a strategy that allows process integration and an interesting tool that can be employed in analytical measurements.

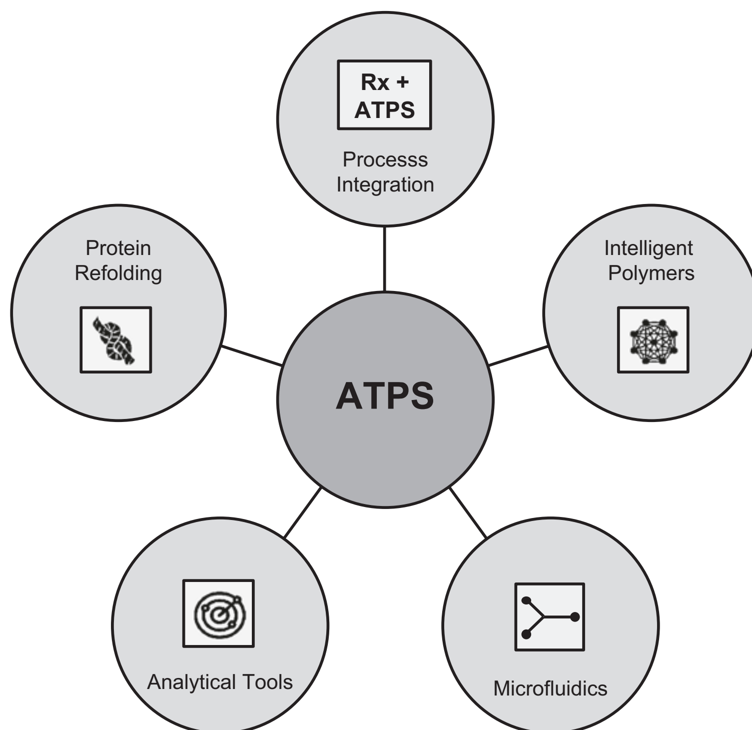

Fig. 2.4 Schematic summary of the novel and forthcoming ATPS-based strategies and applications

References

Aguilar O, Glatz CE, Rito-Palomares M. Characterization of green-tissue protein extract from alfalfa (Medicago sativa) exploiting a 3-D technique. J Separation Sci. 2009;32(18):3223–31.

Amrhein S, Schwab M-L, Hoffmann M, Hubbuch J. Characterization of aqueous two phase systems by combining lab-on-a-chip technology with robotic liquid handling stations. J Chromatogr A. 2014;1367:68–77.

Andersson E, Hahn-Hägerdal B. Bioconversions in aqueous two-phase systems. Enzyme Microbial Technol. 1990;12(4):242–54.

Andrews BA, Schmidt AS, Asenjo JA. Correlation for the partition behavior of proteins in aqueous two-phase systems: effect of surface hydrophobicity and charge. Biotechnol Bioeng. 2005;90(3):380–90.

Atefi E, Mann JA, Tavana H. Ultralow interfacial tensions of aqueous two-phase systems measured using drop shape. Langmuir. 2014;30(32):9691–9.

Benavides J, Rito-Palomares M, Asenjo JA. Aqueous two-phase systems. Comprehensive Biotechnol. 2011:697–713.

Benavides JA, Rito-Palomares MA. Practical experiences from the development of aqueous two-phase processes for the recovery of high value biological products. J Chem Technol Biotechnol. 2008;83(2):133–42.

Bensch M, Selbach B, Hubbuch J. High throughput screening techniques in downstream processing: preparation, characterization and optimization of aqueous two-phase systems. Chem Eng Sci. 2007;62(7):2011–21.

Brooks DE, Jones RN. Measurement of some physical properties of aqueous two-phase systems. Aqueous Two-Phase Systems Methods Protoc. 2000:35–45.

de Oliveira RM, JSDR C, Francisco KR, Minim LA, LHM d S, JAM P. Liquid−liquid equilibrium of aqueous two-phase systems containing poly(ethylene) glycol 4000 and zinc Sulfate at different temperatures. J Chem Eng Data. 2008;53(4):919–22.

Diamond AD, Hsu JT. Aqueous two-phase systems for biomolecule separation. Adv Biochem Eng Biotechnol. 1992;47:89–135.

Diamond AD, Hsu JT. Fundamental studies of biomolecule partitioning in aqueous two-phase systems. Biotechnol Bioeng. 1989;34(7):1000–14.

Forciniti D. Preparation of aqueous two-phase systems. Aqueous Two-Phase Systems Methods Protoc. 2000:23–33.

González-Valdez J, Rito-Palomares M, Benavides J. Effects of chemical modifications in the partition behavior of proteins in aqueous two-phase systems: a case study with RNase a. Biotechnol Prog. 2013;29(2):378–85.

González-Valdez J, Rito-Palomares MA, Benavides JA. Quantification of RNase a and its PEGylated conjugates on polymer-salt rich environments using UV spectrophotometry. Anal Lett. 2011;44(5):800–14.

Kaul A. The phase diagram. Aqueous Two-Phase Systems Methods Protoc. 2000:11–21.

Kuboi R, Morita S, Ota H, Umakoshi H. Protein refolding using stimuli-responsive polymer-modified aqueous two-phase systems. J Chromatogr B Biomed Sci App. 2000;743(1):215–23.

Lebreton B, Walker SG, Lyddiatt A. Characterization of aqueous two-phase partition systems by distribution analysis of radiolabeled analytes (DARA): application to process definition and control in biorecovery. Biotechnol Bioeng. 2002;77(3):329–39.

Leong YK, Lan JC-W, Loh H-S, Ling TC, Ooi CW, Show PL. Thermoseparating aqueous two-phase systems: recent trends and mechanisms. J Separation Sci. 2016;39(4):640–7.

Lladosa E, Silvério SC, Rodríguez O, Teixeira JA, Macedo EA. (Liquid + liquid) equilibria of polymer-salt aqueous two-phase systems for laccase partitioning: UCON 50-HB-5100 with potassium citrate and (sodium or potassium) formate at 23 °C. J Chem Thermodyn. 2012;55:166–71.

Mayolo-Deloisa K, González-Valdez J, Guajardo-Flores D, Aguilar OA, Benavides JA, Rito-Palomares MA. Current advances in the non-chromatographic fractionation and characterization of PEGylated proteins. J Chem Technol Biotechnol. 2011;86(1):18–25.

Mistry SL, Kaul A, Merchuk JC, Asenjo JA. Mathematical modelling and computer simulation of aqueous two-phase continuous protein extraction. J Chromatogr A. 1996;741(2):151–63.

Montalvo-Hernández B, Rito-Palomares M, Benavides J. Recovery of crocins from saffron stigmas (Crocus sativus) in aqueous two-phase systems. J Chromatoge A. 2012;1236:7–15.

Munchow G, Hardt S, Kutter JP, Drese KS. Electrophoretic partitioning of proteins in two-phase microflows. Lab Chip. 2007;7(1):98–102.

Olivera-Nappa A, Lagomarsino G, Andrews BA, Asenjo JA. Effect of electrostatic energy on partitioning of proteins in aqueous two-phase systems. J Chromatogr B. 2004;807(1):81–6.

Princen HM, Zia IYZ, Mason SG. Measurement of interfacial tension from the shape of a rotating drop. J Colloid Interface Sci. 1967;23(1):99–107.

Rito-Palomares MA, Lyddiatt A. Process integration using aqueous two-phase partition for the recovery of intracellular proteins. Chem Eng J. 2002;87(3):313–9.

Rito-Palomares MA. Practical application of aqueous two-phase partition to process development for the recovery of biological products. J Chromatogr B. 2004;807(1):3–11.

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(16):2296–305.

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.

Sánchez-Trasviña C, González-Valdez J, Mayolo-Deloisa K, Rito-Palomares M. Impact of aqueous two-phase system design parameters upon the in situ refolding and recovery of invertase. J Chem Technol Biotechnol. 2015;90(10):1765–72.
Schmidt AS, Andrews BA, Asenjo JA. Correlations for the partition behavior of proteins in aqueous two-phase systems: effect of overall protein concentration. Biotechnol Bioeng. 1996; 50(6):617–26.
Staicopolus DN. The computation of surface tension and of contact angle by the sessile-drop method. J Colloid Sci. 1962;17(5):439–47.
Trindade IP, Diogo MM, Prazeres DMF, Marcos JC. Purification of plasmid DNA vectors by aqueous two-phase extraction and hydrophobic interaction chromatography. J Chromatogr A. 2005;1082(2):176–84.
Vázquez-Villegas P, Aguilar O, Rito-Palomares M. Continuous enzyme aqueous two-phase extraction using a novel tubular mixer-settler in multi-step counter-current arrangement. Sep Purif Technol. 2015;141:263–8.
Vazquez-Villegas P, Ouellet E, González C, Ruiz-Ruiz F, Rito-Palomares MA, Haynes C, et al. A microdevice assisted approach for the preparation, characterization and selection of continuous aqueous two-phase systems: from micro to bench-scale. Lab Chip. 2016;16:2662–72.
Vázquez-Villegas P, Espitia-Saloma E, Rito-Palomares M, Aguilar O. Low-abundant protein extraction from complex protein sample using a novel continuous aqueous two-phase systems device. J Separation Sci. 2013;36(2):391–9.
Walter H. Aqueous two-phase systems. Berlin: Elsevier; 1994.
Zaslavsky B. Aqueous two-phase partitioning: physical chemistry and bioanalytical applications. New York: Marcel Dekker, Inc.; 1995.

Practical Aspects for the Development of ATPS-Based Processes for Protein Recovery

3

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Abbreviations

ATPS	Aqueous two-phase systems
CI2	Chymotrypsin inhibitor 2
HTS	High-throughput screening
K_P	Partition coefficient
MW	Molecular weight
PEG	Polyethylene glycol
pI	Isoelectric point
PPO-Ph	Poly(propylene oxide)-phenyl
RNase A	Ribonuclease A
TLL	Tie-line length
UCON	Ethylene oxide and propylene oxide
V_R	Volume ratio
α-Lac	α-lactalbumin

3.1 Introduction

Because of their biocompatibility, aqueous two-phase systems (ATPS) have shown to be an interesting primary recovery operation of different biological products from which proteins are probably the most important (Rito-Palomares 2004). Nowadays, a wide range of proteic formulations ranging from household products to pharmaceutical

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therapies can be found in the market. In fact, it could be expected that the number of protein-based products will continue to show a steady increase during the following decades mainly because there are many functions that these molecules can perform. Even more, with the advancements in synthetic biology techniques, proteins with novel properties or functionalities can be easily designed transforming them into very versatile molecules which without doubt will find interesting applications in different aspects of human life (Benavides et al. 2008). The production and purification of proteins will continue to be an important engineering problem for the biotechnological industry and innovations in the way these biomolecules are prepared and processed will always represent advancements in the area. Certainly, in this context, ATPS purification strategies will continue to be an easy-to-implement and attractive alternative for these purposes.

However, the development of aqueous two-phase system strategies for the recovery of proteic products requires, first, a complete understanding of the different stream components that are going to be processed. Being a primary recovery operation, ATPS are usually implemented in the first steps of the downstream process, and therefore the number of molecules that is introduced in them is usually large and/or diverse. Ideally, the target product is sought to partition toward either phase, while the rest of the molecules in the sample or contaminants should partition to the opposite one (Benavides and Rito-Palomares 2011). This, however, is not a trivial task and extensive experimentation, and modeling is required since the different physicochemical properties involved in the partition of molecules within an ATPS are very complex. Furthermore, in most cases there is no complete partition of the target molecule or the contaminants to different phases, and concentrated mixtures of either the target biomolecule and some contaminants or vice versa are presented adding difficulties to the generalized implementation of this strategy. Nonetheless, there is now an extensive knowledge on the different partition behaviors of many molecules in different ATPS making it, to some extent, easier to predict and develop efficient strategies in different engineering contexts.

In our experience, the work with ATPS for the recovery of proteins has represented a very interesting topic with successful outcomes using diverse protein sources and mixtures. In this sense, besides working with protein extraction from crude extracts which is commonly one of the most usual procedures in ATPS, we have also worked with the recovery of these biomolecules from waste streams and different chemical reactions. Table 3.1 shows some of our works in the recovery of proteins using ATPS. This experience has allowed the devise of efficient ATPS implementation strategies where several common processing steps are always used. In this context, the objective of this chapter is to present a practical overview on the different procedures, challenges, and experiences we have encountered along the way, to lead and advice other people working with ATPS.

Table 3.1 Selected studies on the development of ATPS-based processes for protein recovery

Protein	Type of ATPS	Biological origin	Product recovery (%)	Reference
Albumin	PEG 3350-PO_4	Human serum	92.0	Garza-Madrid et al. (2010)
α-Amylase	PEG 1000-PO_4	*Glycine max*	81.0	Vázquez-Villegas et al. (2013)
β-Glucuronidase	PEG 600-PO_4	*Glycine max*	83.0	Aguilar and Rito-Palomares Aguilar and Rito-Palomares (2008)
β-Phycoerythrin	PEG 1000-PO_4	*Porphyridium cruentum*	90.0	Benavides and Rito-Palomares (2006)
β-Phycoerythrin	PEOS-12 – PO_4	*Porphyridium cruentum*	84.0	Hernandez-Mireles and Rito-Palomares (2006)
Invertase	PEG 1000-PO_4	Spent brewer's yeast	31.4	Vázquez-Villegas et al. (2015)
Invertase	PEG 3350-PO_4	*Saccharomyces cerevisiae*	60.8	Sánchez-Trasviña et al. (2015)
Laccase	UCON – $(NH_4)_2SO_4$	*Pleurotus ostreatus*	98.3	Bertrand et al. (2016)
Laccase	PEG 1000-PO_4	*Agaricus bisporus*	95.0	Mayolo-Deloisa (2009)
Major royal jelly protein	PEG 600 – PO_4	Recombinant *Pichia pastoris*	96.0	Ibarra-Herrera et al. (2014)
Mono-PEGylated α-lactalbumin	PEG 8000-PO_4	Bovine	77.0	González-Valdez (2011)
Mono-PEGylated ribonuclease A	PEG 8000-PO_4	Bovine	98.3	González-Valdez (2011)
HPV16 L1 protein	PEG 1000-PO_4	Recombinant *Escherichia coli*	65.0	Rito-Palomares and Middelberg (2002)
Superoxide dismutase	t-Butanol-$(NH_4)_2SO_4$	*Kluyveromyces marxianus*	80.0	Simental-Martínez et al. (2014)

3.2 General Strategy in the Selection of ATPS

As it has been mentioned, one of the main advantages making ATPS an interesting primary recovery operation for the recovery of proteins is their highly aqueous environment and the possibility they provide to work at neutral pH and other non-denaturing conditions found in traditional liquid-liquid extraction procedures. Furthermore, ATPS exhibit other advantages such as economic attractiveness, scalability, short phase separation, and processing times besides mild-separating conditions that help maintain the biological activities of the molecules being processed (González-González et al. 2011; Ruiz-Ruiz et al. 2012). Although both polymer–salt and polymer–polymer systems have been mainly used for protein recovery, polyethylene glycol (PEG)–salt ATPS are the most commonly used. From this group, the most suitable phase-forming chemical combination for protein extraction is the use of PEG and phosphate salts due to processing advantages such as the low cost, the vast knowledge accumulated in the characterization of these systems, and the range of operating system pH values (from 6 to 9) under which these ATPS are stable (Rito-Palomares 2004). Besides, in these extraction systems, the product of interest is generally concentrated in the PEG-rich phase (top phase) and the contaminants in the salt-rich phase (bottom phase) in different extents. However, since there are several combinations of polymer–polymer or polymer–salt systems available, it can be difficult to decide on an option when working with a protein for which no previous systems have been reported. In such cases, the recommendation would be to start with the general strategy described in Fig. 3.1. This strategy considers four main stages: (1) the characterization of the protein or the extract, (2) partition screening using 16 PEG–phosphate systems, (3) analysis and optimization of the best systems, and (4) protein recovery.

3.2.1 Protein–Extract Characterization

In this first stage, which is generally performed prior to system construction, it is necessary to know the most important characteristics of the target protein such as molecular weight (MW), isoelectric point (pI), hydrophobicity, and concentration. If this molecule is an enzyme, it is very important to measure its activity and how the different contaminants or properties of the extract affect its measurement, the influence of pH on the activity, and the best technique to measure total protein concentration. In this sense, it is important to consider that protein monitoring throughout different quantification methodologies (i.e., UV spectrophotometry, fluorescence, colorimetric assays, etc.) is frequently hindered by the presence of high-polymer and salt concentrations (that commonly reach up to 30% w/w) in the systems (Barbosa et al. 2009; Dumetz et al. 2009; Gonzalez-Gonzalez 2011). Therefore, it is fundamental to determine the most favorable and reliable techniques for this purpose before starting with the operation. It should also be mentioned that another technique widely used to characterize the protein profile of the sample is SDS-PAGE. This technique allows the clear observation of protein profiles and concentration

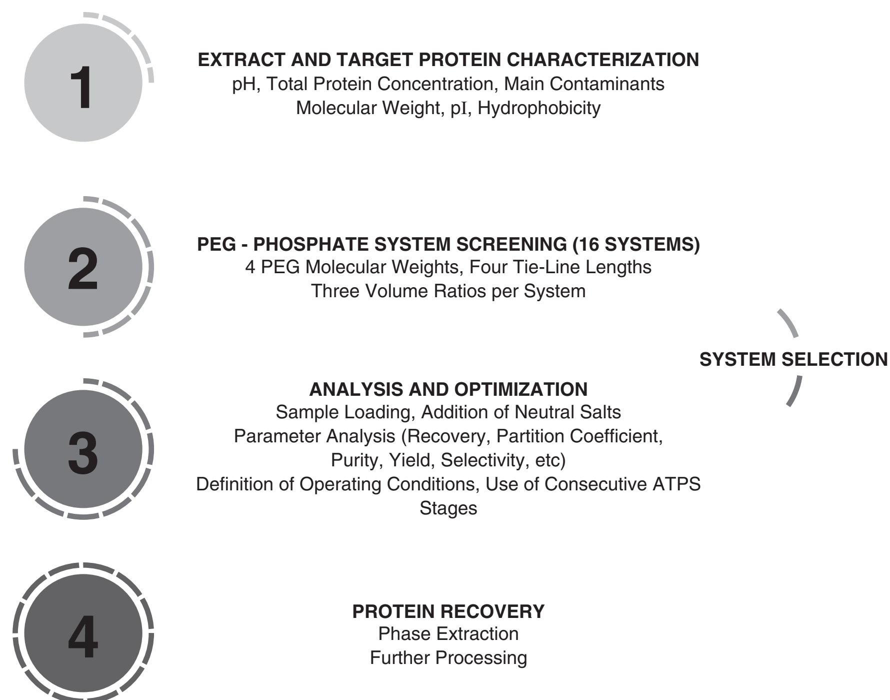

Fig. 3.1 Recommended general strategy for the design of ATPS operation for the recovery of proteins

differences throughout the process. Therefore, it is widely recommended to perform it in each of the extraction stages of the ATPS operation. The selection of the different study parameters and necessary information or physicochemical properties related to the target molecule and the contaminants present in the sample or feedstock will depend on the objective for which the extraction is being carried out and the destination of the protein to be purified.

3.2.2 Partition Screening Using 16 PEG–Phosphate Systems

After determining the most relevant physicochemical characteristics of the sample according to the extraction purposes, the screening of the partition behavior of the different sample components can be initiated using different PEG–phosphate systems. Tables 3.2 and 3.3 show two different system sets that have been previously reported and used successfully for the recovery of proteins (Mayolo-Deloisa et al. 2009; González-Valdez et al. 2011; Sánchez-Trasviña et al. 2015; Bertrand et al. 2016). The idea in this stage is to evaluate the influences of polymer molecular weight (MW) and the tie-line length (TLL) in the partitioning of these components since it is well known that an increase in the polymer MW and its concentration can

Table 3.2 Recommended initial PEG–phosphate system composition for the fractionation of proteic materials

System	PEG MW (g/mol)	TLL (% w/w)	PEG (% w/w)	PO_4 (% w/w)
1	400	15	16.5	16.0
2		25	17.2	15.5
3		35	21.0	16.9
4		45	22.0	17.0
5	1000	15	12.0	19.6
6		25	13.3	15.0
7		35	16.0	16.0
8		45	19.2	17.1
9	3350	15	10.0	10.8
10		25	16.9	11.8
11		35	15.0	13.2
12		45	18.8	15.0
13	8000	15	10.2	8.8
14		25	13.0	10.4
15		35	16.1	12.3
16		45	20.2	14.8

The system pH and V_R are kept constant at 7.0 and 1.0, respectively. The composition of each system was estimated using the binodal curves presented by Zaslavsky (1995)

Table 3.3 Composition of systems used for the recovery of laccase from residual compost of *Agaricus bisporus* that might be used when sample complexity increases or phase-forming chemicals should be added separately when phase formation becomes a concern

System	PEG MW (g/mol)	TLL (% w/w)	PEG (% w/w)	PO_4 (% w/w)	Dibasic PO_4 (% w/w)	Monobasic PO_4 (% w/w)
1	1000	40	18.20	15.00	10.80	4.20
2		45	18.90	16.00	11.52	4.48
3		55	22.20	19.00	13.68	5.32
4		59	24.10	20.10	14.47	5.63
5	1450	36	15.10	13.00	9.36	3.64
6		39	17.50	14.30	10.30	4.00
7		51	21.90	18.00	12.96	5.04
8		56	23.00	19.80	14.26	5.54
9	3350	43	22.20	19.00	13.68	5.32
10		56	24.10	20.10	14.47	5.63
11		58	21.90	18.00	12.96	5.04
12		62	18.00	15.00	10.80	4.20
13	8000	30	15.00	11.00	7.92	3.08
14		33	16.50	11.50	8.28	3.22
15		41	19.00	12.50	9.00	3.50
16		63	24.10	20.10	14.47	5.63

affect both the protein hydrophobicity (Franco et al. 1996) and the excluded phase volume (Benavides and Rito-Palomares 2004). The analysis of these systems has then the purpose of finding the preference of the target protein and the contaminants for each phase. The overall goal is to find those conditions in which the target molecule migrates toward the PEG-rich phase and the contaminants to the opposite phase (salt phase). Based on practical experience, it has been observed that the recovery of hydrophilic high molecular weight (>10,000 g/mol) compounds is favored when both low MW PEG (<4000 g/mol) and low or medium TLL values (<40% w/w) are selected. In contrast, the recovery of hydrophobic low molecular weight (<10,000 g/mol) products is favored when high MW PEG (>6000 g/mol) and medium or high TLL (>30% w/w) are used (Benavides and Rito-Palomares 2008). So, the use of these well-defined systems allows the characterization of how different polymer molecular weights and TLL values affect the behavior of the sample while performing a complete partition analysis. Regarding phase volume ratio (V_R) and pH, initial values of 1.0 and 7.0 are recommended to avoid any potential concentration effects or induced electrostatic interactions. It is also important to point out that the systems must be constructed to the most adequate final volume considering the concentration and quantity of sample. For this reason, systems with a total weight of 2, 5, or 10 g with sample concentrations representing no more than 10% of the total system weight can be constructed to perform this screening. High-throughput screening (HTS) techniques can also be used when sample reduction is necessary (Benavides and Rito-Palomares 2008).

3.2.3 Analysis and Optimization of the Best Systems

After obtaining the results from the screening analysis, the best systems can be selected to perform an optimization procedure. Different quantitative and practical criteria can be used to carry out this selection. Noticeably, the quantitative ones will be based on the obtained partition coefficient (K_P), recovery percentages, selectivity, yield, specific activity, and/or purification factor. However, it is also important to take into consideration practical approaches such as the phase viscosity, the formation of an interface and/or a precipitate, and the interference of the phases in the activity or stability of the protein. Again, all these entirely depend on the final use of the product of interest.

If the selected combination of MW of PEG and TLL results in the required product recovery, the system parameters stage is complete (Benavides and Rito-Palomares 2008). Otherwise, different strategies can be used to increase the recovery such as (1) varying the V_R to concentrate the protein in one of the phases, (2) enhance sample loading, and (3) addition of neutral salts. The systems presented in Tables 3.2 and 3.3 are designed to use a $V_R = 1.0$. But if that is not enough to accomplish a suitable protein recovery percentage, it is recommended using lower or higher values than that recommended initial value (*v.gr.* 0.3 and 3). In this context, changes in V_R modify the free volume available in the phases that might promote a change in partition. It has been suggested that as V_R increases, the partition coefficient increases as more target

protein is partitioned into the polymer-rich top phase (Ng et al. 2011). From practical experience, it has been concluded that an increment in V_R usually causes an increment in both the recovery and purity of high-molecular-weight products (>100,000 g/mol), while low-molecular-weight products (<50,000 g/mol) observe an increment in recovery but not always in purity (Benavides and Rito-Palomares 2008). Additionally, sample loading can be modified until the system becomes saturated. Generally, the systems are designed for the incorporation of up to a 10% w/w of sample. But this concentration can be changed using water to reach the total weight of each system. Thus, sample concentration can be modified from 1 to 10% w/w. However, increases up to 40% w/w have been achieved in PEG–salt systems with positive results (Benavides and Rito-Palomares 2008). The increase in sample concentration should however be performed gradually because, in some cases, increasing the loaded mass of a sample into the ATPS may decrease the V_R and alter the composition of the systems. Besides, the components contained in the crude load (when a crude extract is used) may also change the characteristics of an ATPS causing a selected system to no longer present the best conditions for partitioning as seen in screening experiments (Ng 2011). As a final step, the addition of neutral salts to ATPS can be exploited to enhance the recovery of the target protein. Changes in the salt type and its concentration often produce an electrical potential difference between the two phases caused by the preference of one of the ions to a particular phase (Johansson 1970). The supplementary ionic strength may favor the solubilization of compounds accumulated at the interface of the system into either the top or bottom phase. However, the addition of salts, such as NaCl, may also cause reversible or irreversible denaturalization of halo-sensitive biological compounds present in the system. Therefore, it is necessary to evaluate the effect of the addition of neutral salts upon product recovery, purity, and activity. In this sense, experiments using a progressive increase in the concentration of NaCl such as 0, 0.25, 0.50, 1.0, and 2.0 M are recommended (Benavides and Rito-Palomares 2008).

3.2.4 Recovery of the Partitioned Product

The recovery of the target protein can be finally reached separating the phases in the system. Sometimes the phase containing the protein can be passed directly into the next purification step or unit operation. In most occasions, the phase-forming chemicals need to be separated using membrane operations ultrafiltration/diafiltration which also allows the concentration of the product. Another alternative is to scale the selected system to enhance its capacity making it very important to have an established objective since the beginning of the separation using ATPS. As mentioned, this general strategy can be used for the recovery of any biomolecule, but its use has been probed mostly efficient for the recovery of different proteins. Even when PEG–phosphate salt systems have been directly approached, this strategy can be adopted in a similar fashion to polymer–polymer systems or other types of ATPS.

3.3 Experiences in the Primary Recovery of Proteins from Waste Streams

Proteins are one of the fundamental building blocks for the sustainability of life (Gong et al. 2016). As mentioned before, the increasing need to bring new protein-based products to pharmaceutical and industrial markets using scalable and efficient bioprocessing technology has raised the need of establishing different methodologies for their recovery (Sánchez-Trasviña 2015). The major drawbacks associated with the exploitation and procurement of enzymes are, in many cases, the high production costs and the low production yields that are obtained (Makris 2015). In this manner, the possibility of revalorizing waste materials to obtain high added-value products (as is the case of proteins) from them is essential since these by-product sources represent an economical, extensive, and safe source of them (Bertrand et al. 2013).

In this context, it has been reported that roughly one-third of the edible parts of food produced for human consumption gets lost or wasted globally which account for approximately 1.3 billion tons per year (Gustavsson et al. 2011). This figure does not consider the wastes generated by the cultivation or treatment of such products. These streams are an incredible source of raw materials and/or high added-value compounds that need to be processed to take economical advantage from them while reducing their impact on the environment. In this line, there is an actual need to develop novel recovery and reuse technologies, along with the development of sustainable ideas, technologies, and processes to avoid the loss of all these compounds attached to these wastes (Reis et al. 2012). Because of this, we believe it is the duty of researchers to find alternatives for the reuse of such wastes.

For this reason, we have dedicated part of our efforts to the recovery of different enzymes such as laccase using aqueous two-phase systems. Laccases are oxidoreductases commonly secreted out to the medium extracellularly by several fungi (specially white-rot fungi) during their secondary metabolism (Morozova et al. 2007). Laccase can be used in bioremediation, beverage (wine, fruit juice, and beer) processing, ascorbic acid determination, sugar-beet pectin gelation, baking, and biosensors, among other uses. Due to its environmental, industrial, and economic importance, laccase is widely studied, and its purification processes are not the exception (Minussi et al. 2002).

It has been reported that during the growth of edible fungi such as *Agaricus bisporus* and *Pleurotus ostreatus* on composted wheat straw, large amounts of laccase are produced. After fruit-body harvesting, a considerable amount of residual compost is discarded as by-product. The residue is also a potential source of other ligninolytic enzymes besides laccase (Trejo-Hernandez et al. 2001).

The potential of ATPS for the recovery of laccase from fungi has been widely reported (Lladosa et al. 2012; Moreira et al. 2013; Silvério et al. 2013; Prinz et al. 2014a, b; Schwienheer et al. 2015; Rajagopalu et al. 2016). However, most published works extract the enzyme from liquid fermentations and/or use the pure enzyme to show its partition in the systems under studies. The conditions of these

experiments are very different because the complexity of the extract from which the enzyme is obtained is greater when residual compost is used.

In the same line of using industrial waste streams for the recovery of valuable biomolecules, we have reported the recovery of invertase from wasted brewer's yeast using ATPS (León-González et al. 2015). The beer industry generates a very large amount of biomass since the utilized yeast is generally discarded after a certain number of fermentations to assure product quality and consistence. After this life-span, wasted yeast is usually used as animal feed, being a very good source of proteins for ruminants and swine. However, a large amount of valuable proteins and enzymes such as invertase, amylases, and proteases present in these microorganisms could represent an attractive side business or opportunity for the same beer companies since these enzymes are also usually supplemented by separate to their fermentation broths.

3.3.1 Recovery of Laccase from Residual Compost of Agaricus Bisporus Using ATPS

The complexity of the components of residual compost and the lack of information about them is one of the main problems when trying to obtain proteins from compost crude extracts. In fact, this is one of the general problems limiting the use of residues for the recovery of proteins in ATPS. However, by monitoring the enzymatic activity and the total protein concentration throughout the ATPS operation, these strategies have been found to be effective as it has been shown for laccase. To our knowledge, the recovery of laccase from residual compost of *Agaricus bisporus* (Mayolo-Deloisa 2009) was the first work where this protein was partially purified using ATPS. This strategy was chosen since there were no previous reports regarding the topic. As it has been mentioned and suggested as a general strategy, the 16 PEG–phosphate systems with a V_R of 1.0 were used (Table 3.3), varying the TLL, the molecular weight of PEG, and the crude extract concentration. The general strategy consisted first in demonstrating the partition of laccase in one phase and second in trying to increase the recovery percentage of the enzyme in that phase.

In practice, the preparation of each system with a reported binodal curve is relatively easy. Generally, the systems can be prepared by weighing concentrate solutions of each phase component until reaching the exact composition, but in the work mentioned before, each component was weighted individually. To facilitate the reproducibility of the systems, the exact composition of each phase-forming chemical is shown in Table 3.3. It should be mentioned that these systems are robust and the formation of the two phases is not broken when the sample is added. However, it is important to assure that the sample weight does not exceed 10% w/w of the total weight of the system. Overall, laccase was partitioned toward the PEG-rich phase (top phase), and enzymatic activity was not detected in the salt-rich phase (bottom phase). Furthermore, it was clearly observed that the top-phase recovery of laccase decreased with increments in TLL and PEG MW.

3.3.2 Recovery of Laccase from Residual Compost of Pleurotus Ostreatus Using ATPS

For many reasons, *Pleurotus* genus, commonly known as oyster fungus, has been intensively studied in many different parts in the world: it presents a high gastronomic value, it can colonize and degrade a large variety of lignocellulosic residues, it requires shorter growth times when compared to other edible mushrooms, and it can be cultivated in a simple and cheap way (Jwanny et al. 1995; Bonatti et al. 2004). *Pleurotus ostreatus* belongs to a subclass of lignin-degrading microorganisms that produce laccases (Palmieri et al. 1997). The industrial production of *Pleurotus ostreatus* can be carried out using a variety of agricultural lignocellulosic residues as wheat straw. As with the *Agaricus bisporus* compost, this residue may contain a high-laccase concentration. It is also known that the catalytic activity of laccase depends on its source of production. Therefore, and because of the ease of obtaining this residual compost, it was decided to study the recovery of this enzyme from *P. ostreatus* using ATPS. This time, however, the background of the work with the crude extract of *A. bisporus* already existed, and some reports also demonstrated that polymers such as UCON (a random copolymer of ethylene oxide and propylene oxide) and PEG contributed to laccase stabilization (Silvério 2013). It was then decided to repeat the previously used strategy (Fig. 3.1) to corroborate the behavior of the enzyme with PEG–phosphate ATPS and other polymers as UCON, dextran, and ficoll.

As mentioned, the exact composition of the compost was not known, but it was clearly observed that the crude extract of *P. ostreatus* was cleaner, and the brown coloration was less intense than that of the crude extract of *A. bisporus*. When the PEG–phosphate systems were evaluated, no partition of laccase to the bottom phase was observed, as in the extract of *A. bisporus.* In general, the difference in the results obtained in both works was the recovery percentage. But the behavior of the partition and the effect of the molecular weight of PEG were the same. In this work, it was concluded that the PEG 1000 g/mol-phosphate ATPS were the most suitable for the primary recovery of laccase from both extracts.

After evaluating the behavior of PEG–phosphate systems, other previously reported systems for the recovery of laccase composed by UCON and different salts were tested. In all systems, laccase partitioned to the bottom phase (salt phase). For these experiments, the pH influence in the partition was analyzed. For the laccase of *A. bisporus*, a pre-analysis was made to determine the optimum pH for the recovery, since it is known that the laccase activity is better at acidic pH values between 4 and 5. A difference between both laccases was clear while studying the pH effect. For laccase from *A. bisporus*, pH 7 was the optimum for the recovery, and ATPS were prepared under those conditions. On the other hand, laccase from *P. ostreatus* presented higher recoveries in the UCON-NaH_2PO_4 systems with a pH value of 4 and a pH of 5 in the UCON-$(NH_4)_2SO_4$ and UCON-Na_2SO_4 systems. Apparently, the high hydrophobicity of UCON causes the enzyme to be partitioned to the salt phase. Other polymer–polymer ATPS (i.e., ficoll and dextran systems) were also analyzed for the recovery of laccase from *P. ostreatus*, but the obtained yields were lower.

The most important difference between these other polymer–polymer systems and the original polymer–salt systems was the fact that the enzyme was partitioned in both phases at different degrees.

The SDS-PAGE results show that the molecular weight of laccase from *P. ostreatus* is approximately 30 kDa and 60 kDa for that from *A. bisporus*. The general behavior of the enzymes is very similar in PEG-salt ATPS, but their recovery percentage is different. This may be directly related to the molecular weight of the enzymes, the source of production, and the complexity of the residual compost. Nonetheless, these results show that ATPS are an excellent alternative for the recovery of valuable enzymes from these compost waste streams.

3.3.3 Recovery of Invertase from Wasted Brewer's Yeast

It must be noted that industrially waste streams are regarded as disposable and invaluable materials, so to make their processing or "reuse" attractive, economically speaking, easy-to-implement and relatively inexpensive procedures should be devised to obtain profitable strategies from them. In this sense, ATPS are a very attractive operation since their implementation requires a low investment when compared to other strategies such as chromatography. Therefore, in the case of the recovery of invertase from wasted brewer's yeast, ATPS could be regarded as an interesting alternative to promote the extraction of said biomolecule.

In this context, our work was centered in characterizing the partition behavior of this enzyme in the aforementioned 16 PEG–phosphate ATPS and in intensifying the amount of biomass that could be processed in the operation. To do so, different amounts of total yeast extract were also loaded to each one of the tested systems. After screening and optimization, a PEG 400 g/mol ATPS with a TLL of 45% w/w, V_R 1.0, and pH 7.0 loaded with 8% w/w of crude yeast extract proved to be suitable for the primary recovery of the enzyme in the salt-rich phase of the system. After this, the selected system was scaled to a total mass of 15 g allowing the recovery of the enzyme with a yield of 66%. Furthermore, this case is particularly interesting since the recovery invertase was achieved in a very low PEG MW system, making the later processing of the recovered phase even easier in other operations such as ultrafiltration if required.

3.4 ATPS Strategies for the Recovery of PEGylated Proteins

PEGylation is defined as the covalent attachment of at least one PEG chain to the structure of a protein or another molecule (Veronese 2001). This modification confers these molecules with multiple advantages related mostly to the increase in their molecular size after reaction. For example, in pharmaceutical compounds, this polymer attachment increases their circulating lives, makes them have a lower degradability, and allows their administration at lower and more spaced dosages, among other advantages (Harris and Chess 2003). However, common PEGylation reactions

result in the attachment of different number of chains at different sites in the protein. This low selectivity results in a problem since usually, only one of the protein-polymer conjugates presents the adequate activity. Most frequently it is the mono-PEGylated conjugate that presents these characteristics, and even when the reactions have been optimized to promote higher yields of this conjugate, the appearance of other (i.e., di- and poly-PEGylated) conjugates is unavoidable. From an engineering perspective, this situation has generated an important challenge since after reaction, not only do the conjugates must be separated per their PEGylation degree but also by the positional isomerism of the grafted polymeric chains (Fee and Van Alstine 2006).

Traditionally, this problem has been addressed by different chromatographic approaches (Mayolo-Deloisa et al. 2011); however, in the search of more cost-effective and intensified operations, we have studied the use of ATPS strategies to do so with promising results. In this manner, the ATPS partition of the products of the PEGylation reactions of ribonuclease A (RNase A) and α-lactalbumin (α-Lac) was studied (González-Valdez et al. 2011). However, the quantification method used to establish the partition behavior of these proteins had to be tailored since the UV absorbance of the proteins became hindered by both the grafted polymeric chains and the ATPS environment, arising the need of obtaining correction factors and special calibration curves for each of the conjugates (González-Valdez 2011). After establishing the quantification method, the previously described 16 different PEG–phosphate ATPS were tested in the behavior studies of native, mono-, and di-PEGylated RNase A and α-Lac as a first approach. This initial screening showed that the native (unreacted) species were in all cases recovered in the bottom salt-rich phase of high PEG MW systems, while the PEGylated conjugates presented a preference of partition toward the polymeric phase. It had been previously established that PEGylated conjugates presented in general a preferable partition to the polymeric phase which increases per the PEGylation degree of the proteins (Delgado et al. 1994). After obtaining the initial results, the TLL and V_R values were variated to promote a better partition and recovery of the conjugates. By taking the best V_R 1.0 systems, variations were made in this value, and mono- and di-PEGylated RNase A conjugates were recovered with yields of 98% and 88%, respectively, in the top phase of a PEG 8000 g/mol, TLL 25% w/w VR 1.0, and pH 7.0 system, while mono- and di-PEGylated α-Lac were recovered with yields of 77% and 76%, respectively, in the polymeric phase of a PEG 8000 g/mol, TLL 35% w/w, VR 3.0, and pH 7.0 system (González-Valdez 2011). This study described the potential of PEG–phosphate ATPS for the selective fractionation of native unreacted proteins from their PEGylated conjugates after the reaction.

3.5 Protein Refolding Explorations in ATPS

Expression of genetically engineered proteins in bacteria often results in the accumulation of the product in inactive insoluble deposits inside the cells, called inclusion bodies (Basri et al. 1995). The refolding of proteins to their native

conformation is still the limiting step during active protein recovery from these inclusion bodies (Sridhar 1996). In this context, traditional methods for protein reactivation or refolding consist primarily in the solubilization of such inclusion bodies in denaturing concentrations of guanidine hydrochloride or urea, followed by the removal of the denaturant, and refolding assistance by small molecule additives (García-Arellano et al. 2002). Refolding is commonly addressed by a practical approach using several methods that include dialysis, diafiltration, and size exclusion chromatography (Narain 2006). But such methods are time-consuming and, often, recovery yields of active proteins are low, and a trial-and-error process development is required to achieve success (Nagasaki et al. 2007). Additionally, most of the methods involve the use of additives to assist the correct refolding of the obtained proteins.

In this manner, it has been demonstrated that PEG (the most common polymer used in ATPS strategies) inhibits aggregation during the refolding process (as in the case of bovine carbonic anhydrase B) through the formation of a non-associating PEG-intermediate complex, preventing self-association and promoting correct refolding (Lu et al. 2008). And as it has been shown, ATPS are able to provide good and affordable physicochemical conditions to refold several proteins (Narain 2006).

Kuboi et al. developed a protein-refolding process using ATPS modified with stimuli-responsive polymers, which had a chaperone-like function (Kuboi et al. 2000). PEG bound to a thermos-reactive hydrophobic head (poly (propylene oxide)-phenyl group (PPO-Ph)) was used as the functional ligand to modify the PEG phase of ATPS. In general, the principal contribution of this work was the use of PP-Ph-PEG as chaperone agent. In another work, the usage of UCON and dextran T-500 systems was reported for the refolding of chymotrypsin inhibitor 2 (CI2) (Maruyama et al. 2002). The study stated that the partitioning behavior of CI2 in UCON-dextran and UCON-water systems was due to conformational changes between the native and the unfolded states of the protein; the unfolded CI2 could be refolded to the native form with high yields in the UCON solution. The partition and refolding of chymosin using PEG–phosphate and PEO-maltodextrin systems have been also reported (Reh et al. 2007). In this case, inclusion bodies contained prochymosin, and their refolding was carried out at pH 2.0 to facilitate the auto-conversion of prochymosin to recombinant chymosin. This finding agrees with the known PEG capacity to avoid the contact between protein molecules, thus preventing protein aggregation while enhancing correct refolding and increasing in this way the thermodynamic stability of a protein. Furthermore, another study reported an experimental design to determine the optimal conditions for the refolding of a recombinant thermostable and alkaline-active xylanase from *Bacillus halodurans* in PEG–phosphate systems (Sridhar 1996). Results showed that the recovery of active enzyme in the top phase increased with decrements in PEG molecular weight and decreased with increasing enzyme load. The model based on response surface methodology suggested that exposure of the hydrophobic residues and the increased surface of the proteins or denaturation with urea influences their partition in ATPS and predicted that the activity recovery decreased with increasing PEG molecular weight. This is attributed to the increasing hydrophobicity of the polymer, which

causes more aggregation during the refolding process. In the same way, the partition behavior of native and denatured invertase in different PEG–phosphate systems has been evaluated (Narain 2006). The authors observed that recovered protein in the top phase presented in most systems refolding percentages of 100% that increased only in systems formed with PEG 8000 g/mol.

In our experience, we have conducted refolding procedures using as a first approach the 16 different ATPS in the search of an attractive system that promotes the refolding of denatured invertase (Sánchez-Trasviña et al. 2015). In this case, however, variations in V_R using values of 0.33, 1.0, and 3.0 were tested since the beginning giving a total of 48 different systems. The partition behaviors of native and denatured invertase presented differences since the denatured species presented a predominant preference for the salt-rich phase. It was also observed that system design parameters (i.e., PEG MW, TLL, and V_R) presented some sort of influence on both the partition and the refolding of the enzyme. A complete invertase refolding could be observed in the polymeric phases of the systems, but very low enzyme concentrations were achieved in this phase (0.5 mg/mL). On the other hand, refolding percentages in the bottom phase ranged between 50% and 75%. Therefore, a PEG 3350 g/mol, TLL 25% w/w, and V_R 1.0 system was selected as the best option for refolded invertase recovery in the bottom phase with up to 0.73 mg of enzyme recovered from the original 2.0 mg of denatured enzyme loaded. In general, there are few studies about the use of ATPS for the refolding of proteins; however, their potential has been clearly stated. Some of the reports abovementioned used proteins denatured with different agents as guanidine hydrochloride before the refolding studies. So, there is an area of opportunity in the study of protein refolding from inclusion bodies produced in industrial processes and the use of new intelligent polymers as part of the ATPS to enhance the operation.

3.6 Concluding Remarks

Regardless of the biomolecule being processed or the source where it comes from, the successful implementation of aqueous two-phase system strategies can be addressed using a simple algorithm that has shown effectiveness in a large amount of cases. Shortly, this pathway requires the understanding of the physicochemical characteristics and concentrations of the target molecule and the contaminants present in the sample; an initial screening with well-defined ATPS; the optimization of those systems that give the best results regarding recovery yield and purity; and the recovery of the target molecule. In this chapter, we have presented a series of cases where this strategy has been used successfully and furthermore showing the tremendous capability of ATPS as an attractive primary recovery operation.

It should be noted that the industrial implementation of ATPS strategies is usually halted by regulatory situations (like in the case of pharmaceutical products) where a golden standard for processing operations has already been established and approved and little can be made. However, there is an important opportunity area in other fields such as the management of waste streams and the procurement of high

added-value molecules from them. As it has been mentioned, to have a sustainable and profitable operation when handling wastes, it is important for the selected downstream processing operations to be as economic and robust as possible. ATPS operations possess both characteristics making them and interesting option to achieve this. Furthermore, and besides their implementation in the handling of wastes, ATPS present important advantages over other traditional techniques in the processing of different samples as is the case of chemical reactions or the refolding of proteins from inclusion bodies as it has been discussed. In this sense, it is very important for scientists and industrials to identify those niche opportunity areas where this type of operations can be used. With the aid of the available knowledge in the processing of a very large amount of proteins and the identification of the similarities between these processes, easy-to-implement and successful ATPS strategy should be available regardless of the complexity of the sample or matrix where the product is present.

References

Aguilar O, Rito-Palomares M. Processing of soybean (Glycine max) extracts in aqueous two-phase systems as a first step for the potential recovery of recombinant proteins. J Chem Technol Biotechnol. 2008;83(3):286–93.

Barbosa H, Slater NKH, Marcos JC. Protein quantification in the presence of poly(ethylene glycol) and dextran using the Bradford method. Anal Biochem. 2009;395(1):108–10.

Basri M, Ampon K, Yunus WMZW, Razak CNA, Salleh AB. Synthesis of fatty esters by polyethylene glycol-modified lipase. J Chem Technol Biotechnol. 1995;64(1):10–6.

Benavides J, Rito-Palomares M. Bioprocess intensification: a potential aqueous two-phase process for the primary recovery of B-phycoerythrin from Porphyridium cruentum. J Chromatogr B. 2004;807(1):33–8.

Benavides J, Rito-Palomares M. Practical experiences from the development of aqueous two-phase processes for the recovery of high value biological products. J Chem Technol Biotechnol. 2008;83(2):133–42.

Benavides J, Rito-Palomares M. Simplified two-stage method to B-phycoerythrin recovery from *Porphyridium cruentum*. J Chromatogr B. 2006;844(1):39–44.

Benavides J, Rito-Palomares M, Asenjo JA. 2.49 – aqueous two-phase systems. In: Moo-Young M, editor. Comprehensive biotechnology. 2nd ed. Burlington: Academic Press; 2011. p. 697–713.

Bertrand B, Martínez-Morales F, Trejo-Hernández MR. Fungal laccases: induction and production. Re Mex Ing Quím. 2013;12:473–88.

Bertrand B, Mayolo-Deloisa K, González-González M, Tinoco-Valencia R, Serrano-Carreón L, Martínez-Morales F, Trejo-Hernández MR, Rito-Palomares M. Pleurotus ostreatus laccase recovery from residual compost using aqueous two-phase systems. J Chem Technol Biotechnol. 2016;91(8):2235–42.

Bonatti M, Karnopp P, Soares HM, Furlan SA. Evaluation of Pleurotus ostreatus and Pleurotus sajor-caju nutritional characteristics when cultivated in different lignocellulosic wastes. Food Chem. 2004;88(3):425–8.

Delgado C, Malik F, Selisko B, Fisher D, Francis GE. Quantitative analysis of polyethylene glycol (PEG) in PEG-modified proteins/cytokines by aqueous two-phase systems. J Biochem Biophys Methods. 1994;29(3–4):237–50.

Dumetz AC, Chockla AM, Kaler EW, Lenhoff AM. Comparative effects of salt, organic, and polymer precipitants on protein phase behavior and implications for vapor diffusion. Crys Growth Des. 2009;9(2):682–91. doi:10.1021/cg700956b.

Fee CJ, Van Alstine JM. PEG-proteins: reaction engineering and separation issues. Chem Eng Sci. 2006;61(3):924–39.

Franco TT, Andrews AT, Asenjo JA. Use of chemically modified proteins to study the effect of a single protein property on partitioning in aqueous two-phase systems: effect of surface hydrophobicity. Biotechnol Bioeng. 1996;49(3):300–8.

García-Arellano H, Valderrama B, Saab-Rincón G, Vazquez-Duhalt R. High temperature biocatalysis by chemically modified cytochrome C. Bioconjug Chem. 2002;13(6):1336–44.

Garza-Madrid M, Rito-Palomares M, Serna-Saldivar SO, Benavides J. Potential of aqueous two-phase systems constructed on flexible devices: human serum albumin as proof of concept. Process Biochem. 2010;45(7):1082–7.

Gong M, Aguirre AM, Bassi A. Chapter 5 – technical issues related to characterization, extraction, recovery, and purification of proteins from different waste sources A2. In: Dhillon GS, editor. Protein byproducts. London: Academic; 2016. p. 89–106.

González-González M, Mayolo-Deloisa K, Rito-Palomares M, Winkler R. Colorimetric protein quantification in aqueous two-phase systems. Process Biochem. 2011;46(1):413–7.

González-Valdez J, Cueto LF, Benavides J, Rito-Palomares M. Potential application of aqueous two-phase systems for the fractionation of RNase A and α-Lactalbumin from their PEGylated conjugates. J Chem Technol Biotechnol. 2011;86(1):26–33.

Gustavsson J, Cederberg C, Sonesson U, van Otterdijk R, Maybeck A. Global food losses and food waste – extent, causes an prevention. International Congress Save Food. Rome: Food and Agriculture Organization of the United Natios; 2011.

Harris JM, Chess RB. Effect of PEGylation on pharmaceuticals. Nat Rev Drug Discov. 2003; 2(3):214–21.

Hernandez-Mireles T, Rito-Palomares M. New aqueous two-phase systems based on poly(ethylene oxide sulfide) (PEOS) and potassium phosphate for the potential recovery of proteins. J Chem Technol Biotechnol. 2006;81(6):997–1002.

Ibarra-Herrera CC, Torres-Acosta MA, Mendoza-Ochoa GI, Aguilar-Yañez JM, Rito-Palomares M. Recovery of major royal jelly protein 1 expressed in Pichia Pastoris in aqueous two-phase systems. J Chem Technol Biotechnol. 2014;89(7):941–7.

Johansson G. Partition of salts and their effects on partition of proteins in a dextran-poly(ethylene glycol)-water two-phase system. Biochim Biophys Acta Protein Struct. 1970;221(2):387–90.

Jwanny EW, Rashad MM, Abdu HM. Solid-state fermentation of agricultural wastes into food through Pleurotus cultivation. Appl Biochem Biotechnol. 1995;50(1):71–8.

Kuboi R, Morita S, Ota H, Umakoshi H. Protein refolding using stimuli-responsive polymer-modified aqueous two-phase systems. J Chromatogr B. 2000;743(1):215–23.

León-González G, González-Valdez J, Mayolo-Deloisa K, Rito-Palomares M. Intensified fractionation of brewery yeast waste for the recovery of invertase using aqueous two-phase systems. Biotechnol Appl Biochem. 2015;63(6):886–94.

Lladosa E, Silvério SC, Rodríguez O, Teixeira JA, Macedo EA. (Liquid + liquid) equilibria of polymer-salt aqueous two-phase systems for laccase partitioning: UCON 50-HB-5100 with potassium citrate and (sodium or potassium) formate at 23 °C. J Chem Thermodyn. 2012;55:166–71.

Lu Y, Harding SE, Turner A, Smith B, Athwal DS, Grossmann JG, Davis KG, Rowe AJ. Effect of PEGylation on the solution conformation of antibody fragments. J Pharm Sci. 2008; 97(6):2062–79.

Makris DP. Chapter 16 – recovery and applications of enzymes from food wastes A2. In: Galanakis CM, editor. Food waste recovery. San Diego: Academic; 2015. p. 361–79.

Maruyama T, Nagasawa S, Goto M. Poly(ethylene glycol)-lipase complex that is catalytically active for alcoholysis reactions in ionic liquids. Biotechnol Lett. 2002;24(16):1341–5.

Mayolo-Deloisa K, Gonzalez-Valdez J, Guajardo-Flores D, Aguilar O, Benavides J, Rito-Palomares M. Current advances in the non-chromatographic fractionation and characterization of PEGylated proteins. J Chem Technol Biotechnol. 2011;86(1):18–25.

Mayolo-Deloisa K, Trejo-Hernandez MD, Rito-Palomares M. Recovery of laccase from the residual compost of Agaricus bisporus in aqueous two-phase systems. Process Biochem. 2009;44(4):435–9.

Minussi RC, Pastore GM, Durán N. Potential applications of laccase in the food industry. Trends Food Sci Technol. 2002;13(6–7):205–16.
Moreira S, Silvério SC, Macedo EA, Milagres AMF, Teixeira JA, Mussatto SI. Recovery of Peniophora cinerea laccase using aqueous two-phase systems composed by ethylene oxide/propylene oxide copolymer and potassium phosphate salts. J Chromatogr A. 2013;1321(0):14–20.
Morozova O, Shumakovich G, Gorbacheva M, Shleev S, Yaropolov A. "Blue" laccases. Biochem Mosc. 2007;72:1136–50.
Nagasaki Y, Yoshinaga K, Kurokawa K, Iijima M. Thermal- and dispersion-stable lipase-installed gold colloid: PEGylation of enzyme-installed gold colloid. Colloid Polym Sci. 2007; 285(5):563–7.
Narain R. Tailor-made protein–glycopolymer bioconjugates. React Funct Polym. 2006;66(12): 1589–95.
Ng HS, Tan CP, Chen SK, Mokhtar MN, Ariff A, Ling TC. Primary capture of cyclodextrin glycosyltransferase derived from Bacillus Cereus by aqueous two phase system. Sep Purif Technol. 2011;81(3):318–24.
Palmieri G, Giardina P, Bianco C, Scaloni A, Capasso A, Sannia G. A novel white laccase from Pleurotus ostreatus. J Biol Chem. 1997;272(50):31301–7.
Prinz A, Hönig J, Schüttmann I, Zorn H, Zeiner T. Separation and purification of laccases from two different fungi using aqueous two-phase extraction. Process Biochem. 2014a;49(2):335–46.
Prinz A, Koch K, Górak A, Zeiner T. Multi-stage laccase extraction and separation using aqueous two-phase systems: experiment and model. Process Biochem. 2014b;49(6):1020–31.
Rajagopalu D, Show PL, Tan YS, Muniandy S, Sabaratnam V, Ling TC. Recovery of laccase from processed Hericium erinaceus (Bull.:Fr) Pers. fruiting bodies in aqueous two-phase system. J Biosci Bioeng. 2016;122(3):301–6.
Reh G, Spelzini D, Tubio G, Pico G, Farruggia B. Partition features and renaturation enhancement of chymosin in aqueous two-phase systems. J Chromatogr B. 2007;860(1):98–105.
Reis IAO, Santos SB, Santos LA, Oliveira N, Freire MG, Pereira JFB, Ventura SPM, Coutinho JAP, Soares CMF, Lima ÁS. Increased significance of food wastes: selective recovery of added-value compounds. Food Chem. 2012;135(4):2453–61.
Rito-Palomares M, Middelberg APJ. Aqueous two-phase systems for the recovery of a recombinant viral coat protein from *Escherichia coli*. J Chem Technol Biotechnol. 2002;77(9):1025–9.
Rito-Palomares MA. Practical application of aqueous two-phase partition to process development for the recovery of biological products. J Chromatogr B. 2004;807(1):3–11.
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.
Schwienheer C, Prinz A, Zeiner T, Merz J. Separation of active laccases from Pleurotus sapidus culture supernatant using aqueous two-phase systems in centrifugal partition chromatography. J Chromatogr B. 2015;1002:1–7.
Silvério SC, Rodríguez O, Tavares APM, Teixeira JA, Macedo EA. Laccase recovery with aqueous two-phase systems: enzyme partitioning and stability. J Mol Catal B Enzym. 2013;87(0):37–43.
Simental-Martínez J, Rito-Palomares M, Benavides J. Potential application of aqueous two-phase systems and three-phase partitioning for the recovery of superoxide dismutase from a clarified homogenate of Kluyveromyces marxianus. Biotechnol Prog. 2014;30(6):1326–34.
Sridhar P. Modelling of affinity separation by batch and fixed bed adsorption – a comparative study. Chem Eng Technol. 1996;19(4):357–63.
Sánchez-Trasviña C, González-Valdez J, Mayolo-Deloisa K, Rito-Palomares M. Impact of aqueous two-phase system design parameters upon the in situ refolding and recovery of invertase. J Chem Technol Biotechnol. 2015;90(10):1765–72.
Trejo-Hernandez MR, Lopez-Munguia A, Quintero Ramirez R. Residual compost of Agaricus bisporus as a source of crude laccase for enzymic oxidation of phenolic compounds. Process Biochem. 2001;36(7):635–9.
Veronese FM. Peptide and protein PEGylation: a review of problems and solutions. Biomaterials. 2001;22(5):405–17.

Vázquez-Villegas P, Aguilar O, Rito-Palomares M. Continuous enzyme aqueous two-phase extraction using a novel tubular mixer-settler in multi-step counter-current arrangement. Sep Purif Technol. 2015;141(0):263–8.

Vázquez-Villegas P, Espitia-Saloma E, Rito-Palomares M, Aguilar O. Low-abundant protein extraction from complex protein sample using a novel continuous aqueous two-phase systems device. J Sep Sci. 2013;36(2):391–9.

Zaslavsky B. Aqueous two-phase partitioning: physical chemistry and bioanalytical applications. New York: Marcel Dekker Inc.; 1995.

Aqueous Two-Phase Systems for the Recovery of Bioparticles

4

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Abbreviations

ATPAP	Aqueous two-phase affinity partitioning
ATPS	Aqueous two-phase systems
CCD	Countercurrent distribution
CD	Cluster of differentiation
DEX	Dextran
FACS	Fluorescent-activated cell sorter
FFF	Field-flow fractionation
LFA	Lateral flow assay
MACS	Magnetic-activated cell sorter
PBS	Phosphate-buffered saline
pDNA	Plasmid DNA
PEG	Polyethylene glycol
Poly(NIPAM)	Poly-N-isopropylacrylamide
PVA	Polyvinyl alcohol
PVP	polyvinylpyrrolidone
QMS	Quadrupole magnetic cell sorter
RSM	Response surface methodology
TLL	Tie-line length
VLPs	Viruslike particles
V_R	Volume ratio

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4.1 Introduction

Fractionation and purification techniques represent one of the most important areas for research and development of novel technologies involved in the recovery and effective isolation of high-added value compounds (Ruiz-Ruiz et al. 2013). Within these techniques, protocols or unit operations that allow concentration and selective partitioning of a particle or molecule to a specific recovery phase within the employed methodology are preferred (Asenjo and Andrews 2008; Benavides et al. 2008). Considering that during the past five decades, research comprising the deep study of cell physiology has taken place in order to understand its biochemistry for industrial and scientific purposes, a very important number of research groups have devoted time, effort, and resources to develop highly efficient and selective methods for whole cell and cell particle isolation (Albertsson 1961). Up to date, the correct separation and concentration of cell nuclei, mitochondria, microsomes, chloroplasts, cell walls, and cell membranes constitute one of the primary cores of whole-particle isolation procedures (Albertsson 1961; Silva et al. 2014). In this tenor, novel strategies intended for macroparticles' recovery and purification are one of the current trends in bioprocessing development, specifically within the design of new downstream processing trains for the pharmaceutical, cosmetic, and food industries.

Particle isolation procedures can be grouped within several categories, the most studied being the ones that consider the physical properties of the particle or molecule in order to design an effective methodology for its recovery, purification, or concentration. In this sense, isopycnic centrifugation techniques employing density gradients are the current workhorse for particle isolation offering high reproducibility and relatively low processing times (González-González et al. 2012). The separation process within these protocols considers the density and size of the target particle. The traditional process consists of a sample that is loaded on top of a centrifugation tube which is filled with a solution of active components (e.g., Ficoll, Percoll, cesium chloride) that will form a density gradient when subjected to high centrifugal speeds. The complex mixture loaded into the system travels different lengths according to the physical characteristics of each particle in the blend, until each component of the sample is retained in its isopycnic position. Numerous researches have employed this technique for the effective isolation of whole cells (red blood cells, stem cells, tumor cells), cell fragments or organelles (chloroplasts), or cell membranes (Albertsson 1961; González-González et al. 2012). Nevertheless, this technique has important limitations that may hinder its application during industrial-based processes. Reduced sample loading, nonspecific product concentration or separation, intensive labor required for proper technique evaluation, and low resolution are the main drawbacks for this physical separation of target products in biotechnological approaches. In addition, the overall potential for scale-up is practically null since batch procedures are preferred for isopycnic centrifugation and continuous centrifuges dominate the industrial environment nowadays.

Considering these situations, the application and characterization of alternative and effective technologies for the recovery and purification of macroparticles have been a major challenge in the past two decades. In this context, liquid–liquid

extraction technologies based on aqueous two-phase systems (ATPS) have been proposed as a novel and biocompatible alternative for selective product partitioning (Mayolo-Deloisa et al. 2011; Ruiz-Ruiz et al. 2012). These systems form when specific components (e.g., two polymers, a polymer and a salt, alcohol and a salt, ionic liquid and a salt, detergents) are mixed over certain critical conditions, giving a two-phase system with immiscible phases and particular biochemical characteristics in each phase. ATPS have several advantages when compared to traditional downstream processing techniques since they represent economic approaches for product concentration and purification, they provide increased scale-up potential, the requirement of temperature shifts is avoided in almost every case, systems are easy to set up, and finally they offer a biocompatible environment for target products because of the aqueous nature of the system employed (Benavides et al. 2006). Albertsson employed this technique for the first time in 1955 by mistake in an attempt to extract pyrenoids from the algae *Chlorella pyrenoidosa*. He determined that a polyethylene glycol–potassium phosphate system was efficient in correctly isolating chloroplasts in the polymer-rich phase and other components in the bottom salt phase (Albertsson 1985). From this experience, a very important number of research proposals and publications have been made worldwide in order to exploit ATPS advantages in biotechnological applications ranging from the food industry to cosmetics, point of care diagnostics, and pharmaceutical and environmental uses.

In terms of particle partitioning, the most important characteristic that ATPS offer is the possibility of recovering or concentrating a target molecule or particle by means of a biological and biochemical selectivity, in addition to the traditional physical approaches (size, shape, and/or density) of several sedimentation-based operations currently employed (Asenjo and Andrews 2012). One simple example is that of cells, since ATPS can be designed to work at physiological pH levels, thus working with isotonic conditions in order to work with these biological particles (Sousa et al. 2011). Considering the outer structure of a typical cell, which includes a well-defined membrane with phospholipids, transmembrane proteins, and specific carbohydrates, the set of interactions that may be present in a two-phase system can be primarily electrostatic and hydrophobic, depending on the active groups or residues involved in the behavior. In addition, inherent physical properties such as size and density also play a vital role in order to determine the final partitioning behavior of the molecule in the system. In this regard, the use of ATPS has been studied not only for recovery of particles but also for molecular characterization (Mayolo-Deloisa et al. 2011; González-González et al. 2012). One particular research niche in this area is that of integral proteins in prokaryotic and eukaryotic expression systems, since ATPS studies of cell lysates allow a deep characterization of the intracellular and transmembrane components based on the partitioning behavior of the molecules involved, thus providing important conclusions for research in terms of molecular structures, stability, and superficial net charge and hydrophobicity, among others (Schindler and Nothwang 2006).

Considering the potential of ATPS for whole-particle recovery for diverse purposes, this chapter aims to present and discuss the principal areas of study of this liquid–liquid extraction technology for the proper study of cells, cellular

components, and current bioactive compounds such as viral particles or nucleic acids. The impact of these components on their partition in selected ATPS is discussed, as well as the principal challenges and trends in regard to the design of novel downstream processing alternatives aimed to provide additional options for the biotechnology industry in terms of particle characterization and new product development.

4.2 Partitioning of Whole Cells, Stem Cells, and Cellular Components in ATPS

Whole cells have been the separation target of a wide range of technologies, including density gradient, cell culture, fluorescent-activated cell sorter (FACS), magnetic-activated cell sorter (MACS), quadrupole magnetic cell sorter (QMS), cryogels, membranes, pre-plating, dielectrophoretic microdevices, panning, affinity columns, field-flow fractionation (FFF), and aqueous two-phase systems (ATPS). The isolation of a specific purified type of cells has endless applications, from basic science to clinical. Moreover, there are a variety of cell sources, all of them having a complex biological background. Another key feature about cells as the targeting recovery product is how extremely fragile and susceptible they can be to abrupt environmental changes (i.e., pH, osmolarity, temperature, available nutrients, etc.). With all this into account, it is predictable to conclude that ATPS is a suitable separation approach for cells.

ATPS present the following advantages for the recovery and purification of different types of whole cells:

- A simple, low energy input, fast, and highly reproducible technique that does not require sophisticated and costly equipment or specialized personnel.
- Their high water content confers a mild and biocompatible environment for cells. Furthermore, they can be adjusted to provide an isotonic and physiological media suitable for the separation of viable cells.
- Cells are exposed to identical conditions throughout the separation process (pH, temperature, osmolarity, and ion concentration), avoiding abrupt changes that may have a detrimental effect in viability and functionality.
- ATPS are easily miniaturized via microdevices, which require small amounts of sample and reagents plus are highly portable. On the other hand, ATPS are well-known for their scale-up capacity and possibility of continuous operation.
- Polymers provide a protective surrounding for cells, which helps to retain their viability and biological functions.

All these benefits support the idea of employing ATPS as a feasible technique for whole cell separation or their fractionation into subpopulations. Additionally, the gentleness of ATPS for cells has also been exploited in other applications, including extractive bioconversion, studying surface properties (i.e., charge, hydrophobic) or alterations occurring as a result of both normal and abnormal in vivo processes (i.e., differentiation, maturation, aging, etc.), and as an analytical and preparative technique (Hatti-Kaul 2001; Cabral 2007).

Another relevant point to mention regarding ATPS for cell isolation is the multiple factors that enter into play during sample partitioning. These can be divided into three main categories: (1) the intrinsic polymeric and ionic properties of the two hydrophilic aqueous phase-forming solutions and additives (density, viscosity, and interfacial tension); (2) the selected system parameters of volume ratio (V_R), tie-line length (TLL), pH, and temperature; and (3) the surface properties of the sample (i.e., net surface charge, hydrophobicity, affinity interaction, etc.). As it can be expected, the complexity of the factors has prevented researchers to propose a generic guideline for the separation of any kind of cells employing ATPS.

Throughout scientific literature, the following types of polymers forming ATPS have been employed for the separation of cells from a variety of biological sources: polyethylene glycol (PEG), dextran (DEX), Ficoll, polyvinyl alcohol (PVA), polyvinylpyrrolidone (PVP), hydroxypropyl starch, and methoxypolyethylene glycol. Stimuli-responsive soluble–insoluble polymers (temperature or pH), such as ethylene oxide–propylene oxide (UCON), poly-N-isopropylacrylamide [poly (NIPAM)], and methacrylic acid–methyl methacrylate (Eudragit), have also been reported for cell partitioning in ATPS. In polymer–polymer ATPS, the phase separation is attributed to small repulsive interactions between the two types of monomers in the solution. Given that each of the two polymers is composed of several monomers, the total interaction between the polymers is large (Cabral 2007). Polymer–polymer ATPS are preferred over polymer–salt systems in the case of cell separation because biospecific interaction is usually obstructed by high salt concentration (Cabral 2007) and because of the hypertonicity of the salt component, which affects the viability of cells. Regarding two-polymer systems, the PEG–dextran systems are much less susceptible to salt effect when compared to PVP–dextran or Ficoll–dextran systems (Cabral 2007). Furthermore, the most employed solvent for this type of separation is phosphate-buffered saline (PBS, pH 7.4, 150 mM NaCl), which provides a suitable media for the separation of viable cells (González-González and Rito-Palomares 2013). The use of "charged" ATPS is also commonly employed for the isolation of this kind of products, as the interfacial potential between phases may affect their partitioning (Fisher 1981). These "charged" systems are constructed by adding anions, cations, or salts to the systems. In other cases, as, for example, when employing ATPS as extractive bioconversion, authors have also reported the specific cell growing media as a solvent (Zijlstra et al. 1996; Atefi et al. 2015).

The main goal in ATPS is to determine the partition behavior of cells to the top, interface, and bottom phases of the studied system. This can be performed by determining the number of cells and their viability in the top and bottom and attributing the difference from the total number in the system to the interface. Furthermore, phase fractionation has not only been employed to separate the cell product from the contaminants but also to give information on the charge, receptors, and hydrophobic properties of the surfaces of the product cell (Fisher 1981). Ideally, one single partition is necessary to separate the cell of interest from the complex mixture if the product of interest fractionates entirely one of the phases and the contaminants (cell particles, debris, RNA, carbohydrates, lipids, etc.) into the opposite phase. Nevertheless, it is common to use a sequential partition strategy named countercurrent distribution

(CCD) to separate cells. In CCD experiments, the top phase of the ATPS is transferred sequentially to a fresh bottom phase with the same polymer composition as the initial system.

Another common strategy employed for the isolation of cells exploiting ATPS is the addition of affinity ligands into the system to enhance the selectivity of the target product to the phase to which the ligand has more affinity. Examples of ligand types include dyes, metal ions, enzymes, and antibodies. The ligands could be added freely in the ATPS or coupled reversibly to one of the phase-forming polymers. In the case of PEG–DEX systems, the dextran-rich phase is the most suitable for the conjugation of the ligand because of the following reasons: (1) dextran concentration in the PEG-rich phase is lower than the PEG concentration in the dextran-rich phase; (2) dextran has a higher molecular weight than PEG, thus is less affected by the ligand; and (3) dextran molecule has more coupling potential sites.

Diverse types of cells have been reported to be separated employing ATPS, including animal, plant, bacteria, and viruses. More specifically, different types of animal cells such as cancer cells (leukemia), stem cells ($CD34^+$, $CD133^+$), blood cells (erythrocytes, leukocytes, lymphocytes, T lymphoma), and hybridomas have been documented. Table 4.1 presents a summary of the different kinds of cells, their source, and the type of ATPS employed for their isolation.

4.2.1 Blood Components

Since almost 40 decades ago, different blood species of cells have been reported to be separated employing ATPS, such as leukocytes, platelets, lymphocytes, and erythrocytes. For this, diverse sources of blood have been studied, highlighting human, rat, rabbit, and sheep blood. Moreover, authors have tested varying modes of ATPS including CCD, microfluidic devices, affinity ligands, and charged ATPS. PEG–DEX polymer–polymer ATPS are the most exploited systems for this particular application.

ATPS have been reported to selectively fractionate and thus detect difference in erythrocytes from different species, maturation age, and cell membrane abnormalities. For example, ATPS containing DEX, PEG, and PEG fatty acid esters were able to distinguish between erythrocytes from multiple sclerosis patients and the healthy control (Van Alstine and Brooks 1984). In a charge-sensitive DEX–PEG ATPS, human and rat erythrocytes were separated by CCD (Jimeno et al. 1991). Rabbit and human erythrocytes were fractionated in PEG–DEX immunoaffinity ATPS by CCD (Sharp et al. 1986). Likewise, the separation by CCD of small number of cells ($\sim 10^6$) from rabbit, rat, and sheep blood was reported to be successful with the incorporation of fetal bovine serum in PEG–DEX systems (Walter and Krob 1984). A charged PEG–DEX CCD systems containing 0.05 M NaPB and 0.09 M NaCl were also able to partition erythrocytes from rat bone marrow cells as a function of charge-associated cell surface properties (García-Pérez et al. 1987).

A number of studies on the separation of leukocyte cells have been performed in PEG–DEX ATPS. Leukocytes from single-cell suspensions from rat spleen, lymph

Table 4.1 Partition of cells in aqueous two-phase systems

Source	Cell	System		Reference
Blood components				
Multiple sclerosis healthy patients	Erythrocytes	PEG 6000	DEX T-500	Van Alstine and Brooks (1984)
Human and rabbit	Erythrocytes	PEG 200/1900/5000/8000	DEX T-500	Sharp et al. (1986)
Human and rat	Erythrocytes	PEG 6000	DEX T-500	Jimeno et al. (1991)
Sheep, rat, rabbit	Erythrocytes	PEG 6000	DEX T-500	Walter and Krob (1984)
Rat bone marrow	Erythrocytes	PEG 6000	DEX T-500	García-Pérez et al. (1987)
Human whole blood samples	Erythrocytes	PEG 3350/6000/8000	DEX 40,000/500,000	Tsukamoto et al. (2009)
Rat spleen, lymph nodes, thoracic duct	Leukocytes	PEG 6000	DEX T-500	Malmström et al. (1978)
Whole blood samples	Leukocytes	PEG 8000	DEX 500,000	SooHoo and Walker (2009)
Human peripheral blood	Lymphocytes	PEG 6000	DEX T-500	Malmström et al. (1980a)
Human peripheral blood	Lymphocytes	PEG 6000	DEX T-500	Malmström et al. (1980b)
Human peripheral blood	Lymphocytes	PEG 6000	DEX T-500	Walter et al. (1979)
Stem cells				
Human umbilical cord blood	CD133$^+$	Ficoll 400,000 PEG 8000 UCON	DEX 70,000 DEX 70,000 DEX 75000	González-González and Rito-Palomares (2015)
Human umbilical cord blood	CD133$^+$	Ficoll 400,000 PEG 8000 UCON	DEX 70,000 DEX 70,000 DEX 75000	González-González et al. (2014)
Human umbilical cord blood	CD34$^+$	PEG 8000	DEX 500,000	Sousa et al. (2011)

(continued)

Table 4.1 (continued)

Source	Cell	System		Reference
Human acute myeloid leukemia cells (KG-1) separated from human T-lymphoma cells (Jurkat)	$CD34^+$	PEG 8000	DEX T-500	Kumar et al. (2001)
Mouse	Embryonic	PEG 35000	DEX 500,000	Atefi et al. (2015)
Cancer				
Human skin	A431.H9 cancer	PEG 35000	DEX 500,000	Atefi et al. (2015)
Jurkat cells (clone E6–1)	Acute lymphocytic T leukemia	PEG 3350/6000/8000	DEX 40,000/500,000	Tsukamoto et al. (2009)
Mouse	Myeloma NS-1	PEG 8000	DEX T-500	Hamamoto et al. (1996)
Hybridoma				
Mouse–mouse	16-3F	PEG 8000	DEX T-500	Hamamoto et al. (1996)
Mouse–mouse	BIF6A7	PEG 35000	DEX 40,000	Zijlstra et al. (1996)
Organ cells				
African green monkey kidney	CV-1	PEG 8000	DEX T-500	Hamamoto et al. (1996)
Sprague-Dawley rat	Splenocytes and hepatocytes	PEG 8000	DEX T-500	Hamamoto et al. (1996)
Microorganism				
Escherichia coli	Bacterial	PEG 1540/4000/6000	DEX 60,000–90,000/100,000–200,000	Umakoshi et al. (1997)
Saccharomyces cerevisiae	Yeast	PEG 1540/4000/6000	DEX 60,000–90,000/100,000–20,000	Umakoshi et al. (1997)

nodes, and thoracic duct lymph were reported to be fractionated by CCD in ATPS containing PEG–DEX added with 0.094 M sodium phosphate buffer and fetal calf serum (Malmström et al. 1978). Microfluidic PEG–DEX ATPS devices have also been employed to separate leukocyte from whole blood samples (SooHoo and Walker 2009; Tsukamoto et al. 2009). The subfractionation of lymphocytes from human peripheral blood by CCD in a charged PEG–DEX ATPS has been well documented in multiple studies (Walter et al. 1979; Malmström et al. 1980a, b).

4.2.2 Stem Cells

Stem cells have lately been an attractive target of application for ATPS due to the well-known and documented advantages of this technique. The major sources of cells for this type of studies are bone marrow, peripheral blood, umbilical cord blood, and model stem cell lines. The most reported stem cell types are $CD133^+$ and $CD34^+$, as their purified forms have been used as a therapeutic alternative for several incurable and chronic degenerative diseases. Immunoaffinity ATPS have been the most popular mode with the addition of the free antibody or its conjugation to a polymer forming ATPS. For example, Kumar and coworkers reported the separation of $CD34^+$ human acute myeloid leukemia cells from T-lymphoma cells employing a PEG 8000–DEX T-500 additioned with anti-CD34 conjugated with the temperature-sensitive polymer, [poly(NIPAM)] (Kumar et al. 2001). Another report for the purification of $CD34^+$ stem cells was conducted by Sousa and colleagues, where a monoclonal antibody against the CD34 antigen was used for the direct partitioning of the stem cell of interest from human umbilical cord blood to the PEG-rich phase of a PEG 8000–DEX 500,000 ATPS (Sousa et al. 2011). In the case of $CD133^+$ stem cells, the following work has been conducted: traditional partitioning of $CD133^+$ stem cells from human umbilical sample in the following polymer–polymer systems: UCON–DEX 75,000, PEG 8000–DEX 500,000, and Ficoll 400,000–DEX 70,000 (González-González et al. 2014) and the addition of anti-CD133 to the previous studied systems to compare the selectivity of the immunoaffinity ATPS (González-González and Rito-Palomares 2015).

4.2.3 Other Cell Types

The fractionation of hybridoma cells from their culture media has also been studied in PEG–DEX ATPS. One example employs PEG 8000–DEX T-500 additioned with an antigen coupled to Eudragit (a copolymer of methacrylic acid–methyl methacrylate with a reversible soluble–insoluble feature dependent on pH) to separate the mouse–mouse hybridoma 16-3F cells from NS-1 cells. Without the antigen's presence, the hybridoma cells and contaminants are distributed to the bottom and interface. After the addition of the ligand (which has a favorable partition to the top phase), the target product could be recovered in the top phase (Hamamoto et al. 1996). In another investigation, the mouse–mouse hybridoma BIF6A7 cells partitioned almost completely to

the bottom phase of a PEG 35,000–DEX 40,000 system. Moreover, when this system was constructed with culture media, the hybridoma cells were successfully cultured over a period of 2 months (Zijlstra et al. 1996). This scenario elucidates the feasibility of achieving extractive bioconversion using ATPS, where the integration of fermentation and downstream processing is possible.

Other types of cells have also been studied in ATPS with the objective of characterizing specific properties (surface properties or alterations). The physicochemical properties (isoelectric point, surface net, and local hydrophobicity) of *Saccharomyces cerevisiae* and *Escherichia coli* were determined by studying their partitioning behavior in ATPS by using the same method that has been previously employed for the characterization of the surface properties of proteins in ATPS (Umakoshi et al. 1997). In another example, A431.H9 human skin cancer cells were employed to study the effect of interfacial tension on cell partitioning in a PEG 35,000–DEX 500,000 ATPS (Atefi et al. 2015).

4.3 Cell Components

Different cell components have also been isolated using ATPS, including plasma membranes, endosomes, mitochondria, lysosomes, endoplasmic reticulum, Golgi apparatus, small inclusion bodies, and extracellular vesicles, among others. Table 4.2 presents a list of examples of cell components, their source, and the type of ATPS employed for their fractionation. The mechanism of action for the separation of the cell components exploits the differences of charge and hydrophobicity of plasma membranes in order to fractionate cell organelles (Benavides et al. 2008). ATPS present several advantages for the isolation of cell organelles, including high yield and purity in a short processing time without requiring expensive equipment or large amount of tissue sample. Furthermore, the low interfacial tension joined to the non-denaturing conditions and high water content guarantee the conservation of structure and biological activity. The most studied factors for optimizing ATPS for cell organelles are the molecular weight and concentrations of the polymers and salts. Furthermore, affinity ligands (lectins, antibodies, or receptor agonists and antagonist) can also be additioned to increase the selectivity of the systems, as well as countercurrent distribution could be applied in order to rise the yield and purity of the recovered cell organelle.

The isolation of animal and plant plasma membranes has been popularly investigated with ATPS given to their intrinsic membrane surface characteristics, including the hydrophilic/hydrophobic properties and net surface charge. Most applications to membrane purification have centered on plasma membranes, plasma membrane domains, and separation of right-side-out and inside-out plasma membrane vesicles (Morré and Morre 2000). In general, plasma membranes show a higher affinity for the more hydrophobic phase, and salt (composition and concentration) and temperature have a strong influence in the partition of membranes. CCD experiments are often employed for the purification of this type of cell component, as their recovery cannot be achieved in a single-step extraction. Another option is to

Table 4.2 Partition of cell components in aqueous two-phase systems

Cell component/source	System		Reference
Plasma membrane			
Liver/hepatoma/cultured cells rats	PEG 3350	DEX T-500	Navas et al. (1989)
Cultured mammalian HeLa S cells	PEG 3350	DEX T-500	Morré and Morre (2000)
Human peripheral blood mononuclear cells	PEG 8000	DEX 500,000	Everberg et al. (2006)
Small inclusion bodies			
Escherichia coli	PEG 300	Phosphate salt	Walker and Lyddiatt (1998)
Escherichia coli	PEG 8000	Phosphate salt	Walker and Lyddiatt (1998)
Extracellular vesicles			
Mice tumors	PEG 25000 ~ 45,000	DEX 450,000 ~ 650,000	Kim et al. (2015)
Mice tumors	PEG 25000 ~ 45,000	DEX 450,000 ~ 650,000	Shin et al. (2015)
Golgi apparatus			
Cultured mammalian HeLa S cells	PEG 3350	DEX T-500	Morré and Morre (2000)
Endosomes			
Bovine kidney/HeLa S cells	PEG 3350/8000	DEX T-500	Morré et al. (1998)

combine ATPS with another technique in order to obtain the desired fractionation of the sample. In summary, the reported order of affinity of animal cell membranes for the upper phase is plasma membranes > lysosomes and endosomes > Golgi apparatus > mitochondria > endoplasmic reticulum (Morré and Morre 2000).

Plasma membrane vesicles from human peripheral blood mononuclear cells were recovered from the interface of a PEG–DEX system additioned with 90 mM sodium phosphate buffer and 0.1 mM NaCl for further proteomic analysis (Everberg et al. 2006). Another study reports the isolation of plasma membranes vesicles from the liver, hepatomas, and cultured cells in the upper phase of a PEG–DEX ATPS containing 0.25 M sucrose and 5 mM potassium phosphate in a CCD mode. Furthermore, the authors report that they could further fractionate the enriched membranes into mitochondria-, endoplasmic reticulum-, or Golgi apparatus-enriched fractions employing preparative free-flow electrophoresis or sucrose gradient centrifugation (Navas et al. 1989). Plasma membrane protein from HeLa cells was recovered on the top phase of a PEG–DEX ATPS additioned with 0.2 M potassium phosphate; meanwhile, a 3.5-fold relative enrichment of Golgi apparatus fragments was detected in the lower phase (Morré and Morre 2000).

Extracellular vesicles such as exosomes and microvesicles have also been partitioned in PEG–DEX ATPS. For example, extracellular vesicles from tumor

interstitial fluid of a C57BL6/j strain mice were isolated in the DEX-rich phase of a PEG–DEX system in less than 15 min (Shin et al. 2015). In another case, extracellular vesicles from tumor intestinal fluid of C57BL6/j mice were isolated from a complex mixture of extracellular vesicles and serum proteins in the bottom phase of a PEG–DEX system dissolved in PBS (Kim et al. 2015).

Isolation of endosomes from fresh or frozen HeLa and bovine kidney cells was conducted in a PEG–DEX multistep ATPS. Afterward, the recovered endosomes were subjected to preparative free-flow electrophoresis to fractionate early and late endosomes (Morré et al. 1998).

4.4 Partition of Viral Particles: Toward New Therapeutic Strategies

The mass study of viral components has presented a marked growth within academic and industrial research with a final aim of employing and designing new technologies using these particles as active elements. The most common viral molecules used are viruslike particles (VLPs), which are macromolecular structures directly derived from the expression of viral proteins that exhibit a spontaneous behavior for reassembly into their active viral form without the nucleic acid content (Jacinto et al. 2015). Since these particles cannot replicate, they can serve as important drug delivery vehicles and immunogens for vaccination procedures. Currently present in the international market, five VLP vaccines have been approved: Gardasil (Merck & Co.), Cervarix (GlaxoSmithKline), Recombivax HB (Merck & Co.), Engerix-B (GlaxoSmithKline), and Hecolin (Xiamen Innovax Biotech Co., Ltd) (Ladd Effio et al. 2015). Nevertheless, the major problems for VLP production schemes reside in downstream processing operations within industrial processes (Benavides et al. 2006; Ladd Effio et al. 2015). In this tenor, the study and implementation of alternative unit operations to reduce purification steps and increase recovery and purity yields are constantly being studied. In this section, successful examples of recovery of viral particles with ATPS will be discussed in order to establish potential trends and benefits of this liquid–liquid extraction technique for these macromolecules at industrial and research levels.

Nowadays, the scale-up of bioprocesses is one of the major trends in biotech engineering in order to design novel strategies that can satisfy the increased demand of selected bioproducts. Despite this, another important trend and constant challenge in process design is the scale-down of protocols to minimize reagents and time-consuming procedures. As presented by Jacinto et al. (Jacinto et al. 2015), the necessity of novel recovery procedures for human immunodeficiency virus–VLPs (HIV–VLPs) is crucial, since traditional extraction procedures (e.g., ultracentrifugation or polymer precipitation) constantly interfere with the particle final activity or infectivity. In this context, a miniature ATPS-based approach was developed to effectively recover HIV–VLPs covalently coupled to a GFP protein and thus facilitate particle detection in the mini extraction system. PEG–DEX and PEG/potassium phosphate or ammonium sulfate systems were selected to maximize VLP

partitioning toward one of the phases in the system and thus optimize the extraction step. The best system for particle recovery was composed of PEG 1500 and $(NH_4)_2SO_4$ since a partition coefficient of 3.9 was achieved in the continuous mini system. The results were analyzed, and the authors concluded that the primary variables involved in particle partitioning within the system were size (macrostructure, hence volume exclusion profiles are evident) and the hydrophobic character of the studied VLPs. In order to determine system robustness, batch ATPS were also constructed, and a K_p of 4.4 was achieved, giving important information to the authors and thus determine optimization strategies through geometry changes and mixing protocols for their mini continuous system. It was also concluded that these improvements may potentially aid issues regarding mass transfer and phase component concentrations within the developed device. The major contribution of the authors in this research was the use of a miniature ATPS system that proved effective recovery of HIV–VLPs which maintained infectivity (>55%) and were processed in just a couple of minutes (10 min or less per partitioning analysis). Processing time is an important concern in current biotech applications, and with this research, it was concluded that the employment of micro-ATPS may enhance rapid partition characterization of selected systems and thus provide a wide array of feasible scale-up options for VLP recovery at pilot plant and industrial scales without time- and resource-consuming protocols.

As presented by Effio and others (Ladd Effio et al. 2015), in the past decade more than ten VLP therapeutics have entered clinical phases II or III for indications against malaria, gastroenteritis, and influenza. For these molecules, ultracentrifugation techniques are the most employed in order to generate purified samples of VLPs for further study and characterization. In this research, an effective methodology for human B19 parvo-VLPs (parvovirus) recovery from a complex sample was developed to propose an ATPS extraction in a single- or multistage process with scale-up potential. PEG 400/PEG1450 and sodium/potassium phosphate were constructed to maximize viral particle partition toward the polymer phase. One of the most important factors was the influence of pH levels in the selected systems. Considering the macrostructures comprised by the B19 VLPs, electrostatic interactions determined the direction of particle partitioning within the ATPS tested. When a low molecular weight PEG was used, a 100% recovery of VLPs in the top phase was obtained when an untreated sample was loaded into the separation system. This effect was also achieved by the selective addition of sodium chloride (0.01–0.1 M) in order to stabilize the partitioning of the B19 particles. The main challenges of this research were oriented toward the polishing steps needed to further process the VLP-rich phases of the optimal ATPS employed, since protein contaminants from the crude extracts loaded posed an important set of impurities for the molecules recovered. In this sense, the authors proposed a centrifugal partition chromatography step in order to enhance particle isolation; nevertheless, it was determined that a marked decrease in VLP concentration was obtained after this partition strategy. Since particle purity is a vital step in industrial bioprocesses, important research opportunities can be envisioned and derived from the work presented by Effio and collaborators, specifically those steps involving polishing operations with ATPS.

The recovery of bacteriophage particles in ATPS has been done in recent years to concentrate these particles and employ them as marker particles for specific diseases. One of the most promising applications of phage particles is their potential to improve point-of-care diagnostic assays, since they can be used as ultrasensitive viral reporter molecules and thus enhance the limit of detection of lateral flow assays (LFAs) (Adhikari et al. 2013). In this respect, a vital step consists in preconcentration of a phage sample with a simple and effective technology in order to further employ the viral molecules in novel diagnostic protocols. In 2015, Jue et al. (Jue et al. 2014) successfully designed an ATPS strategy as a sample prep stage to concentrate M13 phage particles before an LFA procedure. An experimental design involving systems composed of PEG 8000 and potassium phosphate, pH 7.4, and $V_R = 1, 3, 6,$ and 9 was developed. Considering size particle and hydrophilic nature, the authors suspected that concentration of the target product in salt phases would be favored. With an optimal procedure which included a 30 min extraction time, 37 °C, pH 7.4, and V_R 0.9, a partition coefficient of 0.0001 was obtained allowing >90% recovery of M13 particles and thus improved tenfold the detection limit of the proposed LFA assay when compared to another lateral flow test without the ATPS pretreatment. The most important challenge discussed by the authors and that poses an interesting research opportunity area is regarding phage quantification, since actual protocols involve particle determination by the plaque assay which is considered to be very time- and resource-consuming, with high variation of results in each batch. In this tenor, technologies involving immunodetection (e.g., ELISA) could be positively enhanced in the near future to design time-saving protocols in this area and thus provide feasible and validated methods to facilitate phage studies within ATPS applications.

One of the actual challenges of pharmaceutical companies is the constant and increasing need of rapid and feasible immunization programs, which by definition require high-quality vaccination products, with competent production yields and affordable prices for the human population. Within this aspect, reengineering of classic-established bioprocesses for biological productions needs to be done in order to reduce the actual financial burdens and hence allow product availability worldwide. Typical unit operations involved in downstream processing (DSP) of vaccine-derived products involve precipitation, centrifugation, and chromatographic separation techniques to achieve selected purity and recovery yields. A recent study presented by Vijayaragavan et al. (Vijayaragavan et al. 2014) presented the recovery of a surrogate molecule for hepatitis A and poliovirus, porcine parvovirus particles (PPV), considering also their small size and structural similarity to adeno-associated virus which are commonly used in gene therapy. After an elaborated experimental design, an effective ATPS strategy for infectious PPV in a serum-containing media was designed to discuss industrial potential for vaccine production. A PEG 12,000 and citrate system allowed 63% PPV recovery in the polymer-rich phase. The authors concluded that due to the molecular nature of PPV particles, hydrophobic forces induced viral molecules to partition into the more hydrophobic PEG phase. In addition, the difference in ionic force and electrochemical forces allowed the majority of contaminant proteins from the serum media

Table 4.3 Partition of viral particles in aqueous two-phase systems

Viral particle	Source	System	Reference
HIV–GFP particles	VLP	PEG 1000/8000–DEX T-500	Jacinto et al. (2015)
Human B19 parvovirus	VLP	PEG 1000/8000–K_2PO_4	Ladd Effio et al. (2015)
M13 particles	Bacteriophage	PEG 8000–K_2PO_4	Jue et al. (2014)
Porcine parvovirus	Viral particles	PEG 3000/8000–citrate	Vijayaragavan et al. (2014)
B19 particles	VLP	PEG 1000–$MgSO_4$	Luechau et al. (2011)
M13 particles	Bacteriophage	Triton X114–K_2PO_4	Mashayekhi et al. (2010)
T4 particles	Bacteriophage	PEG 800–K_2PO_4/PEG 600–sulfate	Kamei et al. (2002a, b)
Rotavirus particles	dlVLP	PEG 400–8000–K_2PO_4	Benavides et al. (2006)
P22 particles	Bacteriophage	Water–$C_{10}E_4$ (micellar)	Kamei et al. (2002a)
X174 particles	Bacteriophage	Water–$C_{10}E_4$ (micellar)	Kamei et al. (2002b)
T4 particles	Bacteriophage	Water–$C_{10}E_4$ (micellar)	Kamei et al. (2002a)
P22 particles	Bacteriophage	Water–$C_{10}E_4$ (micellar)	Kamei et al. (2002a)
HIV-I	Viral particles	PEG 6000–DEX T-500	Hammar and Gilljam (1990)

(>85%) to be recovered in the bottom, salt-rich phase. The major challenge in this research was the importance of understanding and controlling the surface tension of the system in order to prevent the viral particles to be concentrated at the interface. This was achieved with strict control in the citrate or NaCl concentration in the employed systems, and the authors concluded that this variable could be determinant in future research comprising viral particle recovery in two-phase systems in order to design feasible protocols for industrial applications.

Analyzing the above-presented examples and the information described in Table 4.3, it can be concluded that the viral particle recovery has been a strong research niche for ATPS strategies in the past decade. If it is considered that typical recovery yields in liquid–liquid extraction methodologies are above 60%, thus this simple technology might suppose an interesting option for actual industrial processes that commonly exhibit recovery rates that range from 20% to 35%. One of the constant challenges that needs to be studied in the future is the polishing of virus-rich phases in ATPS. Adequate removing of phase components and contaminants (e.g., PEG molecules, high salt concentrations, contaminant products such as proteins) is a key step in bioprocess development, since common unoptimized procedures have a direct impact in particle activity/infectivity. In addition, a major disadvantage of ATPS for viral particles recovery is the employed dilution factors. New approaches of protocols must be adequately studied and implemented in order to concentrate target products in small volumes without compromising particle structure. The last elements to be considered are the analytical methodologies employed for VLP or phage quantification, since plaque assays tend to be labor- and resource-consuming. In this subject, more research is needed in terms of more

robust immunodetection and ELISA tests in order to reduce analytical times in the process. It must be considered that phase components may hamper chemical interactions in these analytical procedures, reason that constitutes the primary challenge in this area nowadays.

4.5 Nucleic Acids Recovery in ATPS: Current Innovations

Improved healthcare advances have been possible in the past four decades primarily because of novel and efficient biotech-derived efforts in both academic and industrial research facilities. One of the most studied areas in current medicine is that of human gene therapy. These therapies actually require efficient DSP for the obtention of high-quality vectors (Bhambure et al. 2013; Tateishi-Karimta et al. 2014). Nevertheless, production titers and recovery yields for these molecules, typically nucleic acids, are modest and thus represent one of the limiting steps for bioprocess engineering strategies. In addition, it must be taken into account that the scale-up potential of traditional purification techniques for nucleic acid isolation is limited, hence the importance of considering alternative technologies such as ATPS (Jungbauer 2013). The following section explores selected examples to emphasize the potential of two-phase extraction for nucleic acid-derived molecules.

The spread of viral-related tropical diseases, such as dengue and its lethal hemorrhagic fever, has been a target for development of new treatments around the world. In this context, an improved interest in gene therapy and vaccines composed of nucleic acids (e.g., DNA principally) as active ingredients has arisen, situation that has increased the demand for production and purification of plasmidic DNA (pDNA). An urgent topic in pDNA production schemes is the vital need of novel and efficient DSP technologies that may offer minimal or null final product contamination. An interesting approach for purifying a dengue 2 plasmid DNA vaccine was developed to determine the potential of ATPS as a scale-up technique for product recovery (Moreira et al. 2007). A simple experimental design using PEG/potassium phosphate systems was designed. After several experimental runs, it was determined that a PEG 1000/ PO_4 system allowed almost a 40% total recovery of the plasmid in the top phase. The authors hypothesized that because of the disrupting interactions between PEG and salt molecules, the partition of plasmid molecules would be favored to the polymer-rich phase. Nevertheless, plasmid stability and high recovery yields are the major challenges, since high ionic strengths disrupt the plasmidic structure in high TLL systems. In addition, nucleic acid structure should be considered when designing an ATPS strategy, since selected systems tested by Moreira clearly concentrated the target molecule in the interface but its recovery from this phase was not further studied (Moreira 2007). Despite this, a 40% plasmid recovery was considered as successful, since traditional pDNA recovery kits have efficiencies ranging from 20% to 30%.

Partitioning of complex nucleic acid samples has been carried in the past, considering several variables that might prove a statistical difference regarding macromolecule partitioning behavior. A deep study presented by Luechau et al. (Luechau et al. 2009a, b) identified the effects of neutral salts onto the behavior of different

types of nucleic acids being loaded from a complex crude sample. Considering that pDNA is commonly recovered from a blend of DNA, RNA, and pDNA, this research emphasized the importance of understanding the effect posed by excluded volumes in selected ATPS formed by PEG (300, 1450, and 6000) and potassium phosphate with NaCl addition. The main vision of the authors was to design an extraction system that could concentrate pDNA in one phase and contaminant RNA and DNA in the opposite one. Because of the particle size and electrostatic interactions with the surrounding media, a PEG 300–PO_4 system was able to concentrate pDNA in the salt-rich phase ($K_p > 0.020$) and contaminant RNA in the PEG phase ($K_p > 3$). Even though the results do not include a graphical response of the partitioning behavior of the nucleic acids versus the changes in TLL, the authors discussed the fact that increasing values of TLLs may enhance pDNA partition toward salt-rich phases following their theoretical results based on volume exclusion. In addition, a response surface methodology (RSM) analysis could be developed to accurately predict partitioning behaviors considering several target products and experimental variables. These studies are not common for ATPS, but they could prove important advances in terms of operation design and further industrial feasibility analysis.

As previously mentioned in this chapter, an important variant within ATPS is their potential to accept a chemical or biological modification in order to enhance partition selectivity by the coupling of affinity ligands into the phase-forming chemicals or adding them free in solution, as defined by Ruiz-Ruiz et al. (Ruiz-Ruiz et al. 2012). For example, liquid–liquid affinity partitioning (ATPAP) of pDNA using a PEGylated zinc finger–GST (glutathione-S-transferase) fusion protein as affinity ligand was studied in PEG–DEX ATPAP by Barbosa et al. (Barbosa et al. 2008). Purified pDNA-based therapies emphasize the importance of scalable and cost-effective process development. The coupling of a fusion protein to a polymeric matrix reduces steric hindrance commonly present in resin-coupled proteins. PEG 600–DEX 40 and PEG 1000–DEX 500 systems were used for pDNA isolation. Comparative experiments determined that when the fusion protein was first administered in the system in its unPEGylated form, its K_p was ~38 and the K_p of the plasmid added to the system was calculated as 0.005. Nevertheless, pDNA partition coefficient increased 5900-fold (from K_p 0.005 to 29.42) when adding the PEGylated form of the proposed affinity ligand to system PEG 1000–DEX 500. The authors confirmed that a strong interaction between the PEGylated fusion protein and the pDNA allows the complex to concentrate in a specific phase (PEG-rich phase). PEG 600–DEX 40 systems presented a lower increase in K_p (from 0.0003 to 2.42). However, when increasing the PEGylated fusion ligand concentration to 293 μg/ml, pDNA partitioned completely (100%) toward the PEG-rich phase, thus representing an efficient strategy for its recovery. The authors demonstrated the potential of affinity partitioning of pDNA in ATPAP, emphasizing the effect of polymer characteristics such as concentration and molecular weight on ligand distribution through the system, which can be exploited to develop new concentration and purification strategies.

Nucleic acids have been recovered since ATPS were first proposed by Albertsson as an interesting recovery technique for macromolecules and particles. As presented in Table 4.4, the most recent studies of nucleic acid recovery are referred to plasmid

Table 4.4 Partition of nucleic acids in aqueous two-phase systems

Nucleic acids	Source	System	Reference
pD2 plasmid	Plasmid DNA	PEG 400–K_2PO_4	Moreira et al. (2007)
E. coli DNA	Cell DNA	PEG 6000–K_2PO_4/sulfate	Bhambure et al. (2013)
pDNA	Plasmid DNA	PEG 300–6000–K_2PO_4	Luechau et al. (2009a)
RNA	Pure RNA	PEG 300–6000–K_2PO_4	Luechau et al. (2009b)
pTX0161	Plasmid DNA	PEG 300–1450–K_2PO_4	Luechau et al. (2009a, b)
Retroviral vectors	Plasmid DNA/ SS NA	PEG 1000/8000–K_2PO_4/ citrate	Rodrigues et al. (2007)
pDNA polyplexes	Plasmid DNA	PEG 600/3350–$(NH_4)_2SO_4$	Duarte et al. (2007)
CF-pDNA	Plasmid DNA	PEG 200/8000–K_2PO_4	Ribeiro et al. (2002)
Nano inclusion bodies	Mimic pDNA	DEX 500/PEG 8000–K_2PO_4	Braas et al. (2000)
pDNA	Plasmid DNA	PEG 400–8000–DEX 70/T-500	Barbosa et al. (2008)

DNA variants of this type of molecules. Considering the important increase in vector-based therapies, the constant study and development of novel and alternative technologies that may enhance particle recovery, without substantially increasing overall processing cost, are an actual constant trend in the bioprocess engineering research environment. Regarding ATPS strategies, structured studies are needed in which relations between pH, volume ratios, TLLs, and ionic force shifts and the use of naturally occurring phase-forming chemicals might positively trigger the particle partition of nucleic acid-derived structures. In addition, no scale-up procedures for these molecules (i.e., nucleic acids) have been carried out, and this is of extreme importance considering that almost all of the actual published research papers state that ATPS technologies could be positively coupled in actual industrial bioprocessing DSP trains. In this same line, no economic projections have been presented for this type of particle recovery using ATPS. This is an important opportunity area since the economic dimension of a bioprocess tends to be considered a valid source of information for well-established pharmaceutical companies that may look into alternative technologies in order to expand their current production schemes into a more robust manufacturing plan within their capabilities. In this tenor, software-aided economic projections for nucleic acid–ATPS could pose an important advantage for future research that seek to exploit the relation between this recovery technique and macromolecular structures.

4.6 Macroparticle Recovery with ATPS: Challenges and Future Trends

Considering the information presented in the past sections, interesting trends regarding the use of aqueous two-phase systems for the recovery and purification of high-added value particles can be envisioned.

As discussed by several authors, one of the major advantages of liquid–liquid extraction consists in the ease of setup, robustness, and scale-up feasibility. In this sense, ATPS strategies for macroparticles may be easily coupled to fermentation, filtration, precipitation, or centrifugation steps and hence significantly increase the recovery and/or purity yields of novel designed bioprocesses with important potential in industrial applications. Taking this into account, one of the major trends in ATPS protocols in terms of cell recovery is the engineering of in situ and continuous extraction systems. The first advances in this field started in South America (Professor Juan Asenjo in Chile) and Europe (Professor Joaquim Cabral in Portugal), and now a solid research line in continuous extraction of high-added value biologicals has also been developed in our research group (Professor Marco Rito-Palomares in Mexico). An important aspect that represents an important advantage of continuous processing, as opposed to a batch-based protocol, is that regulatory aspects of the designed strategies can be more easily fulfilled at industrial scales, providing ATPS-based approaches a theoretical easier inclusion into the biotech environment (i.e., pharmaceutical applications).

Within continuous operation, two additional aspects that constitute another marked trend within recovery of particles in ATPS are the manufacturing and design of miniaturized and fully automated lab-on-a-chip devices capable of mixing and separating in real-time complex samples. In this context, new designs based on microfluidics keep to prove the positive potential of two-phase extraction of whole cells and proteins. Despite this fact, no commercial applications with this technology have been developed since system selectivity has not been optimal in these devices. It has been suggested by several authors that a microscale separation platform constructed with polydimethylsiloxane (PDMS) devices covalently coupling antibodies targeted to specific cell epitopes may prove to be a successful strategy for highly specific stem cell separations. Nevertheless, because of the challenges and physical limitations of this technology, this approach has not been truly proved, and hence an interesting opportunity area for cell recovery in miniature-scale ATPS still remains.

Bioprocess engineering strategies and novel designs based on ATPS technology remain to be applied at industrial scales because of several constant challenges this technology poses. One of the most discussed opportunity areas for two-phase extraction of biomolecules resides in the understanding of the precise molecular mechanisms involved in product/particle partitioning. Even though several authors, such as Aires-Barros (Silva et al. 2014; Jacinto et al. 2015), Asenjo (Asenjo and Andrews 2012), Benavides and Rito-Palomares (Benavides and Rito-Palomares 2008), Zaslavsky (Zaslavsky 1992), and Albertsson (Albertsson 1961, 1985), have thoroughly discussed the molecular and thermodynamic mechanisms of solutes partitioning in ATPS, up to date no general mathematical models and rules can be generally applied to fractionate random biological products. Considering the partitioning of macroparticles, as presented in this chapter, Fig. 4.1 presents the governing interactions that enable selective recovery of whole cells, viral particles, cell fragments/organelles, and nucleic acids. Viral particles and DNA variants, for example, exhibit a marked partition behavior based on their physical size, hence

AQUEOUS TWO-PHASE SYSTEMS	BIOPARTICLE	PARTITIONING MECHANISM
Polymer-Polymer	Cells Stem cells Cell components	Surface charge Hydrophobicity Receptors
Salt-Polymer	Nucleic Acids Viral particles	Size Surface charge

Fig. 4.1 Type of aqueous two-phase system employed and the partitioning mechanism exploited for the recovery of bioparticles

supporting the theory of excluded volumes within the systems. Nevertheless, research analyzing complex biological samples has proved that depending on the nature of the added components to the selected ATPS, ionic force shifts, and hence electrochemical charge potential, can dramatically improve partition of double-stranded DNA molecules toward a polymer (typically PEG)-rich phase. This is a constant challenge that must be overcome and constantly discussed in order to favor ATPS implementation at pilot plant and industrial scales.

One of the most important aspects of biotechnology applications is that of analytical techniques. Considering the research done in the past decades in ATPS design, characterization, and implementation, efficient and reliable identification techniques for target molecules in these liquid–liquid systems have constituted one of the primary challenges. Even though the most studied molecules in ATPS are proteins, traditional detection methods (e.g., Bradford assay, Lowry method, ninhydrin assay, or Smith assay [BCA]) are severely affected by the high concentration of phase-forming components, such as PEG or salts (e.g., phosphate, citrate, ammonium sulfate). Regarding aliquot analysis, phase samples need to be highly diluted up to 1% PEG or 10% salt (final concentration) in order to favor protein detection without compromising more than 10% the final sensitivity of the assay (González-González et al. 2011). In this same subject, detection of whole cells and viral particles represents an important opportunity area since in most of the studied cases, the analytical techniques involved in particle quantification take more time than the actual ATPS processing lapse. Besides this important fact, the complex protocols or equipment (i.e., flow cytometry or plaque assays for phage quantification) can significantly decrease the technical or economic industrial feasibility for a proposed two-phase strategy from the analytical point of view.

Further into the separation process, the polishing steps required in this liquid–liquid technology can be a limiting agent. Since a particular product is to be recovered in a specific phase regarding its physicochemical and biochemical properties, effective protocols for polymer/salt removal and concentration are very common. For viral particles, it has been reported that ultrafiltration, centrifugal partition chromatography, or precipitation steps can dramatically destabilize the particle, and thus

up to 50% of the recovered infectivity in the ATPS can be lost. Despite the fact that polishing steps might reduce the final isolated quantity of active particles, the overall benefits of employing a simple and scalable ATPS might overcompensate this and might prove to be a feasible adaptation to actual industrial applications.

The above-discussed challenges should be carefully considered in order to design novel downstream processing methodologies that could possibly trigger the application of ATPS at industrial levels within specific industries, especially in the pharmaceutical sector.

4.7 Concluding Remarks

ATPS have demonstrated to be a promising technique for the recovery of cells, cell organelles, viruses, and nucleic acids, thanks to the multiple advantages this method has demonstrated. Furthermore, a better understanding of the principle parameters that drive ATPS partitioning for this type of biomolecules has resulted in the development of more robust strategies for their recovery. However, existing challenges such as the development of reliable methods for the quantification and polishing of the target biomolecules in these systems must be attended to promote the implementation of ATPS at industrial scale.

References

Adhikari M, Dhamane S, Hagström AEV, Garvey G, Chen WH, Kourentzi K, Strych U, Willson RC. Functionalized viral nanoparticles as ultrasensitive reporters in lateral-flow assays. Analyst. 2013;138(19):5584–7.

Albertsson PÅ. Fractionation of particles and macromolecules in aqueous two-phase systems. Biochem Pharmacol. 1961;5(4):351–8.

Albertsson PÅ. History of aqueous polymer two-phase partition. In: Walter H, Brooks DE, Fisher D, editors. Partitioning in aqueous two-phase system. Orlando: Academic; 1985. p. 1–10.

Asenjo JA, Andrews BA. Challenges and trends in bioseparations. J Chem Technol Biotechnol. 2008;83(2):117–20.

Asenjo JA, Andrews BA. Aqueous two-phase systems for protein separation: phase separation and applications. J Chromatogr A. 2012;1238:1–10.

Atefi E, Joshi R, Mann JA, Tavana H. Interfacial tension effect on cell partition in aqueous two-phase systems. ACS Appl Mater Interfaces. 2015;7(38):21305–14.

Barbosa H, Hine AV, Brocchini S, Slater NKH, Marcos JC. Affinity partitioning of plasmid DNA with a zinc finger protein. J Chromatogr A. 2008;1206(2):105–12.

Benavides J, Rito-Palomares M. Practical experiences from the development of aqueous two-phase processes for the recovery of high value biological products. J Chem Technol Biotechnol. 2008;83:133–42.

Benavides J, Mena JA, Cisneros-Ruiz M, Ramírez OT, Palomares LA, Rito-Palomares M. Rotavirus-like particles primary recovery from insect cells in aqueous two-phase systems. J Chromatogr B. 2006;842(1):48–57.

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(6):838–45.

Bhambure R, Sharma R, Gupta D, Rathore AS. A novel aqueous two phase assisted platform for efficient removal of process related impurities associated with *E. coli* based biotherapeutic protein products. J Chromatogr A. 2013;1307:49–57.

Braas GMF, Walker SG, Lyddiatt A. Recovery in aqueous two-phase systems of nanoparticulates applied as surrogate mimics for viral gene therapy vectors. J Chromatogr B. 2000;743(1–2):409–19.

Cabral J. Cell partitioning in aqueous two-phase polymer systems. In: Kumar A, Galaev I, Mattiasson B, editors. Cell separation. Berlin/Heidelberg: Springer; 2007. p. 151–71.

Duarte SP, Fortes AG, Prazeres DMF, Marcos JC. Preparation of plasmid DNA polyplexes from alkaline lysates by a two-step aqueous two-phase extraction process. J Chromatogr A. 2007;1164(1–2):105–12.

Everberg H, Peterson R, Rak S, Tjerneld P, Emanuelsson C. Aqueous two-phase partitioning for proteomic monitoring of cell surface biomarkers in human peripheral blood mononuclear cells. J Proteome Res. 2006;5(5):1168–75.

Fisher D. The separation of cells and organelles by partitioning in two-polymer aqueous phases. Biochem J. 1981;196(1):1–10.

García-Pérez AI, Recio MN, Sancho P, Luque J. Partitioning behaviour of rat bone marrow cells in aqueous two-phase systems. Dependence of cell partition on the interfacial tension and electrical potential difference between the phases. J Chromatogr A. 1987;403(C):131–43.

González-González M, Rito-Palomares M. Aqueous two-phase systems strategies to establish novel bioprocesses for stem cells recovery. Crit Rev Biotechnol. 2013;34(4):318–27.

González-González M, Rito-Palomares M. Application of affinity aqueous two-phase systems for the fractionation of CD133+ stem cells from human umbilical cord blood. J Mol Recognit. 2015;28(3):142–7.

González-González M, Mayolo-Deloisa K, Rito-Palomares M, Winkler R. Colorimetric protein quantification in aqueous two-phase systems. Process Biochem. 2011;46(1):413–7.

González-González M, Vázquez-Villegas P, García-Salinas C, Rito-Palomares M. Current strategies and challenges for the purification of stem cells. J Chem Technol Biotechnol. 2012;87(1):2–10.

González-González M, Rito-Palomares M, Méndez Quintero O. Partition behavior of CD133+ stem cells from human umbilical cord blood in aqueous two-phase systems: in route to establish novel stem cell primary recovery strategies. Biotechnol Prog. 2014;30(3):700–7.

Hamamoto R, Kamihira M, Iijima S. Specific separation of animal cells using aqueous two-phase systems. J Ferment Bioeng. 1996;82(1):73–6.

Hammar L, Gilljam G. Extraction of HIV-1 in aqueous two-phase systems to obtain a high yield of gp120. Aids Res Hum Retroviruses. 1990;6(12):1379–88.

Hatti-Kaul R. Aqueous two-phase systems – a general overview. Mol Biotechnol. 2001;19(3): 269–77.

Jacinto MJ, Soares RRG, Azevedo AM, Chu V, Tover A, Conde JP, Aires-Barros MR. Optimization and miniaturization of aqueous two phase systems for the purification of recombinant human immunodeficiency virus-like particles from a CHO cell supernatant. Sep Purif Technol. 2015;154:27–35.

Jimeno P, Garcia-Perez AI, Luque J, Pinilla M. Changes in glycolytic enzyme activities in aging erythrocytes fractionated by counter-current distribution in aqueous polymer two-phase systems. Biochem J. 1991;279(1):237–43.

Jue E, Yamanishi CD, Chiu RYT, Wu BM, Kamei DT. Using an aqueous two-phase polymer-salt system to rapidly concentrate viruses for improving the detection limit of the lateral-flow immunoassay. Biotechnol Bioeng. 2014;111(12):2499–507.

Jungbauer A. Continuous downstream processing of biopharmaceuticals. Trends Biotechnol. 2013;31(8):479–92.

Kamei DT, King JA, Wang DIC, Blankschtein D. Understanding viral partitioning in two-phase aqueous nonionic micellar systems: 2. Effect of entrained micelle-poor domains. Biotechnol Bioeng. 2002a;78(2):203–16.

Kamei DT, Liu C-L, Haase-Pettingell C, King JA, Wang DIC, Blankschtein D. Understanding viral partitioning in two-phase aqueous nonionic micellar systems: 1. Role of attractive interactions between viruses and micelles. Biotechnol Bioeng. 2002b;78(2):190–202.
Kim J, Shin H, Kim J, Kim J, Park J. Isolation of high-purity extracellular vesicles by extracting proteins using aqueous two-phase system. PLoS One. 2015;10(6):e0129760.
Kumar A, Kamihira M, Galaev IY, Mattiasson B, Iijima S. Type-specific separation of animal cells in aqueous two-phase systems using antibody conjugates with temperature-sensitive polymers. Biotechnol Bioeng. 2001;75(5):570–80.
Ladd Effio C, Wenger L, Ötes O, Oelmeier SA, Kneusel R, Hubbuch J. Downstream processing of virus-like particles: single-stage and multi-stage aqueous two-phase extraction. J Chromatogr A. 2015;1383:35–46.
Luechau F, Ling TC, Lyddiatt A. Partition of plasmid DNA in polymer-salt aqueous two-phase systems. Sep Purif Technol. 2009a;66(2):397–404.
Luechau F, Ling TC, Lyddiatt A. Selective partition of plasmid DNA and RNA in aqueous two-phase systems by the addition of neutral salt. Sep Purif Technol. 2009b;68(1):114–8.
Luechau F, Ling TC, Lyddiatt A. Recovery of B19 virus-like particles by aqueous two-phase systems. Food Bioprod Process. 2011;89(4):322–7.
Malmström P, Nelson K, Jönsson Å, Sjögren HO, Walter H, Albertsson PÅ. Separation of rat leukocytes by countercurrent distribution in aqueous two-phase systems. Cell Immunol. 1978; 37(2):409–21.
Malmström P, Jonsson A, Hallberg T, Sjogren HO. Countercurrent distribution of lymphocytes from human peripheral blood in an aqueous two-phase system. I. Separation into subsets of lymphocytes bearing distinctive markers. Cell Immunol. 1980a;53(1):39–50.
Malmström P, Jonsson A, Sjogren HO. Countercurrent distribution of lymphocytes from human peripheral blood in an aqueous 2-phase system. II. Separation into subsets of lymphocytes with distinctive functions. Cell Immunol. 1980b;53(1):51–64.
Mashayekhi F, Chiu RYT, Le AM, Chao FC, Wu BM, Kamei DT. Enhancing the lateral-flow immunoassay for viral detection using an aqueous two-phase micellar system. Anal Bioanal Chem. 2010;398(7–8):2955–61.
Mayolo-Deloisa K, Gonzalez-Valdez J, Guajardo-Flores D, Aguilar O, Benavides J, Rito-Palomares M. Current advances in the non-chromatographic fractionation and characterization of PEGylated proteins. J Chem Technol Biotechnol. 2011;86(1):18–25.
Moreira KA, Chaves AC, Marques ET, Prazeres DMF, De Azevedo WM, Porto ALF, De Filho JLL. Extraction of dengue 2 plasmid DNA vaccine (pD2) from cell lysates by aqueous two-phase systems. Biotechnol. 2007;6(4):520–6.
Morré DM, Morre DJ. Aqueous two-phase partition applied to the isolation of plasma membranes and Golgi apparatus from cultured mammalian cells. J Chromatogr B. 2000;743(1–2):377–87.
Morré DJ, Morré DM, Van Alstine JM. Separation of endosomes by aqueous two-phase partition and free-flow electrophoresis. J Chromatogr B. 1998;711(1–2):203–15.
Navas P, Nowack DD, Morré DJ. Isolation of purified plasma membranes from cultured cells and hepatomas by two-phase partition and preparative free-flow electrophoresis. Cancer Res. 1989;49(8):2147–56.
Ribeiro SC, Monteiro GA, Cabral JMS, Prazeres DMF. Isolation of plasmid DNA from cell lysates by aqueous two-phase systems. Biotechnol Bioeng. 2002;78(4):376–84.
Rodrigues T, Carrondo MJT, Alves PM, Cruz PE. Purification of retroviral vectors for clinical application: biological implications and technological challenges. J Biotechnol. 2007;127(3):520–41.
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.
Ruiz-Ruiz F, Benavides J, Rito-Palomares M. Scaling-up of a B-phycoerythrin production and purification bioprocess involving aqueous two-phase systems: practical experiences. Process Biochem. 2013;48(4):738–45.
Schindler J, Nothwang HG. Aqueous polymer two-phase systems: effective tools for plasma membrane proteomics. Proteomics. 2006;6(20):5409–17.

Sharp KA, Yalpani M, Howard SJ, Brooks DE. Synthesis and application of a poly(ethylene glycol)-antibody affinity ligand for cell separations in aqueous polymer two-phase systems. Anal Biochem. 1986;154(1):110–7.

Shin H, Han C, Labuz JM, Kim J, Kim J, Cho S, Gho YS, Takayama S, Park J. High-yield isolation of extracellular vesicles using aqueous two-phase system. Sci Rep. 2015;5:13103.

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.

SooHoo J, Walker G. Microfluidic aqueous two phase system for leukocyte concentration from whole blood. Biomed Microdevices. 2009;11(2):323–9.

Sousa A, Andrade P, Pirzgalska R, Galhoz T, Azevedo A, da Silva C, Raquel Aires-Barros M, Cabral J. A novel method for human hematopoietic stem/progenitor cell isolation from umbilical cord blood based on immunoaffinity aqueous two-phase partitioning. Biotechnol Lett. 2011;33(12):2373–7.

Tateishi-Karimta H, Sugimoto N. Control of stability and structure of nucleic acids using cosolutes. Methods. 2014;67(2):151–8.

Tsukamoto M, Taira S, Yamamura S, Morita Y, Nagatani N, Takamura Y, Tamiya E. Cell separation by an aqueous two-phase system in a microfluidic device. Analyst. 2009;134(10):1994–8.

Umakoshi H, Kuboi R, Komasawa I. Control of partitioning of bacterial cells and characterization of their surface properties in aqueous two-phase systems. J Ferment Bioeng. 1997;84(6):572–8.

Van Alstine JM, Brooks DE. Cell membrane abnormality detected in erythrocytes from patients with multiple sclerosis by partition in two-polymer aqueous-phase systems. Clin Chem. 1984;30(3):441–3.

Vijayaragavan KS, Zahid A, Young JW, Heldt CL. Separation of porcine parvovirus from bovine serum albumin using PEG–salt aqueous two-phase system. J Chromatogr B. 2014;967:118–26.

Walker SG, Lyddiatt A. Aqueous two-phase systems as an alternative process route for the fractionation of small inclusion bodies. J Chromatogr B. 1998;711(1–2):185–94.

Walter H, Krob EJ. Separation and subfractionation of small numbers of cells (~106) by countercurrent distribution in dextran-poly(ethylene glycol) aqueous-phase systems. Cell Biophys. 1984;6(4):253–62.

Walter H, Webber TJ, Michalski JP, McCombs CC, Moncla BJ, Krob EJ, Graham LL. Subfractionation of human peripheral blood lymphocytes on the basis of their surface properties by partitioning in two-polymer aqueous phase systems. J Immunol. 1979;123(4):1687–95.

Zaslavsky BY. Bioanalytical applications of partitioning in aqueous polymer two-phase systems. Anal Chem. 1992;64(15):765A–73A.

Zijlstra GM, de Gooijer CD, van der Pol LA, Tramper J. Design of aqueous two-phase systems supporting animal cell growth: a first step toward extractive bioconversions. Enzyme Microb Technol. 1996;19(1):2–8.

5 Aqueous Two-Phase System Strategies for the Recovery and Partial Purification of Bioactive Low Molecular Weight Compounds

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Abbreviations

ATPS	Aqueous two-phase systems
diCQA	Dicaffeoylquinic acids
[EMIM][Ac]	1-ethyl-3-methylimidazolium acetate
HPLC	High-performance liquid chromatography
LMWCs	Low molecular weight compounds
SFE	Supercritical fluid extraction
SPE	Solid-phase extraction
TLL	Tie-line length
V_R	Volume ratio

5.1 Introduction

In recent years, the interest in nutraceutical compounds (i.e., chemicals with both nutritional and pharmaceutical effect) has increased significantly (Das et al. 2012; Nasri et al. 2014). In fact, the global nutraceutical market was valuated in USD 250

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billion in 2014, and an increase of USD 385 billion in the next 7 years is estimated (Mordor Intelligence Report 2016). Many of these nutraceuticals are low molecular weight compounds (LMWCs; <1000 g mol^{-1}), such as garlic acid, resveratrol, chlorogenic acid, gallic acid, salicin, and carotenoids, among others (Chavez-Santoscoy et al. 2010; Gómez-Loredo et al. 2014; Simental-Martínez et al. 2014; Badhani et al. 2015). Particularly, phytochemicals including phenolic acids, hydroxycinnamic acids, stilbenes, flavonols, resveratrol, quercetin, and crocins have been extensively studied due to the wide beneficial effects on human health including antioxidant, antiallergenic, antiviral, antiatherogenic, antifungal, anti-inflammatory, antimicrobial, and cardioprotective (Quideau et al. 2011; Montalvo-Hernández et al. 2012). For example, 3-caffeoylquinic acid, also known as chlorogenic acid, is the most studied member of the chlorogenic acid family mainly for its antioxidant activity (El-Seedi et al. 2012). 3,4-Dicaffeoylquinic acid is another member of this family, and there is a special interest in this compound due to its activity against the integrase of the human immunodeficiency virus (Robinson et al. 1996). On the other hand, resveratrol has been extensively studied because of its protection against cardiovascular disease, cancer, diabetes mellitus, and other aging-related diseases (Wang et al. 2008a). Crocins are water-soluble carotenoids having many biological activities including antioxidant, hypolipidemic, neuroprotective, and antidepressive, among other activities (Montalvo-Hernández et al. 2012).

Because of their multiple nutraceutical effects, bioactive LMWCs have potential uses in pharmaceutical, food, and dietary supplement industries. This has motived the development of efficient production and downstream process strategies in order to obtain the highest yields of the compound of interest (Cisneros-Zevallos 2003; Wang et al. 2008a; Jiang et al. 2009; Dai and Mumper 2010; Jacobo-Velázquez and Cisneros-Zevallos 2012; Sánchez-Rangel et al. 2016).

The first step to isolate bioactive LMWCs is to extract them from their expression system. For this purpose, solid–liquid extraction approaches remain the most used strategies (Kim and Lee 2002; Pérez-Magariño et al. 2008; Tsao and Deng 2004). To select the appropriate solvent for extraction, it is important to have information about the physicochemical properties of the compound of interest, the nature of matrix source, and the contaminants present in the sample (Dai and Mumper 2010). In order to extract nutraceutical compounds, the sample is homogenized, resulting in an increase of the contact area between the solvent and the sample (Kim and Lee 2002; Dai and Mumper 2010). In addition to solid–liquid extraction, there are other strategies to extract compounds from different sources including microwave-assisted extraction, ultrasound-assisted extraction, supercritical fluid extraction, pressurized fluid extraction, or accelerated solvent extraction (Tsao and Deng 2004; Wang and Weller 2006; Dai and Mumper 2010; Garcia-Salas et al. 2010; Khoddami et al. 2013; Cuéllar-Villarreal et al. 2016) (Fig. 5.1).

Specifically, microwave-assisted extraction involves the application of microwaves for heating the sample in a solvent. The heating increases the rate of extraction of the compounds from the cell. However, the high temperature involved in this approach limits its use to extract compounds with low thermostability, such as tannins and anthocyanins, which are degraded at relative mild temperatures (Dai and

	Extraction strategies					Purification strategies			
Parameters	Homogenization	Microwave-assisted extraction	Supercritical fluid extraction	Pressurized fluid extraction	ATPS	Solid phase extraction	HPLC	SFE	ATPS
Low cost	Y	Y	N	N	Y	N	N	N	Y
Amount of solvent	M	M	M	M	M	M	H	H	M
Extraction rate	H	M	M	H	H	M	H	H	H
Sample pretreatment	Y	Y	Y	Y	N	Y	Y	Y	N
Integration process	N	N	N	N	Y	N	N	N	Y
Scalable process	Y	N	N	N	Y	Y	Y	Y	Y
Complex instruments	Y	Y	Y	Y	N	Y	Y	Y	N

Fig. 5.1 Comparison of some parameters between different extraction and purification strategies and ATPS approach. *HPLC* high-performance liquid chromatography, *SFE* supercritical fluid extraction. *Y* yes, *N* no, *L* low, *M* medium, *H* high

Mumper 2010; Garcia-Salas et al. 2010; Delazar et al. 2012). Ultrasound-assisted extraction comprises the implosion of cavitation bubbles generated by acoustic waves, resulting in the release of compounds due to the disruption of the cells. Although this strategy can be used to extract nutraceutical compounds from different matrices, the yield of extraction is lower compared to hot solvent extractions (Kim and Lee 2002; Garcia-Salas et al. 2010). Supercritical fluid extraction (SFE) method involves the application of high pressure (3.3–20.3 MPa), which produces a faster extraction time and lower solvent used. However, the application of this approach is limited to chemical compounds of low or medium polarity, whereas in pressurized fluid extraction, the high temperatures used may degrade the nutraceutical compounds. Furthermore, the scaling up of these methodologies at industrial scale is, in some cases, still unfeasible, and the cost related to their operation is high (Wang and Weller 2006; Dai and Mumper 2010; Garcia-Salas et al. 2010).

Once the compound of interest has been extracted from its source, it needs to be fractionated and isolated to achieve the required purity for its final application. In order to concentrate and collect fractions enriched in the target compounds, the extract obtained from any of those extraction techniques previously mentioned is commonly processed using solid-phase extraction (SPE) or high-performance liquid chromatography (HPLC). Specifically, SPE comprises the interaction between the compound of interest and a specific matrix. Using organic solvents such as hexane, dichloromethane, methanol, chloroform, or their combinations, it is possible to eliminate the contaminants and elute the nutraceutical compound. This approach is rapid, and specific due to can be used different cartridges (Pérez-Magariño et al. 2008; Dai and Mumper 2010). The purification of the extracted sample also can be

performed by HPLC, which is an improved column chromatography. In HPLC, the liquid phase (i.e., the sample and the solvents) is forced through the solid phase (the column) under high pressures (up to 400 atm), resulting in a faster fractioning of the sample (Kim and Lee 2002). Supercritical fluid chromatography is a recent approach to fractioning the sample. This involves moderate temperature (31.1 °C) and high pressures (73 MPa) using CO_2 as the extracting solvent. This strategy is nontoxic, inflammable, and chemically stable but, due to low polarity of CO_2, is no use for fractioning many polyphenols (Wang and Weller 2006; Garcia-Salas et al. 2010). Nonetheless, these strategies have some disadvantages including the pretreatment of the sample and the use of hazard organic solvents.

Aqueous two-phase systems (ATPS) are liquid–liquid fractioning strategy that has proved to be efficient for the recovery and partial purification of biomolecules (Aguilar and Rito-Palomares 2010). The phases are formed when two hydrophilic constituents (i.e., alcohol, polymer, ionic liquid, or salt solution) are mixed at specific concentration. This results in the formation of two liquid phases which equilibrium can be represented by a binodal curve diagram. When a mixture of compounds (including the product of interest) is fed into the system, those molecules fractionate between phases based on their respective physicochemical and biochemical properties (Benavides et al. 2011). Once fractionated the phase in which the product of interest is partitioned is recovered in order to be further processed. The advantages of ATPS include biocompatibility with most types of molecules and particles, low cost (depending on the constituents of the system), scaling-up feasibility, and process intensification capability. Furthermore, ATPS have not only demonstrated to be a technique used for fractionation but also for the extraction of bioactive compounds directly from solid tissue by applying a process integration strategy called extractive fractionation (Chethana et al. 2007; Montalvo-Hernández et al. 2012; Gómez-Loredo et al. 2014; Sánchez-Rangel et al. 2016). In this approach, the homogenized solid tissue containing the molecule of interest is fed into the ATPS, and all constituents are mixed and then let to settle to achieve thermodynamic equilibrium. This results in the formation of two liquid phases and a solid phase depleted of the product of interest, as the composition of the system promotes the release and fractionation of the LMWC. In this way, one single unit operation can be used in order to extract the product from the solid tissue and fractionate from some contaminants between liquid phases.

The use of ATPS has primordially focused on the fractionation, recovery, and partial purification of large molecular weight compounds (proteins, nucleic acids, etc.) and bioparticles (cells, organelles, viruslike particles, etc.). Nevertheless, in recent years the interest in the application of ATPS for the recovery and partial purification of LMWC has steadily increased, demonstrating the potential of this technique for the fractionation of such bioactive compounds (Benavides et al. 2011; Simental-Martínez et al. 2014; Iqbal et al. 2016). A comparison between different extraction and purification techniques, including ATPS approach, is shown in Fig. 5.1. For instance, both ATPS and homogenization approaches have low cost and high extraction rate of LMWC; however, in the latter, a pretreatment of the sample is necessary, but in ATPS approach, LMWC can be extracted directly from

the raw sample. Besides this, ATPS can be scaled up at industrial level without the need of using costly or sophisticate equipment. Additionally, ATPS also have some advantages in purification strategies, being the most important its low cost compared to HPLC, SFE, or SPE, the high extraction rate of the compound of interest, the less hazard constituents, and the wide range of systems that can be explored to recover LMWC.

This chapter is dedicated to revise the application of ATPS for the extraction, recovery, and partial purification of low molecular weight compounds. Selected studies presenting the state-of-the-art are discussed, and finally future trends in the use of ATPS for the fractionation of LMWC are envisioned.

5.2 Evolution of the Use of Aqueous Two-Phase Systems for the Recovery and Partial Purification of Low Molecular Weight Compounds

ATPS have been used mainly for the recovery of proteins, nucleic acids, cells, and organelles (Benavides et al. 2011). Nevertheless, the number of studies related to the recovery and purification of LMWC has increased significantly in the last 20 years. ATPS have been used for the recovery and partial purification of a wide range of LMWCs including natural dyes, phytochemicals, alkaloids, and hormones, among others (Fig. 5.2).

One of the first reports of the recovery of LMWC using ATPS technique was penicillin-G by PEG–salt system from *Penicillium chrysogenum*. Using

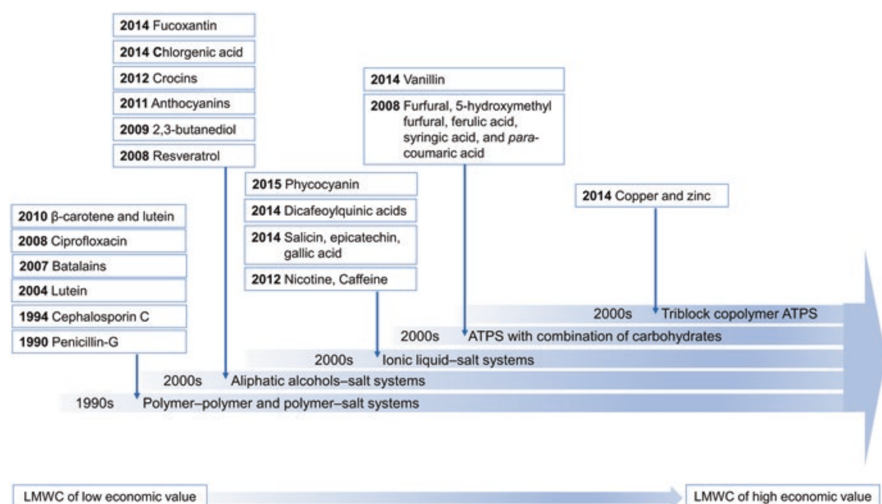

Fig. 5.2 Timeline of the recovery and purification of LMWC in different ATPS. The recovery of LMWC has gone from compounds of low economic value to compounds with significant economic value

PEG–potassium phosphate, a 97% recovery yield was achieved (Yang and Chu 1990). Yang et al. (1994) recovered cephalosporin C (63.2%) from *Cephalosporium acremonium* by PEG–ammonium sulfate system. Other antibiotics such as vancomycin, cephalexin, and ciprofloxacin have been purified using ATPS strategies (Yixin et al. 1994; Marcos et al. 1999; Wei et al. 2002; Mokhtarani et al. 2008). PEG–salt systems have also been used for the recovery and purification of phytochemicals. Cisneros et al. (2004) recovered lutein in a PEG–salt system in the top phase. Chavez-Santoscoy et al. (2010) also recovered lutein and β-carotene simultaneously using PEG–dextran system. In this case, each compound was partitioning in a different phase. Chethana et al. (2007) reported the purification of betalains, a natural food colorant, from beetroot using PEG–ammonium sulfate, with a yield about 70–75%.

Although polymer–polymer and polymer–salt systems (i.e., PEG, dextran, and polypropylene glycol) are two of the most commonly used ATPS, these types of systems may present a major drawback in some cases. Due to their high ionic strength, particularly in the salt-rich phase, the fractionation of ionic-sensitive molecules could result in low recovery yields. Furthermore, the use of some polymers may be costly, the segregation of the phases is usually slow due to viscosity, and the removal of the polymer from the phase at which the product partitions could present technical challenges (Benavides et al. 2011; Yau et al. 2015). Some of these disadvantages are overcome using alcohol–salt ATPS. These ATPS have low viscosity, high polarity, low cost, and low toxicity, and the removal and reuse of the system constituents presents less technical challenges when compared to polymeric systems (Greve and Kula 1991; Ooi et al. 2009). Alcohol–salt ATPS have been used for the recovery and partial purification of a large number of phytochemicals. For instance, using ethanol–ammonium sulfate, Wang et al. (2008a) recovered resveratrol, piceid, and emodin from *Polygonum cuspidatum*, with recovery yields of 82%, 84%, and 53%, respectively. Jiang et al. (2009) recovered 2,3-butanediol (98.1%), an organic compound used as precursor for the production of diverse plastics and pesticides, in ethanol–dipotassium hydrogen phosphate system. In addition to this compound, salvianolic acid B, anthocyanins, crocins, fucoxanthin, and chlorogenic acid have been recovered, some of them with purification yields greater than 90% (Zhi and Deng 2006; Wu et al. 2011; Montalvo-Hernández et al. 2012; Simental-Martínez et al. 2014; Gómez-Loredo et al. 2014; Sánchez-Rangel et al. 2016). Though ethanol–salt ATPS type has been used in the recovery of LMWC, the main disadvantage of this system is its incompatibility with molecules that may denature in alcohol-rich environments (Ooi et al., 2009; Benavides et al. 2011). Fortunately, most LMWC are stable under such conditions.

Ionic liquid–salt ATPS represent another alternative for the recovery and purification of LMWC. These systems have low volatility, a wide range of phase viscosities, and high thermal stability (Yau et al. 2015). Alkaloids, such as caffeine and nicotine, hormones, antibiotics, and some aromatic compounds including vanillin, nitrobenzene, gallic acid, and chlorogenic acids have been partitioned using ionic liquid ATPS type (Freire et al. 2012; Simental-Martínez et al. 2014; Sánchez-Rangel et al. 2016; Zhang et al. 2015).

On the other hand, in micellar ATPS two immiscible phases are formed, one of them with more micelles than the other, from a surfactant solution (Mazzola et al. 2008). The main advantage of this ATPS type is that micelles offer an isolate hydrophobic environment to recover nonpolar molecules (Liu et al. 2011). Recently, other ATPS types have been developed. One of them is ATPS with combination of carbohydrates, particularly mono- and disaccharides to form two immiscible phases. Using this type of system, Wang et al. (2008b) reported the distribution coefficient of five organic compounds (i.e., furfural, 5-hydroxymethyl furfural, ferulic acid, syringic acid, and *para*-coumaric acid) in a sugar acetonitrile–water ATPS type. This system was used to extract and purify vanillin, with recovery yields between 75% and 91% (Cardoso et al. 2013). Another ATPS type is the triblock copolymer system, which involves the formation of three phases, two blocks of ethylene oxide and one of propylene oxide block. This system is generated when copolymers are mixed at specific concentration and temperature in aqueous solution, resulting in the formation of micelles with an inside hydrophobic environment and outside hydrophilic surface (Yau et al. 2015). This type of ATPS has been used to fractionate low molecules and even ions such as copper and zinc (de Lemos et al. 2013).

5.3 Selected Studies on the Extraction, Recovery, and Partial Purification of Low Molecular Weight Compounds Using Aqueous Two-Phase Systems

As part of this section, selected studies are presented in order to give an overview of the development of ATPS-based strategies for the downstream processing of LMWC. Table 5.1 summarizes some studies focused on the extraction, recovery, and partial purification of LMWC using different ATPS.

5.3.1 Polymer–Polymer and Polymer–Salt ATPS

Lutein is a nonpolar compound (XLogP3, 11) of economic interest due to its antioxidant activity and its uses as natural dye. This compound was recovered from the green microalgae *Chlorella protothecoides* through polymer–salt ATPS (Cisneros et al. 2004). According to the authors, the best system to recover lutein was PEG 8000–potassium phosphate system (22.9–10.3% w/w, TLL 49.5%, V_R 1, pH 7.0), in which a recovery yield of 81% was achieved at the top phase (Table 5.1). Chavez-Santoscoy et al. (2010) also recovered lutein (76%) in a PEG–dextran 66,900 system (6.5–8.4% w/w, TLL 17.3%, V_R 1, pH 7.0), but unlike what is reported by Cisneros et al. (2004), in PEG–dextran system, lutein was recovered at the bottom phase (dextran-rich phase). Chavez-Santoscoy et al. (2010) also recovered β-carotene, another highly hydrophobic compound (XLogP3, 13.5), through the same polymer–polymer ATPS, although in this case, the compound was retrieved (87%) at the top phase (PEG-rich phase).

Table 5.1 Select examples of bioactive LMWC recovered using ATPS strategies

Compound	MW (g mol^{-1})	XLogP3	Nutraceutical effect	ATPS type	Constituents (%w/w)	TLL (%)	V_R	pH	Recovery yield (%) (and recovery phase)	Reference
β-carotene	536.87	13.5	Antioxidant	Polymer–polymer	PEG 3350 (6.6)–dextran 6690 (8.4)	17.3	1.0	7.0	96% (top)	Chavez-Santoscoy et al. (2010)
Lutein	568.87	11.0	Antioxidant, natural dye	Polymer–salt	PEG 8000 (22.9)–potassium phosphate (10.3)	49.4	1.0	7.0	81% (top)	Cisneros et al. (2004)
				Polymer–polymer	PEG 3350 (6.6)–dextran 6690 (8.4)	17.3	1.0	7.0	76% (bottom)	Chavez-Santoscoy et al. (2010)
Salvianolic acid B	718.61	4.0	Treatment of hypertension, hepatoprotective activity	Alcohol–salt	*n*-propanol (34)–potassium phosphate (8)	–	1.1	7.8	95% (top)	Zhi and Deng (2006)
Papaverine	339	3.9	Treatment of impotence and as a vasodilator, especially for cerebral vasodilation	Ionic liquid–salt	([C_4mim] Cl)–potassium phosphate	–	–	–	85% (top)	Li et al. (2005)
Glycyrrhizin	822.93	3.7	Anti-inflammatory	Alcohol–salt	Ethanol (60)–potassium phosphate (15)	–	–	7.0	98% (top)	Tianwei et al. (2002)
Testosterone	288.42	3.3	Hormone	Ionic liquid–salt	[C_4mim] Cl–potassium phosphate	–	0.6	–	90% (top)	He et al. (2005)
Epitestosterone	288.42	3.3	Hormone	Ionic liquid–salt	[C_4mim] Cl–potassium phosphate	–	0.6	–	85% (top)	He et al. (2005)

Resveratrol	232.26	3.1	Anti-inflammatory, anticancer, cardioprotective	Alcohol–salt	Ethanol (25)–ammonium sulfate (21)	–	1.7	–	86% (top)	Wang et al. (2008a)
Emodin	270.23	2.7	Antibacterial, antitumor activities	Alcohol–salt	Ethanol (25)–ammonium sulfate (21)	–	1.7	–	55% (top)	Wang et al. (2008a)
Piceid	390.38	1.7	Anti-inflammatory, anticancer, cardioprotective	Alcohol–salt	Ethanol (25)–ammonium sulfate (21)	–	1.7	–	86% (top)	Wang et al. (2008a)
3,5-dicaffeoylquinic acid	516.45	1.5	Antioxidant, anti-HIV, hypoglucemic, antimutagenic, anti-inflammatory, antihypertensive	Ionic liquid–salt	[EMIM][Ac] (30.0)–potassium phosphate (15.2)	–	4.1	9.0	73% (top)	Sánchez-Rangel et al. (2016)
4,5-dicaffeoylquinic acid	516.45	1.5	Antioxidant, hypoglucemic, antimutagenic, anti-inflammatory, antihypertensive	Ionic liquid–salt	[EMIM][Ac] (30.0)–potassium phosphate (15.2)	–	4.1	9.0	85% (top)	Sánchez-Rangel et al. (2016)
Vanillin	152.14	1.2	Flavoring agent in foods, beverages, and pharmaceuticals	ATPS with carbohydrates	Acetonitrile (40%)–glucose (20%)	58.9	–	6.8	91% (top)	Cardoso et al. (2013)
Nicotine	162.23	1.2	Stimulant, ergogenic, and restricted drugs	Ionic liquid–salt	[OHC_2mim]Cl (40)–K_3PO_4 (15) and [C_7H_7mim]Cl (25)–K_3PO4 (15)	–	–	–	100% (top)	Freire et al. (2010)
Codeine	299.36	1.1	Tread mild or moderate pain	Ionic liquid–salt	([C_4mim] Cl)–potassium phosphate	–	–	–	85% (top)	Li et al. (2005)

(continued)

Table 5.1 (continued)

Compound	MW (g mol^{-1})	XLogP3	Nutraceutical effect	ATPS type	Constituents (%w/w)	TLL (%)	V_R	pH	Recovery yield (%) (and recovery phase)	Reference
Gallic acid	170.12	0.7	Antioxidant, apoptosis-inducing agent	Ionic liquid–salt	[EMIM][Ac] (30.0)–potassium phosphate (15.2)	–	7.5	7.0	93% (top)	Simental-Martínez et al. (2014)
Epicatechin	290.27	0.4	Antioxidant, inhibition of tumorigenesis, carcinogenesis, and mutagenesis	Ionic liquid–salt	[EMIM][Ac] (30.0)–potassium phosphate (15.2)	–	7.5	7.0	99% (top)	Simental-Martínez et al. (2014)
Caffeine	194.19	−0.1	Stimulant, ergogenic, and restricted drugs	Polymer–salt	PEG 400 (26.9%)–sodium sulfate (9.7%)	41.8	–	–	–	Sampaio et al. (2016)
				Ionic liquid–salt	[OHC_2mim]Cl (40)–K_3PO_4 (15) and [C_7H_7mim]Cl (25)–K_3PO4 (15)	–	–	–	100% (top)	Freire et al. (2010)
Chlorogenic acid	354.31	−0.4	Antibacterial, antifungal, antiviral, antithrombotic, hepatoprotective	Polymer–salt	PEG 1000 (19.0)–potassium phosphate (17.1)	45.0	1.0	7.0	94% (top)	Simental-Martínez et al. (2014)
				Alcohol–salt	Ethanol (19.0)–potassium phosphate (25.5)	50	1	7.0	81% (top)	Sánchez-Rangel et al. (2016)
Salicin	286.28	−1.2	Analgesic, antipyretic, rheumatism treatment	Ionic liquid–salt	[EMIM][Ac] (15.8)–potassium phosphate (30.5)	–	1.0	7.0	99% (top)	Simental-Martínez et al. (2014)

Crocins	976.96	−2.5	Antioxidant, hypolipidemic, neuroprotective, antidepressive, anticholesterolemic, antitumoral, anticarcinogenic	Alcohol–salt	Ethanol (19.8)–potassium phosphate (16.5)	25	3.2	7.0	75% (top)	Montalvo-Hernández et al. (2012)
Iohexol	821.14	−3.0	Nonionic contrast medium	Ionic liquid–salt	[EMIM][Ac] (30.0)–potassium phosphate (15.2)	–	7.5	7.0	98% (top)	Simental-Martínez et al. (2014)

One of the most studied phenolic compounds is 3-caffeoylquinic acid, known as chlorogenic acid, which is the most abundant chlorogenic acid in nature (El-Seedi et al. 2012). Simental-Martínez et al. (2014) performed the partitioning of this compound, which is slightly hydrophilic (XLog3, −0.4), by PEG 1000–potassium phosphate system (19.2–17.1% w/w, TLL 45%, V_R 1, pH 7.0), reaching a recovery yield of 92% in the top phase (PEG-rich phase) (Table 5.1). Other types of compounds have also been recuperated by ATPS including hormones (i.e., testosterone and epitestosterone) and some alkaloids such as caffeine, nicotine, papaverine, and codeine (Table 5.1).

5.3.2 Alcohol–Salt ATPS

Alcohol–salt system has been used in the recovery of salvianolic acid B and glycyrrhizin. These two compounds have similar molecular weight and hydrophobicity; specifically, glycyrrhizin was recovered (98%) using ethanol (60% v/v)–potassium phosphate (15% w/v, pH 7.0) (Tianwei et al. 2002), whereas salvianolic acid was recovered (95%) by *n*-propanol-phosphate (36–8% w/w) (Zhi and Deng 2006). Resveratrol, piceid, and emodin, like chlorogenic acids, are derived from the shikimate pathway, and they also have multiple biological functions. These compounds were extracted from *Polygonum cuspidatum* by microwave-assisted ATPS, specifically in ethanol–ammonium sulfate (25–21%w/w) (Wang et al. 2008a). Resveratrol, piceid, and emodin have different molecular weights and hydrophobicities, which may explain the recovery yields obtained, being emodin the bioactive compound that presented the lowest recovery yield (55%), whereas resveratrol and piceid showed the same yield (86%). On the other hand, Montalvo-Hernández et al. (2012) performed the recovery of crocins (97%) in an optimized ethanol–potassium phosphate system (14.5–24.7% w/w, TLL 25%, V_R 3.2, pH 7.0, 0.1 M NaCl) from saffron stigmas. Chlorogenic acid was also recovered (81%) at the top phase by Sánchez-Rangel et al. (2016) from stressed carrot tissue, using ethanol–potassium phosphate system (19–25.5% w/w, TLL 50%, TLL 50%, V_R 1, pH 7). These and some other examples are shown in Table 5.1.

5.3.3 Ionic Liquid–Salt ATPS

Many phytochemical compounds are recovered by different ATPS types due to their wide physicochemical properties. For instance, epicatechin, gallic acid, and salicin are compounds with a lower hydrophobicity compared to lutein or β-carotene. These three compounds were partitioned by 1-ethyl-3-methylimidazolium acetate ([EMIM][Ac])–potassium phosphate (30–15.2% w/w, V_R 7.5), obtaining a recovery yield of 93% for both epicatechin and gallic acid and 99.8% for salicin (Simental-Martínez et al. 2014). On the other hand, Sánchez-Rangel et al. (2016) extracted and performed the partial purification of some members of the chlorogenic acid family,

particularly 3,4- and 4,5-dicaffeoylquinic acids (diCQA) from stressed carrot tissue. Using [EMIM][Ac]–potassium phosphate ATPS type (pH 9.0, V_R 4.1), these authors recovered 3,4-diCQA and 4,5-diCQA with a yield of 73% and 85%, respectively.

Caffeine and nicotine fractionation in ATPS was studied by Freire et al. (2010) using different ionic liquid–salt systems. These authors reported a complete extraction of both bioactive compounds in [OHC_2mim]Cl (40%w/w)–K_3PO_4 (15%w/w) and [C_7H_7mim]Cl (25%w/w)–K_3PO4 (15%w/w), indicating that the %w/w of ionic liquid should not exceed 25% for an economic viable recovery of these compounds. Papaverine and codeine are two of the main compounds found in opium (*Pericarpium papaveris*). These compounds were recovered using a 1-butyl-3-methylimidazolium chloride ([C_4mim]Cl)–potassium phosphate system, achieving a recovery yield of 81% for codeine and 85% for papaverine (Li et al. 2005). Also some hormones were partitioned by ATPS, specifically testosterone and epitestosterone using [C_4mim]Cl–potassium phosphate with recovery efficiencies of 90% and 85%, respectively (He et al. 2005).

5.3.4 Product Properties Versus ATPS Type

Figure 5.3 depicts the distribution of LMWC mentioned in Table 5.1, according to their molecular weight (*x*-axis) and XLogP3 value (*y*-axis), which represents the predicted value of the partition coefficient between *n*-octanol and water, and it is used to express the hydrophobicity of molecular compounds. As it is shown in Fig. 5.3, polymer–polymer and polymer–salt ATPS are used to recover LMWC with high XLogP3 value (i.e., lutein and β-carotene), whereas alcohol–salt and ionic liquid–salt systems are applied to recuperate compounds with lower hydrophobicity and polar compounds. Interestingly, alcohol–salt ATPS are used to recover compounds with a wide range of molecular weights, while ionic liquid systems are utilized in partitioning chemicals with lower molecular weight. Based on Fig. 5.3, it seems that in alcohol–salt and ionic liquid–salt ATPS strategies, it is more relevant to take into account both the hydrophobicity and the molecular weight of the compound of interest to achieve its partial purification. In polymer–salt ATPS, it seems that hydrophobicity is a much more relevant parameter for tuning the fractionation of LMWC, at least when compared to polymer molecular weight. With respect to the recovery yield (%), which is represented by the area of the circle, LMWC were purified with yields greater than 80%, except crocins (75%), 3,5-dicaffeoylquinic acid (73%), and emodin (55%) (Table 5.1), indicating that it is possible to reach high recovery values using any of the ATPS strategies (Fig. 5.3). In general terms it can be concluded that alcohol–salt and ionic liquid–salt ATPS strategies are more used to recover LMWC than polymer–polymer and polymer–salt ATPS.

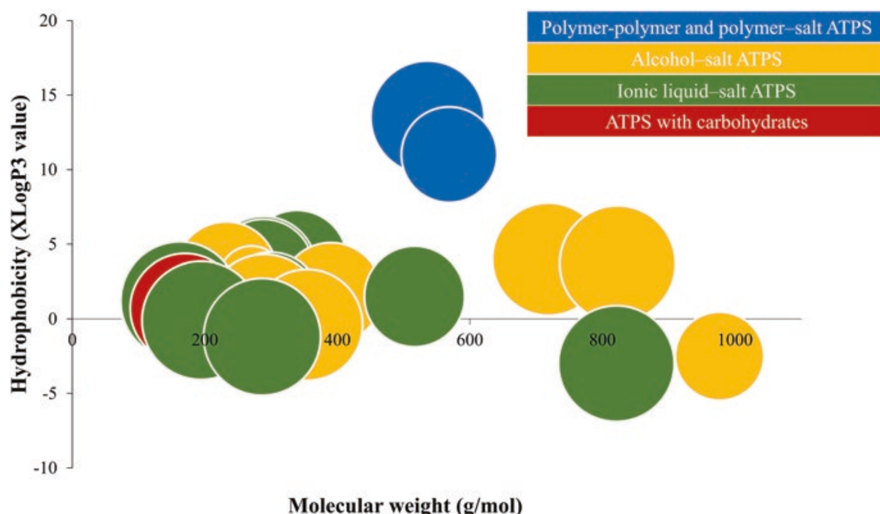

Fig. 5.3 Distribution of LMWC according to their molecular weight and hydrophobicity of the compounds mentioned in Table 5.1. The area of the *circle* represents the recovery yield (%) of the LMWC in specific ATPS strategies

5.4 Future Trends in the Recovery of Low Molecular Weight Compounds Using Aqueous Two-Phase Systems

Regarding the scientific and technological development around the use of ATPS for the recovery of low molecular weight compounds in the near future, three main topics will probably be addressed: (1) novel high-value low molecular weight products to be recovered, (2) new ATPS constituents exploiting affinity and other physicochemical properties for a tuned fractionation, and (3) efficient strategies for the removal and reuse of ATPS constituents.

Biotechnology has a significant aspect in most industries and human activities. As a result, new bioactive compounds are identified, characterized, and commercialized on a daily basis. In this context, many of such bioactives are low molecular weight compounds that require to be efficiently produced, recovered, and purified with cost-effective and time-effective processes. In this regard, studies focused on the recovery and partial purification of bioproducts such as transcription factors, custom-made drugs, low molecular weight antimicrobial peptides, and newly discovered phytochemicals which are envisioned within the next 10 years.

One of the main drawbacks in the use of ATPS is that, in general terms, since the systems have a large number of degrees of freedom, the understanding of the partition behavior is limited. Furthermore, the capability of prediction of the partition behavior is poor, resulting in the need of proving a large variety of ATPS types and system parameters in order to find optimal fractionation and recovery conditions. In

this regard, the identification and characterization of new system constituents with "tuned" fractionation capabilities are of major interest in the ATPS development research area. One simple way to achieve predictability would be identifying natural or synthetic polymers with affinity for the particular product of interest. In this way, the product of interest would mainly migrate to that phase in which the affinity polymer is rich in. This polymer–product interaction should be reversible since as part of the process, the product should be released from the ligand and further processed. Besides affinity, there are other properties that may be exploited in order to tune the partition of LMWC in ATPS. For instance, the development of polymers with adjustable hydrophobicity and electrochemical charge may allow a much more predictable design based on the characteristics of the products to be recovered. In such scenario, a highly positively charged constituent may be used in order to fractionate a negatively charged biomolecule, or an amphipathic polymer could be used in order to match the polarity of the product of interest within the biphasic system. The resulting increase in predictability would reflect in a significant decrease in the number of experiments needed to characterize fractionation in ATPS, saving time and resources in the process. This will further promote the use of ATPS as an alternative to traditional fractionation techniques.

As it was already mentioned as part of the chapter, one of the main technical difficulties when using ATPS is the removal and reuse of system constituents, primordially polymers. The most used strategy for removing polymeric constituents once the fractionation occurs is ultrafiltration. In the case of large molecular weight products (for instance, proteins), the polymer passes through the membrane, while the product remains in the retentate. This allows removing of the polymer while giving the opportunity of concentrating the product of interest. However, in the case of LMWC, as the polymer is larger in size, it remains in the retentate, while the product passes through the membrane. Unfortunately, as the polymer concentrates in the retentate, the flux significantly decreases due to the increase in viscosity and deposition of the polymer over the surface of the membrane. This results in large processing times. In this context, the development of effective strategies for removing and reusing polymer constituents is of great interest. In this regard, the use of "intelligent" polymers in ATPS has been explored recently, and it is expected to increase in the future. In this way, thermo-sensible or pH-sensible polymers are used to form the biphasic system, and once the partition takes place, the conditions are changed in order to promote polymer precipitation, allowing further processing of the product and recovery of the polymer for reuse. Although this kind of strategies is already been characterized, further studies are still needed in order to optimize this kind of approaches.

5.5 Concluding Remarks

In this chapter, the application of ATPS-based approaches for the recovery and partial purification of LMWCs, which have a wide variety of potential applications in most industries, is presented. According to the increased number of reports on the recovery and purification of LMWC using ATPS, it is expected that the use of this

methodology will continue growing in the following years. The incorporation of new inexpensive and environmentally friendly constituents such as complex carbohydrates and gums and the use of novel copolymers will open the possibility to optimize the partitioning of specific compounds under specific operational parameters while favoring the efficiency/cost ratio. Additionally, to carry on more studies with the aim of characterizing the partitioning behavior of LMWC in the different ATPS systems, it is an important task to obtain greater recovery yields and increase the possibility of developing an economically viable process using this approach.

References

Aguilar O, Rito-Palomares M. Aqueous two-phase systems strategies for the recovery and characterization of biological products from plants. J Sci Food Agric. 2010;90:1385–92.

Badhani B, Sharma N, Kakkar R. Gallic acid: a versatile antioxidant with promising therapeutic and industrial applications. RSC Adv. 2015;5:27540–57.

Benavides J, Rito-Palomares M, Asenjo JA. Aqueous two-phase system. In: Moo-Young M, editor. Comprehensive biotechnology. 2nd ed. Burlington: Elsevier; 2011. p. 697–713.

Cardoso GB, 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.

Chavez-Santoscoy A, Benavides J, Vermaas W, Rito-Palomares M. Application of aqueous two-phase systems for the potential extractive fermentation of cyanobacterial products. Chem Eng Technol. 2010;33:177–82.

Chethana S, Nayak CA, Raghavarao KSMS. Aqueous two phase extraction for purification and concentration of betalains. J Food Eng. 2007;81:679–87.

Cisneros M, Benavides J, Brenes CH, Rito-Palomares M. Recovery in aqueous two-phase systems of lutein produced by the green microalga *Chlorella protothecoides*. J Chromatogr B. 2004;807:105–10.

Cisneros-Zevallos L. The use of controlled postharvest abiotic stresses as a tool for enhancing the nutraceutical content and adding-value of fresh fruits and vegetables. J Food Sci. 2003;68:1560–5.

Cuéllar-Villarreal MR, Ortega-Hernández E, Becerra-Moreno A, Welti-Chanes J, Cisneros-Zevallos L, Jacobo-Velázquez DA. Effects of ultrasound treatment and storage time on the extractability and biosynthesis of nutraceuticals in carrot (*Daucus carota*). Postharvest Biol Technol. 2016;51:242–9.

Dai J, Mumper RJ. Plant phenolics: extraction, analysis and their antioxidant and anticancer properties. Molecules. 2010;15:7313–52.

Das L, Bhaumik E, Raychaudhuri U, Chakraborty R. Role of nutraceuticals in human health. J Food Sci Technol. 2012;49(2):173–83.

Delazar A, Nahar L, Hamedeyazdan S, Sarker SD. Microwave-assisted extraction in natural products isolation. In: Sarker SD, Nahar L, editors. Natural products isolation, Methods in molecular biology. New York: Springer; 2012. p. 89–115.

de Lemos LR, Campos RA, Rodrigues GD, da Silva LHM, da Silva MCH. Green separation of copper and zinc using triblock copolymer aqueous two-phase systems. Sep Purif Technol. 2013;115:107–13.

El-Seedi HR, El-Said AMA, Khalifa SAM, Göransson U, Bohlin L, Borg-Karlson AK, et al. Biosynthesis, natural sources, dietary intake, pharmacokinetic properties, and biological activities of hydroxycinnamic acids. J Agric Food Chem. 2012;60:10877–95.

Freire MG, Neves CMSS, Marrucho IM, Lopes JNC, Rebelo LPN, Coutinho JAP. High-performance extraction of alkaloids using aqueous two-phase systems with ionic liquids. Green Chem. 2010;12:1715–8.

Freire MG, Cláudio AFM, Araújo JMM, Coutinho JAP, Marrucho IM, Canongia JN, et al. Aqueous biphasic systems: a boost brought about by using ionic liquids. Chem Soc Rev. 2012;41:4966–95.

Garcia-Salas P, Morales-Soto A, Segura-Carretero A, Fernández-Gutiérrez A. Phenolic-compound-extraction systems for fruit and vegetable samples. Molecules. 2010;15:8813–26.

Gómez-Loredo A, Benavides J, Rito-Palomares M. Partition behavior of fucoxanthin in ethanol-potassium phosphate two-phase systems. J Chem Technol Biotechnol. 2014;89:1637–45.

Greve A, Kula MR. Phase diagrams of new aqueous phase systems composed of aliphatic alcohols, salts and water. Fluid Phase Equilib. 1991;62:53–63.

He C, Li S, Liu H, Li K, Liu F. Extraction of testosterone and epitestosterone in human urine using aqueous two-phase systems of ionic liquid and salt. J Chromatogr A. 2005;1082:143–9.

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.

Jacobo-Velázquez DA, Cisneros-Zevallos L. An alternative use of horticultural crops: stressed plants as biofactories of bioactive phenolic compounds. Agriculture. 2012;2:259–71.

Jiang B, Li Z-G, Dai J-Y, Zhang D-J, Xiu Z-L. Aqueous two-phase extraction of 2,3-butanediol from fermentation broths using an ethanol/phosphate system. Process Biochem. 2009;44:112–7.

Khoddami A, Wilkes MA, Roberts TH. Techniques for analysis of plant phenolic compounds. Molecules. 2013;18:2328–75.

Kim DO, Lee CL. Extraction and isolation of polyphenolics. In: Wrolstad RE, Acree TE, Decker EA, Penner MH, Reid DS, Schwartz SJ, Shoemaker CF, Smith DM, Sporns P, editors. Current protocols in food analytical chemistry. New York: Wiley; 2002. p. I1.2.1–I1.2.12.

Li S, He C, Liu H, Li K, Liu F. Ionic liquid-based aqueous two-phase system, a sample pretreatment procedure prior to high-performance liquid chromatography of opium alkaloids. J Chromatogr B. 2005;826:58–62.

Liu Y, Yu YL, Chen MZ, Xiao X. Advances in aqueous two-phase systems and applications in protein separation and purification. Can J Chem Eng. 2011;2:1–7.

Marcos JC, Fonseca LP, Ramah MT, Cabral JMS. Partial purification of penicillin acylase from *Escherichia coli* in poly(ethylene glycol)–sodium citrate aqueous two-phase systems. J Chromatogr B. 1999;734:15–22.

Mazzola PG, Lopes AM, Hasmann FA, Jozala AF, Penna TCV, Magalhaes PO, et al. Liquid–liquid extraction of biomolecules: an overview and update of the main techniques. J Chem Technol Biotechnol. 2008;83:143–57.

Mokhtarani B, Karimzadeh R, Amini MH, Manesha SD. Partitioning of ciprofloxacin in aqueous two-phase system of poly(ethylene glycol) and sodium sulphate. Biochem Eng J. 2008;38:241–7.

Montalvo-Hernández B, Rito-Palomares M, Benavides J. Recovery of crocins from saffron stigmas (*Crocus sativus*) in aqueous two-phase systems. J Chromatogr A. 2012;1236:7–15.

Mordor Intelligence. Global nutraceuticals market – growth, trends and forecasts (2016–2021). http://www.reportlinker.com/p04360237-summary/global nutraceuticals-market-growth-trends-and-forecasts.html (2016). Accessed Nov 2016.

Nasri H, Baradaran A, Shirzad H, Rafieian-Kopaei M. New concepts in nutraceuticals as alternative for pharmaceuticals. Int J Prev Med. 2014;5:1487–99.

Ooi CW, Tey BT, Hii SL, Kamal SMM, Lan CW, Ariff A, et al. Purification of lipase derived from *Burkholderia pseudomallei* with alcohol/salt-based aqueous two-phase systems. Process Biochem. 2009;44:1083–7.

Pérez-Magariño S, Ortega-Heras M, Cano-Mozo E. Optimization of a solid-phase extraction method using copolymer sorbents for isolation of phenolic compounds in red wines and quantification by HPLC. J Agric Food Chem. 2008;56:11560–70.

Quideau S, Deffieux D, Douat-Casassus C, Pouységu L. Plant polyphenols: chemical properties, biological activities, and synthesis. Angew Chem Int Ed. 2011;50:586–621.

Robinson WE, Reinecket MG, Abdel-malekt S, Jiat Q, Chow SA. Inhibitors of HIV-1 replication that inhibit HIV integrase. Proc Natl Acad Sci USA. 1996;93:6326–31.

Sampaio DA, Mafra LI, Yamamoto CI, de Andrade EF, de Souza MO, Mafra MR, et al. Aqueous two-phase (polyethylene glycol + sodium sulfate) system for caffeine extraction: equilibrium diagrams and partitioning study. J Chem Thermodyn. 2016;98:86–94.
Sánchez-Rangel JC, Jacobo-Velázquez DA, Cisneros-Zevallos L, Benavides J. Primary recovery of bioactive compounds from stressed carrot tissue using aqueous two-phase systems strategies. J Chem Technol Biotechnol. 2016;91:144–54.
Simental-Martínez J, Montalvo-Hernández B, Rito-Palomares M, Benavides J. Application of aqueous two-phase systems for the recovery of bioactive low-molecular weight compounds. Sep Sci Technol. 2014;49:1872–82.
Tianwei T, Qing H, Qiang L. Purification of glycyrrhizin from *Glycyrrhiza uralensis Fisch* with ethanol/phosphate aqueous two phase system. Biotechnol Lett. 2002;24:1417–20.
Tsao R, Deng Z. Separation procedures for naturally occurring antioxidant phytochemicals. J Chromatogr B. 2004;812:85–99.
Wang L, Weller CL. Recent advances in extraction of nutraceuticals from plants. Trends Food Sci Technol. 2006;17:300–12.
Wang H, Dong Y, Xiu Z-L. Microwave-assisted aqueous two-phase extraction of piceid, resveratrol and emodin from *Polygonum cuspidatum* by ethanol/ammonium sulphate systems. Biotechnol Lett. 2008a;30:2079–84.
Wang B, Ezejias T, Fenga H, Blascheka H. Sugaring-out: a novel phase separation and extraction system. Chem Eng Sci. 2008b;63:2595–600.
Wei D, Zhu J, Cao X. Enzymatic synthesis of cephalexin in aqueous two-phase systems. Biochem Eng J. 2002;11:95–9.
Wu X, Liang L, Zou Y, Zhao T, Zhao J, Li F, et al. Aqueous two-phase extraction, identification and antioxidant activity of anthocyanins from mulberry (*Morus atropurpurea* Roxb.). Food Chem. 2011;129:443–53.
Yang WY, Chu IM. Extraction of penicilin-G by aqueous two-phase partition. Biotechnol Tech. 1990;3:191–4.
Yang WY, Lin CD, Chu IM, Lee CJ. Extraction of cephalosporin C from whole broth and separation of desacetyl cephalosporin C by aqueous two-phase partition. Biotechnol Bioeng. 1994;43:439–45.
Yau YK, Ooi CW, Ng E-P, Lan JCW, Ling TCW, Show PL. Current applications of different type of aqueous two-phase systems. Bioresour Bioprocess. 2015;2:1–13.
Yixin G, Lehe M, Ziqiang Z. Recovery of antibiotics by aqueous two-phase partitioning behavior of pure acetylspiramycin solution in polyethylene glycol potassium phosphate aqueous two-phase systems. Biotechnol Tech. 1994;8:491–6.
Zhang X, Zhang F, Luo G, Yang S, Wang D. Extraction and separation of phycocyanin from *Spirulina* using aqueous two-phase systems of ionic liquid and salt. J Food Nutr Res. 2015;3:15–9.
Zhi W, Deng Q. Purification of salvianolic acid B from the crude extract of *Salvia miltiorrhiza* with hydrophilic organic/salt-containing aqueous two-phase system by counter-current chromatography. J Chromatogr A. 2006;1116:149–52.

Affinity ATPS Strategies for the Selective Fractionation of Biomolecules

6

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Abbreviations

AL	Affinity ligand
ATPAP	Aqueous two-phase affinity partitioning
ATPS	Aqueous two-phase systems
CMC	Critical micelle concentration
DEX	Dextran
DS	Degree of saturation
EBA	Expanded bed adsorption chromatography
EF	Extractive fermentation
K_p	Partitioning coefficient
mAbs	Monoclonal antibodies
MACS	Magnetic-activated cell sorting
mPEG	Monomethylether PEG
PAP	Papain
PEG	Polyethylene glycol
TLL	Tie-line length

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TM Target molecule
UCB Umbilical cord blood
V_R Volume ratio

6.1 Introduction

The past 20 years have been decisive for biotechnology in terms of technology development and transfer, novel protocols, optimization tools based on software utilization, and new unit operations in order to establish alternative approaches for final product design and further commercialization. In this context, the biotech industry has experienced a series of challenges that have evolved in the past decades, particularly in the downstream processing area, commonly known as the bioseparation methods division. One of the most important opportunity areas within the actual established processes is the extremely high purity required for specific bioactive compounds (e.g., human therapeutics that require purities >99.9%) (Knäblein 2005). In addition, these products need to be separated from a complex matrix of contaminants typically including whole cells, cell fragments such as membranes and organelles, polysaccharides, proteins, and nucleic acids. These aspects contribute directly to an important increase in bioprocessing costs, since a high number of unit operations are usually required to ensure product quality (i.e., purity, final presentation).

Literature reports state that the typical bioprocess cost is divided into upstream and downstream operations, the latter accounting for up to 80% of the total capital invested (Cunha et al. 2003; Knäblein 2005). In this context, there is a constant need to establish scalable and bioselective methods for product recovery and purification which finally result in the implementation of novel bioengineering strategies in order to accomplish new technical objectives and designs in bioprocesses in the biotech industry. Two common strategies that seek to contribute to the potential of a bioprocessing design are intensification and integration. Bioprocess intensification intends to maximize process efficiency increasing the throughput given in a single or group of unit operations by a careful characterization and selection of process parameters (Benavides and Rito-Palomares 2004; Hernández-Mireles and Rito-Palomares 2006; Schügerl and Hubbuch 2005). Following this strategy consumables and raw materials quantities can be reduced, thus favoring the economic and technical dimensions of the operation. On the other hand, bioprocess integration attempts to merge two or more unit operations in one, thus reducing the total number of stages in the downstream section and finally improving overall recovery and purity yields (Benavides and Rito-Palomares 2008; Schügerl and Hubbuch 2005).

Considering the advances and importance of process integration and intensification, research efforts have developed important techniques that present important benefits for novel bioprocess engineering designs. One of the most employed technologies is extractive fermentation (EF), which consists in the in situ removal of selected components with the aid of a biocompatible extracting agent (e.g., typically

an ad hoc solvent) during a fermentation step (Costa and Badino 2015). As stated by Gutiérrez et al., EF allows microbiological processes carried out in batch mode to significantly increase production yields by constantly removing end products like acetic acid or lactate in commercial fermentation steps, hence improving the effective growth rate of the host cell employed (Gutiérrez et al. 2013). A similar approach has been reported for in situ purification of high-added value products, in which a two-phase extraction system formed with polyethylene (PEG) and dextran was used to grow *Synechocystis* sp. PCC 6803 and concentrate the biomass and β-carotene in the top phase and lutein in the bottom dextran phase. The results of this study demonstrated the potential application of a two-phase extraction procedure that allowed production and in situ recovery of these cyanobacterial products, reducing the necessary steps for their final purification (Chavez-Santoscoy et al. 2010).

Another whole-broth processing technique that is fairly recent and allows recovery and purification of biomolecules from crude extracts (e.g., fermentation broths, natural sources such as vegetable or food matrixes) is expanded bed adsorption (EBA) chromatography. According to Lihme et al., if the proper adaptations are given to this technology in the current biotech industry, successful high-throughput methodologies for mass production of highly sophisticated pharmaceuticals and even low-cost industrial enzymes could be implemented (Lihme et al. 2000). The use of EBA-related approaches is attractive since it allows, in many cases, the combination of traditional separation effects obtained by centrifugation, filtration, concentration, and purification in a single step, thus reducing the number of steps and production time, increasing yields, and reducing bioprocessing costs.

Liquid-liquid extraction techniques such as aqueous two-phase systems (ATPS) constitute also an important alternative to traditional fractionation and purification techniques, primarily chromatographic-based procedures, in order to develop a scalable and economic process with integration and intensification potential. These systems are obtained when two phase-forming components (e.g., two polymers, polymer-salt, ionic liquid-salt, alcohol-salt, detergents) are mixed over certain critical conditions, thus forming a two-phase system with immiscible phases, each with different physicochemical and biochemical properties (Park et al. 2007; Silvério et al. 2008). Noticeable advantages of this technique include improved biocompatible environment for biological products, scalability and ease of operation, possibility of continuous operation for high-throughput recovery of target products, and positive bioprocess economy (Benavides and Rito-Palomares 2008; Cordes et al. 1987; Rito-Palomares 2004). In this context, ATPS have been used for the primary recovery, concentration, and purification of different types of bioproducts such as proteins, nucleic acids, whole cells and cell fragments, and molecular weight metabolites. The molecular mechanisms underlining the partition behavior in ATPS are complex, and a delicate balance between process parameters (e.g., temperature and pH, volume ratio (V_R), tie-line length (TLL), system constituents selection/concentration) and solute characteristics (e.g., molecular weight, hydrophobicity, superficial charge, isoelectric point, solubility) determines the efficiency of a particular system within an specific extraction and/or purification procedure (Benavides and Rito-Palomares 2008; Schindler and Nothwang 2006).

One of the major disadvantages of ATPS partitioning is their lack of specificity. Even though numerous researches have established effective partitioning of high-added value molecules by carefully designed experiments, the manipulation of system parameters does not usually provide high enough specificity to maximize target product separation from a complex sample for advanced biotechnological applications. In this tenor, several strategies could be employed to improve this major drawback, and one that has exhibited potential and positive feedback within the scientific community will be discussed in the following sections.

The objective of this chapter is to present the concept of affinity-based two-phase partitioning in order to overcome selectivity issues in traditional ATPS. The main definitions of affinity-enhanced liquid-liquid partitioning, additional variables, and challenges to be considered within these systems, along with successful examples and challenges of actual applications of this technology, will be thoroughly discussed.

6.2 Affinity Partitioning

6.2.1 Definition and Conventional Applications

Biomolecule partitioning in two-phase systems can be carried out if the preference of these molecules toward the phases in the systems is unequal, i.e., when their partitioning coefficient (K_p), the concentration of target product in top phase divided by the concentration of target product in the bottom phase, is different than 1. In most cases K_p values of target biomolecules partitioned in ATPS are not extremely high or low ($10 < K_p > 0.1$), and therefore, the application of such systems for processes oriented to high-purity products (such as molecular diagnostics and point-of-care assays) is hampered.

In order to enhance ATPS selectivity toward recovery and purification of high-added value products, aqueous two-phase affinity partitioning (ATPAP) has emerged. This technique, as presented in Fig. 6.1, consists in the chemical modification of at least one of the phase-forming components (e.g., typically polymers such as PEG or dextran) in order to attach an affinity ligand (AL) which will exhibit high specificity toward a particular molecule (Cordes et al. 1987). A second approach consists in the addition of ligands free in solution (Fig. 6.1B), thus avoiding a phase-component activation protocol, which will also have specific affinity to the target product (Azevedo et al. 2009). The final outcome of these strategies is an improved selectivity of the system between the selected ALs and the particle to be partitioned to the modified or AL-enriched phase, thus significantly increasing/decreasing K_p values and providing a different scope for novel downstream processing applications.

The first theoretical and practical approach of ATPAP was developed by Flanagan and Barondes in 1975. They proposed a scheme called "affinity partitioning" which included modified versions of polyethylene oxide (PEG) with ethylenediamine and dinitrofluorobenzene in order to have an active dinitrophenyl-PEG (dnp-PEG).

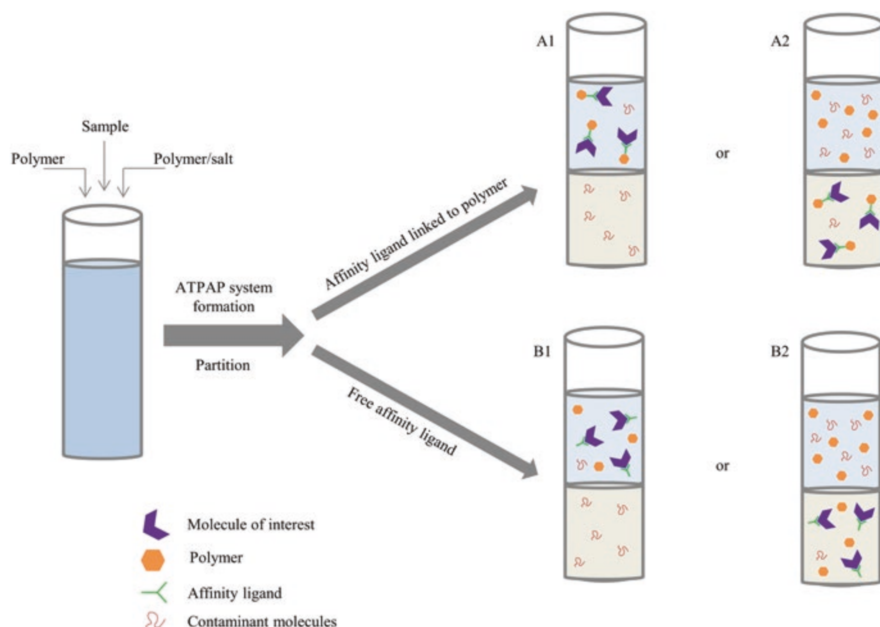

Fig. 6.1 (*A1* and *A2*) ATPAP systems with affinity ligand (AL) chemically coupled to phase-forming chemicals in top and bottom phases, respectively. (*B1* and *B2*) ATPAP systems with ALs free in solution concentrating in top or bottom phase, respectively

This active derivative allowed a 150% increase in the partitioning coefficient of the target product, S-23 myeloma proteins, in a PEG 6000-dextran 500 system. In addition, one of the most important conclusions of the authors was that affinity interactions were easily reversible, since the addition of a simple competitor of dnp-PEG, e.g., *N*-Dnp-lysine, could reverse the effect of the partitioning behavior (Flanagan and Barondes 1975). From this point on, there has been a notable increase in ATPAP applications for the recovery of high-added value compounds in order to propose commercial applications for them or as sample-preparation protocols to be used in further analytical assays.

6.2.2 New Implications and Variables in ATPAP

Affinity partitioning exhibits a partitioning behavior similar to the one traditional ATPS have; nevertheless a more complex set of equilibria and variables are present in ATPAP systems, as presented in Fig. 6.2. Since the final objective of ATPAP is to modify the "natural" partition behavior of solutes, a simple thermodynamic approach may be considered in order to determine the final potential for particle partition toward a specific phase. The association/dissociation constants between all the solutes must be considered (Fig. 6.2), which include the following: (i) Polymer-AL, (ii) Polymer-AL-target molecule (TM), and (iii) AL-TM when ligands

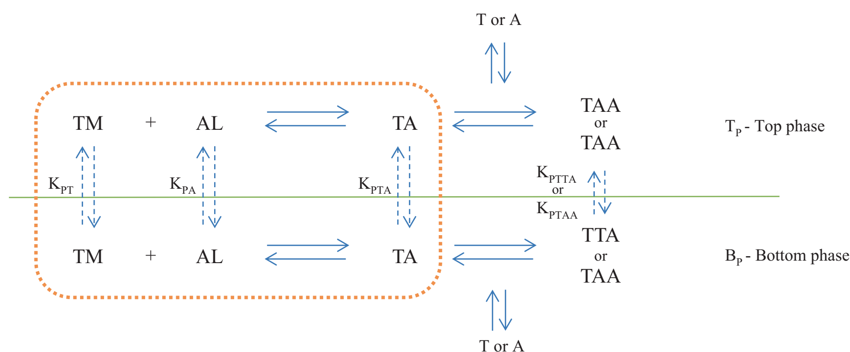

Fig. 6.2 Scheme that highlights the possible interactions of a target product (TM or T) and an affinity ligand (AL or A) in a two-phase system with an interface in between. These reactions can apply to a single interaction between a ligand and target product (information inside the *dotted box*) or additional ligand or target product molecules can be bound consequently. The partition coefficients (K_p) of the complexes (TA, TAA, or TTA) require a net change in Gibbs free energy (ΔG), which takes into account the concentration of both the AL, the TM, the employed phase-forming chemicals, and the conditions taken place in the ATPAP procedure, in order to finally determine the energy needed to transfer 1 mole of complex from one phase to the other

free in solution are used. If it is assumed that no products are adsorbed in the interface, the free energy required (ΔG_7 in Fig. 6.2) to transfer 1 mole of complex (e.g., polymer-AL-TM or AL-TM) from one phase to the other can be calculated from the values of the ΔG changes for dissociating the complex in one phase, transferring the involved species across the interface, and finally reassembling the complex in the opposite phase (Asenjo and Andrews 2008; Johansson 2000). Following the same thermodynamic path presented by Flanagan and Barondes, the estimation of the partition coefficient of a particle in an ATPAP system can be obtained and is presented here in Eq. (6.1).

$$K_p = \frac{[P_f]^{TP} + \sum_{n=1}^{N} [P_n AL]^{TP}}{[P_f]^{BP} + \sum_{n=1}^{N} [P_n AL]^{BP}} \tag{6.1}$$

The first term, P_f, in Eq. (6.1) is defined as the concentration of target product free in solution in either the top or bottom phase, and the second terms are the complex aggregates between the products coupled to a selected AL, where *N* is defined as the total number of ligands (recognition groups) in the affinity molecule. A simplified model would consider the basic interaction of 1 target molecule bound to 1 AL ($N = 1$), but this scenario does not always take place.

Considering the information previously presented for ATPAP systems, these protocols provide a biocompatible environment for biological molecules and samples which can be manipulated in order to develop more predictable purification strategies. As a result, ATPAP techniques combine the bioselectivity of an affinity technique with the robustness and scalability of well-known liquid-liquid fractionation

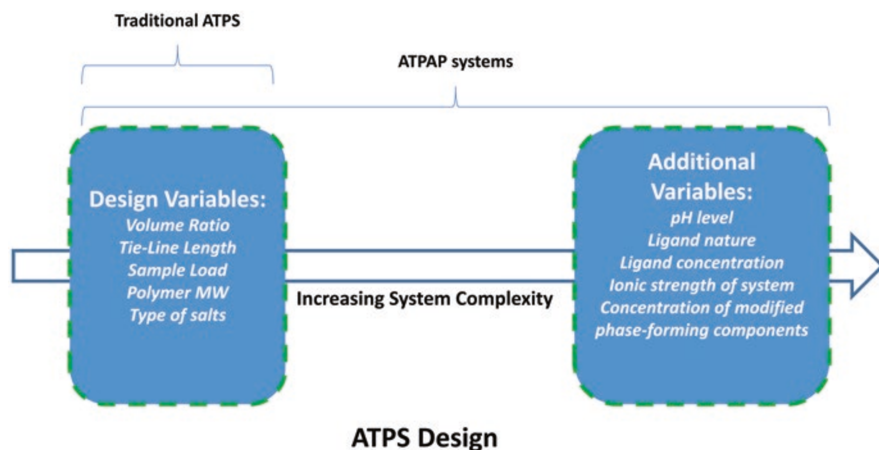

Fig. 6.3 Variables involved in ATPS design. In the *left section*, traditional variables needed for system characterization and product partitioning. In the *right section*, important additional variables that our research group has identified in order to design a novel and effective ATPAP partitioning strategy are presented

systems, hence representing a valuable approach for the recovery and purification of high-added value biologicals. Nevertheless, a new set of process parameters must be taken into account in ATPAP protocols in order to consider the affinity interactions present. In a recent research conducted by our group, we defined key elements to be considered in liquid-liquid affinity partitioning, and now along with additional variables, these are presented in Fig. 6.3. Based on scientific literature, it can be stated that affinity-enhanced interactions in ATPAP systems are directly affected by the ionic state of both the selected ALs and the target products to be recovered (González-González and Rito-Palomares 2014). In this tenor, the pH level and ionic strength of selected systems must be carefully monitored and studied, in order to design an efficient extraction protocol. In the same subject, information regarding AL concentration and modified phase-component concentration needs to be considered. ATPAP strategies include an important term, defined as the degree of saturation (DS) (i.e., number of ligand molecules/number of polymer molecules) which constitutes a pivotal role in target product capture in a selected phase of the system. The major impact that DS poses within product capture resides in the establishment of the selective basis for product capture, since a high saturation content may inhibit system efficiency by means of a hook-type effect. Considering this, a high concentration of ALs or modified phase components could introduce shielding effects toward target molecules, generating crowding behaviors (i.e., steric hindrance) which could finally represent a problem between target products and active receptor sites. These scenarios need to be considered always if an efficient ATPAP strategy is to be designed and implemented.

6.3 Phase-Forming Components Activation Procedures

In order to obtain higher affinity on the target molecule, it is convenient to use specific ligands compatible with the nature of the biomolecule. The use of affinity ligands to increase the efficiency of purification can be used in two ways: (1) free affinity ligands or (2) covalently linked affinity ligands. Free affinity ligands in solution are used in order to avoid the chemical surface functionalization of the polymer (Fig. 6.1). In the case of free ligands, it is highly recommended to consider the partitioning behavior of the ligand in the ATPS before the interaction analysis between the ligand and the target molecule. On the other hand, one of the main strategies is the chemical modification of the polymer used. The surface functionalization of the polymer (e.g., polyethylene glycol (PEG) and/or dextran (DEX)) allows a covalent binding with the affinity ligand. Typically, the covalent binding between the polymer and the target molecules is achieved through hydroxyl groups of the polymer. In the case of PEG, the monomethylether of PEG (mPEG) is frequently used to cleave one target molecule per polymer chain and/or to link multiple PEG chains to avoid a cross-linking reaction (Zalipsky 1995). In this regard, the first step of the downstream processing of a biomolecule is to find a suitable functionalization of the polymer.

There are different methodologies that can be used in surface functionalization of the polymer (Fig. 6.4). In the particular case of PEG and DEX, surface functionalization is achieved by the activation of hydroxyl groups present in those polymers. There are two main approaches for the functionalization of the polymer's surface: (1) direct transformation of OH groups and (2) reaction of the molecule with a bifunctional molecule which means that one function will form the attachment to the polymer and the other remains available for further chemical reactions (Zalipsky 1995). The most accurate methodology should be selected according to the nature and properties of the target molecule. For molecules with tryptophan residues, the benzoate activation methodology seems more suitable because the benzoyl groups in the polymer act as an affinity ligand and recognize the tryptophan residues from the protein. This process is appropriate due to the integration of the affinity ligand to the polymer with the activation of it, which will lead to the reduction of chemical reactions.

One of the main drawbacks of surface functionalization techniques is the number of reactions and the time required to achieve the activation of functional groups in the polymer. The ethylenediamine and butanediol diglycidyl methodologies require from 65 to 110 h of consecutive reactions in order to activate the surface. The activation of hydroxyl groups thought these techniques facilitates the binding of proteins, peptides, and amino acids (Flanagan and Barondes 1975; Persson and Jergil 1995). On the contrary, methodologies such as periodate and tresyl-chloride provide a shorter protocol for surface functionalization (less than 24 h), but they facilitate the coupling of nucleophilic substitutions and additions to the polymer such as proteins, cofactors, or carbohydrate-binding modules (De Gouveia and Kilikian 2000; Ekblad et al. 1998; Persson and Jergil 1995). In the case of the benzoate activation methodology, it takes just up to 5 h to functionalize the surface.

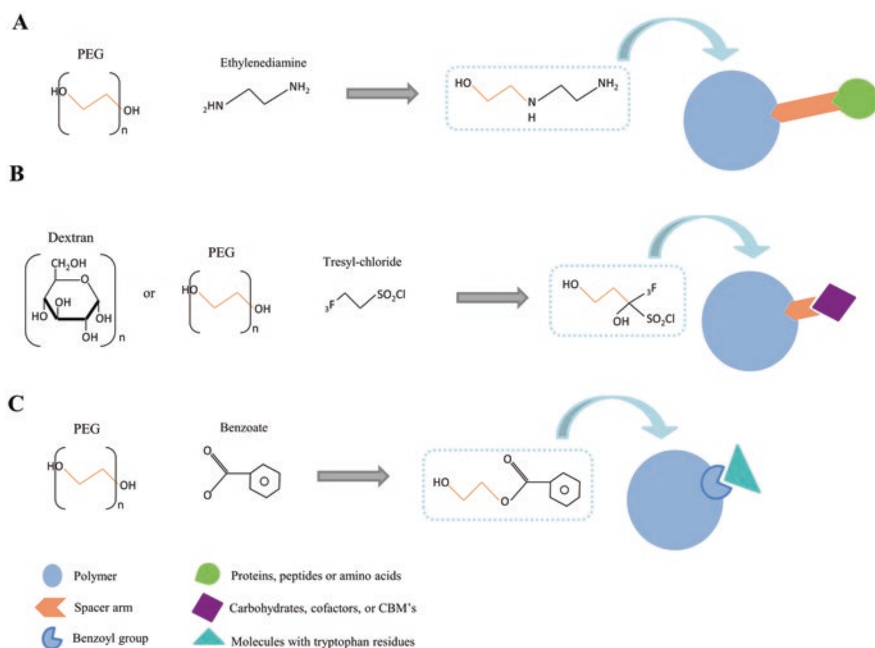

Fig. 6.4 Polymer surface functionalization. Three of the most common and used techniques to activate OH groups phase-forming polymers are presented. (**a**) Ethylenediamine technique provides a longer spacer arm between the polymer and the affinity ligand, which enhances the bioseparation process due to the higher interaction of the affinity ligand and the target molecules such as proteins, peptides, and amino acids. (**b**) Tresyl-chloride technique generates shorter spacer arms between the polymer and the affinity ligand. Nevertheless, the main advantage of this kind of technique is the short time required to activate the polymer. This technique allows the binding of carbohydrates, carbohydrate-binding modules (CBMs), and cofactors. (**c**) Benzoate techniques involve an integration affinity process because it enables the polymer to act as an affinity ligand itself. Additionally, this specific technique has a second advantage of short reaction time (5 h) in comparison of the above techniques that can take from 20 to 110 h reaction activation processes. Polymers activated with this technique will be able to link proteins with tryptophan residues. Hence, the benzoate technique is a feasible methodology for the activation of polymers for ATPAP systems

Another relevant aspect to be considered is the selection of the spacer arm between the polymer and the affinity ligand, as its length dictates at least in some degree the level of interaction between both constituents. Despite that ethylenediamine and butanediol diglycidyl methodologies have longer reaction times; they provide a longer arm between the polymer and the affinity ligand which will allow to the target molecule to have a better interaction with the polymer. In contrast to this, periodate and tresyl-chloride methodologies provide a shorter space arm which decreases the flexibility of the polymer-affinity ligand complex and reduces the efficiency of the separation process.

It must be emphasized that modifications of other functional groups can be achieved in order to enhance the efficiency of purification processes through ATPAP

protocols. The biochemical configuration of the target molecule will govern the addition or modification of certain functional group. The most common group modifications can be done to hydroxyl groups, carboxyl groups, guanido groups, carboxamido groups, and specific amino residue modification in proteins (Bonara and Drioli 2009). In this tenor, it should be considered the operational conditions of each methodology such as pH, temperature, and salt concentration in order to maintain the stability of the protein. Additional information of the advantages of these groups modifications are described in the literature (Bonara and Drioli 2009; Yang et al. 1995; Zalipsky 1995). Rocha et al. studied the feasibility to use alginate as a macroligand to recover papain (PAP) from *Carica papaya.* The aim of this macroligand is to direct the target molecule to the polymer-rich phase. Specifically, alginate was used as a free affinity ligand; hence no polymer surface functionalization was done. The results obtained from this study revealed that ATPS formed with PEG-salt sodium citrate could recover only 20% of PAP in the PEG-rich phase. The addition of alginate (0.1% w/w) enhanced the recovery of the enzyme to 72% (Rocha et al. 2016). Other interesting study focuses on the separation of histidine, histidine derivatives, and amino acids using metal affinity (Oshima et al. 2015). The separation of histidine derivatives was due to the specific compatibility between functional groups of the biomolecules and divalent metal ions such as Co (II), Ni (II), Cu (II), or Zn (II). Oshima and colleagues used an intermediary (chelating ligand) to create a stronger hydrophobic complex which will distribute in the PEG-rich phase. Among these divalent metal ions, the best extraction was reached with Cu (II) due to the strong hydrophobic interaction with the intermediary. The chelating ligand alizarin complexone (3-[N,N-bis(carboxymethyl)amino methyl]-1,2-dihydroxy anthraquinone, AC) and the metal affinity ligand were used in a free form. The extraction of histidine in the presence of Cu (II)-AC was 73% in comparison with the 13% of extraction using Cu (II)-free. With this novel protocol, histidine and histidine derivatives can be separated with a high selectivity over other amino acids due to the specific interactions with the imidazole group of histidine.

6.4 Affinity Liquid-Liquid Partitioning: Polymer-Polymer Systems

Considering the typical phase-forming components of ATPS, traditional polymer-polymer systems are among the most employed and versatile for product isolation (selected examples in Table 6.1). Systems formed by PEG and DEX have been exploited to recover high-value molecules that justify the cost of the chemical-forming phases, particularly dextran. In addition, affinity-enhanced polymer-polymer systems provide a wide scope for the recovery of biological products. In these affinity-based strategies, the polymer-ligand complex added to the system mainly partitions toward the phase rich on that specific polymer (e.g., DEX-ligand molecules partition to the DEX-rich phase). Purified or isolated molecules in these systems include monoclonal antibodies (mAbs) (Azevedo et al. 2009; Rosa et al. 2007), nucleic acids (Barbosa et al. 2008, 2010), industrial enzymes (Ekblad et al.

Table 6.1 Selected examples of affinity partitioning in polymer-polymer aqueous two-phase systems

Product	System	Modification/AL	Performance	Reference
Plasma membranes	DEX 500/ PEG 3350	DEX-wheat agglutinin	>91% recovery BP	Ekblad et al. (2000)
β-Galactosidase	DEX 500/ Valeryl DEX 500	DEX-benzoyl	4X increase in K_p	Lu and Tjerneld (1997)
Therapeutic plasmid	DEX 40/PEG 600	GST+ zinc finger	5900X increase in K_p	Barbosa et al. (2008)
Red blood cells	DEX 500/ PEG 6000	MPEG-IDA (II)	>95% recovery BP	Laboureau and Vijayalakshmi (1998)
$CD133^+$ stem cells	DEX 70/ Ficoll 400	Anti-CD133 AB	>60% recovery TP	González-González and Rito-Palomares (2015)
Human IgG	DEX 500/ PEG 1500	PEG-HRP	90% recovery in TP	Hye-Mee et al. (2007)
Trypsin	DEX 500/ PEG 8000	PEG-trypsin inhibitor	32X increase in K_p	Andrews et al. (1990)
Proteases from *Penicillium restrictum*	NaPA/PEG 2000	PEG-benzoyl	18X increase in K_p	Gomes Barros et al. (2014)

1998; Ling et al. 2010; Teotia et al. 2004), cellular membranes (Barinaga-Rementeria Ramírez et al. 2002), human cells (Laboureau and Vijayalakshmi 1998), and recently human stem cells (González-González and Rito-Palomares 2014; González-González et al. 2016; Sousa et al. 2011). In this section selected examples of bioproducts effectively recovered with ATPAP technologies are described and discussed.

One of the most important areas in which recent breakthroughs in bioseparations engineering have been proposed regarding liquid-liquid extraction techniques is that of stem cells separation. Research by Joaquim Cabral and Raquel Aires-Barros placed the platform for stem cell partitioning in ATPAP systems (Sousa et al. 2011) back in 2011. Since the development of an effective and robust separation system for large-scale cell separation with high throughput, purity, and yield was not yet established, an imminent challenge in this area was identified. In this research, an ATPAP system composed of PEG 8000 and DEX 500,000 was used for the selective isolation of $CD34^+$ stem cells from umbilical cord blood (UCB). In order to favor cell partition toward one of the phases, a macroligand free in solution (monoclonal antibody against the CD34 antigen marker) was employed. Since the selected ligand exhibited a partition toward the PEG-rich phase, a recovery yield of 95% and a purification factor of 245 were obtained for the studied stem cells. The authors determined that the presence of the affinity ligand demonstrated separation efficiencies comparable to the most common techniques for hematopoietic stem cell

purification upon a mononuclear cell fraction such as magnetic-activated cell sorting (MACS). One of the major concerns within this research is that of cell viability and pluripotency, since the selective separation of stem cells through the use of a mAbs targeted for outer protein markers could enhance cell differentiation in the separation process. Up to date, this particular effect has not been characterized effectively in ATPAP partitioning studies and thus represents an important study area in order to establish effective methodologies for stem cells isolation.

In the past 5 years, extensive research has established a defined path toward the recovery of CD 133$^+$ stem cells with means of establishing a novel, robust, scalable, and economic recovery platform for these target products. CD 133$^+$ stem cells have been successfully transplanted in humans for treatment of cirrhosis, leukemia, and ischemia (González-González and Rito-Palomares 2015), results that emphasize the importance of this particular line of cells. A recent study presented an ATPAP process for the selective recovery of CD 133$^+$ stem cells (González-González and Rito-Palomares 2015) from UCB. The use of traditional (PEG-DEX and Ficoll-DEX) and novel (UCON-DEX) two-phase systems was studied in the presence and absence of a macroligand free in solution, a CD 133/2 antibody, in order to favor partition of cells to a specific phase in the system. The novel system employed showed an increased partition of the ligand to the bottom phase (DEX), result that was validated with SDS-PAGE electrophoresis, and thus provided evidence to support the idea of allowing increased partition of CD 133$^+$ stem cells to the dextran phase. Traditional systems did not present a marked preference of the antibody toward neither phase. Even though the UCON systems selected exhibited ligand partitioning, the results did now presented an enhanced decrease or increase of the partition coefficient of stem cells. The authors concluded that additional strategies are needed to be considered in order to concentrate the antibodies more effectively in the top phase, which could finally result in the partitioning of stem cells to the target phase. In this tenor, one important characteristic that has to be considered is the DS, since the number of molecules/target product might play a vital role in selective partitioning. Considering the approach herein discussed, the use of cheap and recombinant mAbs might be a solution in order to reduce expense costs of these ATPAP strategies if they are to be implemented in the future.

The intensified partition of cell membranes and liposomes with a biotin spacer arm was studied in liquid-liquid affinity systems with DEX 40/DEX 2000 and PEG 3350, respectively (Barinaga-Rementeria Ramírez et al. 2002). Purification of membranes (outer/inner) represents an important research area for drug delivery strategies (Barinaga-Rementeria Ramírez et al. 2002). Target products were recovered in ATPAP systems which had deglycosylated avidin (D-avidin) as affinity ligand coupled to DEX molecules that partitioned to the bottom phase. Liposomes partition was first studied in order to determine optimal parameters for further purification of biotinylated membrane fractions. In systems with the absence of D-avidin, more than 80% of the liposomes partitioned to the PEG-rich phase. Addition of chemically active DEX phase induced a notable shift of biotinylated liposomes to the dextran-rich phase, from 85% in PEG-rich phase (ligand absence) to 90% in the dextran bottom phase. The authors concluded that the interaction between the biotin

spacer arm and D-avidin in liposomes and membranes determines the partitioning behavior of these molecules. This biological interaction represents a valuable approach for the purification of biotinylated products and also an important positive result for ATPAP for the recovery of high-added value cell fragments intended for therapeutic processes.

Polymer-polymer ATPAP systems offer enhanced versatility for biotechnology-based applications since both phases could be chemically active in order to further attach affinity ligands. In this tenor, an important characteristic within polymer activation techniques and ligand coupling is the nature of the spacer arm between the active phase component and the selected ligand. These spacers tend to have different lengths according to the protocols employed in chemical activation, and careful selection of the derivatization methodology would give an increased interaction of the ligand and the desired target molecule by means of positive mobility of the molecular bridging at hand. Also important to consider is the ionic force and pH of the systems in order to favor product partition. Even though marked shifts in salt concentration or pH may favor affinity interactions between target molecules and affinity ligands, these phenomena have not been deeply studied in ATPAP applications, and thus an important research niche can be identified in this area since positive effects considering these variables have been reported for protein partitioning in the past.

Even though polymer-polymer systems offer increased versatility for ligand coupling strategies, the most important protocols for ATPAP systems include free ligands in solution (González-González et al. 2016; Sousa et al. 2011). Nevertheless, a proper study of ligand partitioning must be undertaken in order to characterize its partitioning behavior and thus determine its true potential in affinity extraction procedures. Biochemical modification of affinity ligands can be carried out in order to favor their partitioning to a specific phase in the system. One of the most recent and common modification techniques in ATPAP systems is PEGylation, since it has been positively used in the past for efficient molecule modification. PEGylation reactions have been well studied and characterized and allow the coupling of a PEG moiety to a particular compound, in this case an affinity ligand (Barbosa et al. 2008). Because of the chemical nature of the modified ligand, it has been demonstrated that partitioning behaviors toward PEG-rich phases are greatly favored (Barbosa et al. 2008). This methodology can exploit modification of protein molecules (used as ALs) and thus aid in the design of efficient operations for the recovery of high-added value compounds. It is important to emphasize that no in-depth studies about PEGylation in ATPAP have been reported; however, new applications of this technology with ATPAP can be expected. Due to the stability of the systems and low ionic force, polymer-polymer systems offer important versatility for ATPAP strategies in recovery of whole particles and macromolecules (Table 6.1). One of the principal aspects to be considered when using these systems is the final cost, since PEG and particularly DEX may increase the final bioprocessing cost at pilot or industrial scales. Potential chemical-forming phases recycling strategies could minimize the negative perception of these systems for the generic application at large scale.

6.5 Affinity Liquid-Liquid Partitioning: Polymer-Salt Systems

The use of polymer-salt systems has been reported for several decades for the purification of macromolecules, specifically proteins. Regarding ATPAP strategies, phase-forming components including salt phases are not very common for the recovery of a wide array of molecules, the most notable ones being industrial-grade enzymes (Table 6.2). The major drawbacks of these ATPAP systems lie within their highly ionic nature. The use of certain salts has proven to increase partition of molecules in ATPS, but above certain critical concentrations, a negative pattern of solubility for proteins and other biological products can be observed. In this context, important shifts in the ionic force of the system might hamper essential ionic interactions between ALs and target molecules, thus significantly reducing the affinity potential of ATPAP protocols. Nevertheless successful research has been reported in this area, and some important examples are here presented and discussed.

One of the most important areas in biotechnology engineering is that of food development including designs with new or novel ingredients. Asenjo and Andrews performed the selective purification of thaumatin, a flavor enhancer and sweetener protein from the *Thaumatococcus daniellii* fruit. Since thaumatin and some derivatives can exhibit a 2000-fold increase in sweetness when compared to dextrose, efficient operations for protein characterization and isolation have been proposed. PEG 8000-potassium phosphate systems were employed in a protocol which included functionalized PEG molecules through an epoxy-oxirane method in order to couple glutathione as AL. The addition of 8% of active PEG in the ATPAP system proved enough to increase the protein concentration in the PEG phase from 0.82 to 2.46 g/L (threefold increase). This result demonstrated that the purification and concentration of active proteins can be dramatically improved when using biospecific ligands attached to a phase-forming component. The authors also concluded that the DS plays a vital role in product partitioning, since in this research it was found that high concentrations of coupled glutathione further reduced the amount of recovered thaumatin (Asenjo and Andrews 2008).

Production of pharmaceutical compounds is a major research area within bioprocess engineering. During the past three decades, antibiotic synthesis procedures have been optimized through the efficient use of enzymes. One important example is that of penicillin acylase (PA) presented by Gavasane and Gaikar. This enzyme is involved in the production of 6-amino-penicillianic acid, a key intermediate in the production of semisynthetic penicillins (Hamilton-Miller 1966). Four active PEGs were developed: PEG-phenylacetamide (PEG-paa), PEG-trimethylamine (PEG-tma), PEG-benzoate (PEG-bz), and PEG-palmitate (PEG-pmt). Interaction between the target enzyme and PEG-bz or PEG-paa favored product recovery. The authors determined that high affinity was favored since the intermolecular energy between the active PEGs and PA was lower to the interaction energy between PA and its natural substrate. The enzyme exhibited a 15-fold increase in partition coefficient and 60% product recovery when PEG-paa was used. These functionalized PEGs proved increased interaction between the amide or phenyl groups with specific

Table 6.2 Affinity partitioning of bioproducts in polymer-salt two-phase systems

Product	ATPS	Affinity approach	Performance	Reference
Penicillin acylase	PEG 4000/KPO_4	PEG-palmitate	>81% recovery in TP after optimization runs, K_p 15X increase	Gavasane and Gaikar (2003)
Thaumatin	Glucoamylase	PEG-glutathione	3.5X increase in K_p after modification	Andrews et al. (1990)
Histidine derivatives	PEG 8000/Li_2SO_4	Alizarin as AL	>75% recovery in TP when the chelating ligand was present in the ATPS	Oshima et al. (2015)
Phospholipase	PEG 6000/KPO_4	Alginate as AL	~80-fold increase in purification factor and 85% global recovery in TP	Teotia and Gupta (2004)
Phenolic acids	PEG 200–600/ Na_2SO_4	CMIM derivatives	5% w/w of CMIM allowed up to 99% recovery of gallic and vanillic acid	Almeida et al. (2014)
Glucoamylase	PEG 300–400/ KPO_4	Starch as AL	9X K_p decrease when partitioned to the salt phase	Gouveia and Kilikian (2000)
Papain	PEG 8000/ Sodium Citrate	Alginate as AL	>70% recovery and 2.41 purification factor achieved	Rocha et al. (2016)

residues in PA (Gavasane and Gaikar 2003). One key element discussed and emphasized in this research was the molecular simulation of ligand interactions, in order to provide enhanced bioselectivity for product retrieval in future applications. This approach can be exploited for future experiments using other types of ligands and target products in order to design robust, economical, and efficient downstream operations based on ATPAP technology.

Scientific advances in medicine are often possible due to availability of novel substrates or molecules that will help design further researches. The purification of phospholipase D (PLD) from *Arachis hypogaea* and *Daucus carota* with ATPAP systems was presented by Teotia and Gupta. This enzyme is implicated in several vital cellular processes including membrane transport and cytoskeletal recognition in mammalian cells. PEG 6000-potassium phosphate systems were constructed adding alginate as a ligand free in solution that partitioned almost exclusively to the PEG-rich phase (>95%). It was determined that, when alginate (0.2% w/v) was added to the system, an increase in the purification factor of PLD of 78-fold with more than 80% recovery was observed. These values represent a significant improvement when compared with those achieved in traditional ATPS (30 purity fold and 50% recovery). The main benefits of this research were that a high-value product could be recovered without preprocessing or clarification steps, with the

implementation of an economical PEG-salt system (Teotia and Gupta 2004). The most important contribution of this research is the affinity interaction between alginate, a naturally occurring anionic polysaccharide, and the target product. The interaction of molecules with phase-forming components has not been extensively studied in two-phase partitioning protocols. Hence, this could provide an interesting approach toward the design of novel separation techniques in APTAP through the visualization of potential affinity interactions of molecules and natural polymers.

Partitioning in polymer-salt systems presents important advantages for affinity-based strategies. Regarding the physicochemical characteristics of the systems, reduced viscosity and density for the phases can be seen, which increases the industrial potential and feasibility for ATPAP strategies to be scaled-up in commercial processes. In addition, the bulk cost per gram of phase-forming component/final phase is significantly lower when compared to polymer-polymer systems, specifically those which include dextran. Nevertheless, a set of future challenges can also be described for these systems. At a first glance, a deep and adequate characterization of the biospecific, electrostatic, and hydrophobic interactions between PEG molecules, ions, ALs employed, and target products to be recovered or purified is needed. Very few studies or comments have been reported in literature in this subject (Hamilton-Miller 1966; Teotia and Gupta 2004), situation that clearly highlights an important research niche in this area. Also to be considered is the complexity of the molecules or components that might aid or hinder the partition of a selected particle, since hydrophobic, ionic, or pH shifts can be achieved using a vast array of reagents, small molecules, or macroligands.

An important approach that must be considered when designing ATPAP strategies is the implementation of ordered and efficient statistical tools that provide positive information regarding unit operation performance. Thus, appropriate experimental designs coupled to optimization methodologies (i.e., response surface analysis) might prove useful in order to identify key elements or variables in ATPAP operations (e.g., Fig. 6.3) and thus establish feasible strategies to be applied at industrial level. The primary benefits from these studies would reside in the elaboration and elucidation of predictive mathematical models which could greatly reduce capital expenses, human hours, and time spent on research experiments involving the design and characterization of the operation at hand. In addition, if enough attention is given to the information presented in Table 6.2 and Fig. 6.4, polymer-salt affinity systems may provide interesting solutions for the recovery and purification of industrial enzymes that commonly withstand harsh processing conditions, such as shifting pH and temperature settings. Hence, cheaper and selective liquid-liquid systems for these bioproducts could be designed and massively implemented at industrial scale if the abovementioned challenges or suggestions are to be considered and addressed by biotechnology companies worldwide.

6.6 Affinity Liquid-Liquid Partitioning: Alternative Systems

One area that has not been intensely studied, in the context of ATPAP, is that related to the characterization and use of natural (i.e., starch or gums) or alternative/intelligent polymers (thermosensitive polymers such as UCON). These systems have been poorly studied in their traditional two-phase configuration; hence, evidence for their applicability in affinity partitioning is limited. Nevertheless, considering that these alternative phase-forming components can offer important advantages over traditional two-phase-forming chemicals, strategies for the recovery and purification of several biological particles have been reported such as proteins or whole cells. This brief section will present a couple of successful cases in which ATPAP efficiently recovered biological products, along with a discussion of the primary challenges within this research area.

Affinity chromatography for monoclonal antibodies recovery constitutes the primary purification method of the biotechnological industries nowadays involved in mAbs production and commercialization. Traditional ATPS for mAbs recovery have been presented in the past with efficient yields (>90% recovery). An ATPAP strategy for human immunoglobulin (IgG) from CHO cells was reported by Ferreira et al., using non-conventional phase-forming components for the systems studied. The approach developed involved a system comprised by 6% w/w DEX 500,000, 8% w/w ethylene oxide/propylene oxide (UCON), and an AL free in solution in the form of triethylene glycol-diglutaric acid. With addition of TEG-COOH, an improvement on both extraction yield and purity of human IgG in the top phase was clearly observed. The significant increase in log K_p (from −0.2 to 3.5) of IgG was attributed to electrostatic interactions between the positively charged IgG and the negative carboxylic groups of TEG-COOH (Ferreira et al. 2008). According to the optimization strategies employed by the authors (response surface methodologies), the optimal extraction system for the purification of the target product was determined to contain 5.5% w/w dextran, 10% w/w UCON, and 20% w/w ligand (TEG-COOH). In this particular case, the information provided by the authors supports one of the strategies previously mentioned in this chapter, which involves the elucidation of mathematical models for predictive behaviors of product partitioning and optimization.

Antov et al. employed a novel system formed by guar gum (a renewable polysaccharide galactomannan) and starch, in which carbohydrate-binding modules (CBMs) were used as affinity ligands since these tags are present in some proteins and enzymes and thus facilitate substrate location and coupling. The target product in this research was β-mannanase from *Cellulomonas fimi*, an enzyme with high affinity toward specific mannans because of its native CBM. The strategy consisted in understanding the affinity of the protein-binding module to the galactomannan polymer in the aqueous extraction system in order to determine the optimal partitioning conditions for mannanase. The natural polymers hydroxypropyl starch PES 200 and guar gum galactomannan (9% w/w and 3.6% w/w, respectively) were used as forming components. The selected system allowed a top phase recovery yield of 90% with a K_p of 7.06 for β-mannanase. With the obtained results, the authors noted

that diverse CBMs could be employed for protein purification (Antov et al. 2006). Hence, a general extraction procedure for proteins or ad hoc molecules could be developed if each target molecule is fused with a specific binding module. This would represent an important advantage in the development of novel downstream processing strategies, in which an additional affinity behavior could represent an alternative for product purification.

Additional research results are presented in Table 6.3 in which successful applications of affinity partitioning have employed alternative phase-forming components. Among the results presented in this table, it must be considered that an interesting area that could prove important advantages in product recovery and

Table 6.3 Affinity partitioning of bioproducts in alternative phase-component two-phase systems

Product	ATPS	Affinity mechanism	Performance	Reference
β-Mannanase	PES 200-Guar Gum	Galactomannan as AL	5.1X–fold increase in K_p	Antov et al. (2006)
His-tag: GFP	Triton X114/water	Nickel-chelated TX	20X–fold increase in K_p when modified TX was used	Wang et al. (2013)
GFP	Triton X114/Ni-Triton X114	Nickel-chelated TX	With use of EDTA to back-extract, 83% recovery yield and 70% purity achieved	Wang et al. (2014)
IgG	PEG 4000/ hydroxypropyl starch	NaCl in solution	Purification factor of 5.28 and extraction yield of 99.2%	Wu et al. (2013)
Cutinase	BREOX 100/PES 200	TX 100 as AL	>11X–fold increase in K_p when recombinant cutinase was purified	Mannesse et al. (1995)
Phenylalanine in racemic mixture	Chiral tropine ionic liquids (ILs)	Chirally active ILs	65% of D-enantiomer partitioned to the TP rich in Cu^{2+} backbone	Wu et al. (2015)

purification is that of micellar preparations. These systems are formed when surfactants (ionic or no ionic detergents) are mixed with an organic/aqueous phase over a critical concentration known as the “critical micelle concentration” (CMC). When the resulting system is formed, the micelles can exhibit a hydrophobic or hydrophilic behavior (depending on the phase-forming chemicals). These particles can be chemically activated, as presented by Lam et al., in order to couple ALs and thus present potential for an ATPAP strategy. Nevertheless, the stability of the systems and the activation procedures for micelles have not been deeply studied, situation which highlights an important opportunity area for these protocols. The major challenge these systems suppose for product extraction resides in biocompatibility of the molecules to be recovered and the modified ATPAP system. In addition, since micellar systems can also significantly increase system costs (concentration and nature of surfactants), modification procedures to be employed in these strategies must be very selective and cheap and require little reaction time, as presented in Fig. 6.4, in order to maximize interaction between selected ligands and potential binding sites in the active micelles in the system. Considering the amphipathic nature of these systems, a careful and deep analysis of the interactions between the active sites, the involved ligands, and the target products should be first envisioned step in order to characterize positive strategies using this technology (Lam et al. 2005).

Considering the additional variables to be studied in affinity fractionation procedures as presented in Sects. 6.1 and 6.2, the idea of using a phase component (e.g., natural polymers) that may serve as an affinity molecule toward a target product, and at the same time constitute one of the phases in the system, could represent a significant approach for integration and intensification of unit operations. Nevertheless, certain challenges must be addressed within natural polymers and ATPAP partitioning. Considering the information discussed by Sturesson et al., some versions of industrial starches that are commercially available exhibit lower economical costs when compared to dextran or other synthetic polymers. However, the functional time of these natural polymers may be limited due to the biodegradable potential (Sturesson et al. 1990). Another important aspect that must be considered with natural polymers is their high viscosity. Even though these polymers (i.e., starch, guar gums) can be considered environmentally friendly in low concentrations, their high viscosities must be reduced in order to use them in ATPAP and traditional ATPS strategies. The most used approach in order to pretreat viscous polymers is that of enzymatic protocols (Antov et al. 2006), and heating in a minor scale, but the cost-effective approach of using a natural non-expensive polymer could be completely hampered. Finally, the industrial production schemes of such components must be standardized in order to produce quality batches with proved chemical stability, since current natural polymers solutions cannot be prepared and stored for prolonged periods of time.

6.7 Current and Future Challenges for Liquid-Liquid Affinity Partitioning

Considering the major advances in bioprocess engineering regarding unit operations, purification protocols, and production schemes, one of the constant challenges and trends in this area is that of process integration and intensification. Considering the information presented throughout this chapter, the selective recovery of proteins (Effio et al. 2015; Flanagan and Barondes 1975), enzymes (Gomes Barros et al. 2014; Ling et al. 2010; Ramakrishnan et al. 2016; Teotia et al. 2004), recombinant proteins, cellular membranes (Laboureau and Vijayalakshmi 1998), or even human stem cells (González-González and Rito-Palomares 2015; González-González et al. 2016; Silva et al. 2014; Sousa et al. 2011) has been achieved with high recovery yields. Some of the major advantages of these success cases are the strong biocompatible environment, ease of operation, and economic feasibility of the designed ATPAP operations. In addition, the authors conclude that the reduction of chromatographic steps is a key element in these emerging bioprocessing areas. Nevertheless, considering the vast amount of information regarding chromatographic-based operations for biological recovery and purification, the well-established techniques and protocols at industrial level of these operations could possibly hinder ATPAP applications at commercial scales in the near future if some challenges (e.g., new phase-forming components, mathematical modeling, novel polymer modification techniques, versatile ligands) are not adequately studied and validated.

An assessment between ATPAP and chromatographic techniques was presented by our research group in the past (Ruiz-Ruiz et al. 2012). The main comparison among these operations must include the overall efficiency for recovery/purity yields and the economic burden of the protocol in the bioprocess at hand. One of the most interesting cases in which ATPAP posed an increased potential when compared to chromatographic-based separations is that of whole cells (CD 34^+ stem cells) separation. Recovery yields tend to be above 90% after the employed protocols, but current separation techniques based on cell sorting methodologies (i.e., magnetic-activated cell sorting, MACS) will be insufficient in the future in order to meet the needs of stem cell therapies and treatments due to their low scalability potential and sample loading capacities (Huettmann et al. 2014). Since recovery yields are very comparable with those obtained with MACS devices, several reports have emphasized the simplicity and robustness ATPAP protocols pose within bioprocess design, including scale-up feasibility (González-González and Rito-Palomares 2015). The most important challenge in whole-cell recovery in ATPAP systems is the establishment of purity standards for the correct isolation of the target particles (e.g., red blood cells, stem cells), since these objectives have not been correctly addressed. Despite this, ATPAP methodologies seem promising since 96% recovery of CD 34^+ stem cells as reported by Sousa et al. was obtained compared to 85% total extraction with a commercially available MACS procedure (Sousa et al. 2011).

Even though liquid affinity partitioning has not been extensively studied and characterized, important advantages such as feasible scale-up potential and intensification capabilities could position this recovery technique as a valuable component

of industrial bioprocess designs. In order to favor ATPAP coupling in current bioprocesses, an important challenge that must be addressed relies on the strict control and understanding of the electrostatic interaction between charged groups in target molecules and affinity ligands employed in the system at hand. This situation can be characterized by studying the optimal pH levels for protein/product stability and activity in a selected ATPAP system, since each molecule exhibits a particular partitioning behavior based on this variable. Another important aspect that must be considered is product partitioning maximized through hydrophobic interaction between ligands, system components, and particle/molecular structure (Benavides and Rito-Palomares 2008). Considering the aspects and practical experiences found in literature, the use of hydrophobic domains in AL could favor recovery of molecules with similar domains or active structures. In addition, this polarity-based effect could be positively studied and enhanced if the extraction procedure is made near the isoelectric point of a protein, since solubility will be low and thus facilitate hydrophobic interactions to take place.

One of the most important trends in ATPAP technology nowadays involves the study and characterization of affinity ligands. The advances and evolution of ALs in affinity separations have experienced dramatic changes in the past four to five decades, including those used in chromatographic and non-chromatographic techniques. Regarding ATPAP protocols, the first generation of ALs consisted in the careful synthesis of molecules in order to exert specific affinity toward a target product. During the past 15 years, research in this area has experienced a marked transition, which constitutes one of the most important trends in ATPAP technology, from chemical molecules to biological structures such as biological substrates, binding modules (i.e., CBMs), enzymes, antigens, or even antibody fragments. It can be expected that third-generation affinity ligands will mark an important increase in ATPAP designs in the near future, in which possibly molecularly engineered products (e.g., proteins, chimeric products, low molecular weight compounds) will be employed in dual-affinity strategies in order to favor product recovery in cheap and robust systems. Regarding also the importance within ligands in ATPAP, it can be expected that future applications of this extraction technique will favor the use of ALs free in solution since no polymer activation procedures would be needed in order to add the molecule to the system. The challenge of this approach resides in the proper and deep characterization of the partitioning of the selected ALs, aiming to not expect a change in the system characteristics such as V_R, TLL, and composition.

One last aspect that must be mentioned in this chapter is that one regarding all the biochemical or biological elution strategies of target products when partitioned with an ATPAP approach. Even though there is an important number of affinity partitioning research papers published in the last four decades (>40), desorption methodologies are rarely studied and discussed. This final step, even though simple and quick, could prove the most important challenge for ATPAP efficiency methods, since no clear methods have been characterized up to date. Some of the traditional methods employed for chromatographic-based separations have been proposed, such as ionic force shifting, pH adjustment, and addition of displacer

molecules (i.e., competitive substrates) to promote desorption of the target molecule from the affinity ligand used in an ATPAP strategy. Nevertheless, these protocols have not been properly studied, and thus an important opportunity area in this technology can be identified within displacing techniques for the final recovery and polishing steps in a selected bioprocess.

6.8 Concluding Remarks

The past two decades have witnessed a significant increase in the number of studies related to liquid-liquid affinity partitioning. It is evident that nucleic acid therapies and stem cell research could be positively enhanced by development of ATPAP technologies since specific interactions and high recovery yields could be expected. In addition, the possibility of designing extraction operations using natural resources, such as starch or gums, may give these systems an environmental-friendly dimension that could prove an advantage and trend within bioprocess engineering designs worldwide. Nevertheless, these systems continue to experience important challenges nowadays, as discussed in the past section, since the manipulation and high viscosity of the components has not yet allowed them to be commonly accepted and employed in fractionation research at lab and pilot scales. In this tenor, new research within material science could trigger the development of novel ATPAP designs in the near future, including at the same time the coupling of new affinity ligands, since the proper conjunction of efficient phase-forming chemicals and affinity-enhanced molecules is the perfect combination for the design of novel bioseparation breakthroughs. A last and important aspect to be considered within this topic involves the bioprocess economics in ATPAP strategies, specifically within ligand selection/coupling/production schemes. Capital investment in two-phase systems has been focused in the direct cost of the phase-forming chemicals. In this case, the relative costs of ligand inclusion should be permanently considered in order to establish competitive bioseparation ATPAP alternatives when compared to traditional chromatographic techniques and thus offer attractive technologies for future development of downstream processing strategies in the biotech industry.

References

Almeida M, Passos H, Pereira M, Lima A, Coutinho J, Freire M. Ionic liquid as additives to enhance the extraction of antioxidants in aqueous two-phase systems. Sep Purif Technol. 2014;128:1–10.

Andrews BA, Head DM, Dunthorne P, Asenjo JA. PEG activation and ligand binding for the affinity partitioning of proteins in aqueous two-phase systems. Biotechnol Tech. 1990;4(1):49–54.

Antov M, Anderson L, Andersson A, Tjerneld F, Stålbrand H. Affinity partitioning of a *Cellulomonas fimi* β-mannanase with a mannan-binding module in galactomannan/starch aqueous two-phase system. J Chromatogr A. 2006;1123(1):53–9. doi:10.1016/j.chroma.2006.05.021.

Asenjo JA, Andrews BA. Challenges and trends in bioseparations. J Chem Technol Biotechnol. 2008;83(2):117–20. doi:10.1002/jctb.1851.

Azevedo AM, Rosa PAJ, Ferreira IF, Pisco AMMO, de Vries J, Korporaal R, Aires-Barros MR. Affinity-enhanced purification of human antibodies by aqueous two-phase extraction. Sep Purif Technol. 2009;65(1):31–9. doi:10.1016/j.seppur.2008.03.006.

Barbosa H, Hine AV, Brocchini S, Slate NKH, Marcos JC. Affinity partitioning of plasmid DNA with a zinc finger protein. J Chromatogr A. 2008;1206(2):105–12. doi:10.1016/j.chroma.2008.07.095.

Barbosa HSC, Hine AV, Brocchini S, Slater NKH, Marcos JC. Dual affinity method for plasmid DNA purification in aqueous two-phase systems. J Chromatogr A. 2010;1217(9):1429–36. doi:10.1016/j.chroma.2009.12.059.

Barinaga-Rementeria Ramírez I, Mebrahtu S, Jergil B. Affinity partitioning for membrane purification exploiting the biotin-NeutrAvidin interaction – model study of mixed liposomes and membranes. J Chromatogr A. 2002;971(1–2):117–27. doi:10.1016/S0021-9673(02)00841-5.

Benavides J, Rito-Palomares M. Bioprocess intensification: a potential aqueous two-phase process for the primary recovery of B-phycoerythrin from *Porphyridium cruentum*. J Chromatogr B. 2004;807(1):33–8. doi:10.1016/j.jchromb.2004.01.028.

Benavides J, Rito-Palomares M. Practical experiences from the development of aqueous two-phase processes for the recovery of high value biological products. J Chem Technol Biotechnol. 2008;83(2):133–42. doi:10.1002/jctb.1844.

Bonara GM, Drioli S. Reactive PEGs for protein conjugation. In: Veronese FM, editor. PEGylated protein drugs: basic science and clinical applications. Basel: Birkhäuser; 2009. p. 33–45.

Chavez-Santoscoy A, Benavides J, Vermaas W, Rito-Palomares M. Application of aqueous two-phase systems for the potential extractive fermentation of cyanobacterial products. Chem Eng Technol. 2010;33(1):177–82. doi:10.1002/ceat.200900286.

Cordes A, Flossdorf J, Kula MR. Affinity partitioning: development of mathematical model describing behavior of biomolecules in aqueous two-phase systems. Biotechnol Bioeng. 1987;30(4):514–20. doi:10.1002/bit.260300408.

Costa CLL, Badino AC. Overproduction of clavulanic acid by extractive fermentation. Electron J Biotechnol. 2015;18(3):154–60. doi:10.1016/j.ejbt.2015.03.001.

Cunha MT, Costa MJL, Calado CRC, Fonseca LP, Aires-Barros MR, Cabral JMS. Integration of production and aqueous two-phase systems extraction of extracellular *Fusarium solani pisi* cutinase fusion proteins. J Biotechnol. 2003;100(1):55–64. doi:10.1016/S0168-1656(02)00225-0.

De Gouveia T, Kilikian BV. Bioaffinity extraction of glucoamylase in aqueous two-phase systems using starch as free bioligand. J Chromatogr B. 2000;743(1–2):241–6. doi:10.1016/S0378-4347(00)00031-1.

Effio CL, Wenger L, Ötes O, Oelmeier SA, Kneusel R, Hubbuch J. Downstream processing of virus-like particles: single-stage and multi-stage aqueous two-phase extraction. J Chromatogr A. 2015;1383:35–46. doi:10.1016/j.chroma.2015.01.007.

Ekblad L, Kernbichler J, Jergil B. Aqueous two-phase affinity partitioning of biotinylated liposomes using neutral avidin as affinity ligand. J Chromatogr A. 1998;815(2):189–95.

Ekblad L, Jergil B, Gierow JP. Purification of rabbit lacrimal gland plasma membranes by aqueous two-phase affinity partitioning. J Chromatogr B. 2000;743(1–2):397–401.

Ferreira IF, Azevedo AM, Rosa PAJ, Aires-Barros MR. Purification of human immunoglobulin G by thermoseparating aqueous two-phase systems. J Chromatogr A. 2008;1195(1–2):94–100. doi:10.1016/j.chroma.2008.04.077.

Flanagan SD, Barondes SH. Affinity partitioning. J Biol Chem. 1975;250(4):1484–9.

Gavasane MR, Gaikar VG. Aqueous two-phase affinity partitioning of penicillin acylase from *E. coli* in presence of PEG-derivatives. Enzyme Microb Technol. 2003;32(6):665–75. doi:10.1016/S0141-0229(03)00032-2.

Gomes Barros KV, Monteiro Souza P, Medeiros Freitas M, Ferreira Filho EX, Pessoa Junior A, Oliveira MP. PEG/NaPA aqueous two-phase systems for the purification of proteases expressed by *Penicillium restrictum* from Brazilian Savanna. Process Biochem. 2014;49(12):2305–12.

González-González M, Rito-Palomares M. Aqueous two-phase systems strategies to establish novel bioprocesses for stem cells recovery. Crit Rev Biotechnol. 2014;34(4):318–27. doi:10.3109/07388551.2013.794125.

González-González M, Rito-Palomares M. Application of affinity aqueous two-phase systems for the fractionation of CD133(+) stem cells from human umbilical cord blood. J Mol Recognit. 2015;28(3):142–7. doi:10.1002/jmr.2374.

González-González M, Willson RC, Rito-Palomares M. Elimination of contaminants from cell preparations using aqueous two-phase partitioning. Sep Purif Technol. 2016;158:103–7. doi:10.1016/j.seppur.2015.12.006.

Gouveia T, Kilikian B. Bioaffinity extraction of glucoamylase in aqueous two-phase systems using starch as free bioligand. J Chromatogr B. 2000;743(1–2):241–6.

Gutiérrez LF, Sánchez ÓJ, Cardona CA. Analysis and design of extractive fermentation processes using a novel short-cut method. Ind Eng Chem Res. 2013;52(36):12915–26. doi:10.1021/ie301297h.

Hamilton-Miller JMT. Penicillinacylase. Bacteriol Rev. 1966;30(4):761–71.

Hernández-Mireles T, Rito-Palomares M. Improved recovery of B-phycoerythrin produced by the red microalga *Porphyridium cruentum*. J Chem Technol Biotechnol. 2006;81:989–96. doi:10.1002/jctb.1503.

Huettmann H, Berkemeyer M, Buchinger W, Jungbauer A. Preparative crystallization of a single chain antibody using an aqueous two-phase system. Biotechnol Bioeng. 2014;111(11):2192–9. doi:10.1002/bit.25287.

Hye-Mee P, Sang-Woo L, Woo-Jin C, Yoon-Mo K. Affinity separation by protein conjugated IgG in aqueous two-phase systems using horseradish peroxidase as a ligand carrier. J Chromatogr B. 2007;856(1–2):108–12.

Johansson G. Affinity partitioning in aqueous two-phase systems. Affinity Sep. 2000;19:235–46. doi:10.1385/MB:19:3:269.

Knäblein J. Modern biopharmaceuticals: design, development and optimization. Weinheim: Wiley-VCH; 2005. p. 525–38.

Laboureau E, Vijayalakshmi MA. Concerning the separation of mammalian cells in immobilized metal ion affinity partitioning systems: a matter of selectivity. J Mol Recognit. 1998;10(1997):262–8.

Lam H, Kavoosi M, Haynes CA, Wang DIC, Blankschtein D. Affinity-enhanced protein partitioning in decyl β-D-glucopyranoside two-phase aqueous micellar systems. Biotechnol Bioeng. 2005;89(4):381–92. doi:10.1002/bit.20300.

Lihme A, Hansen M, Olander M, Zafirakos E. Expanded bed adsorption in the purification of biomolecules. In: Downstream processing of proteins: methods and protocols. 1st ed. Totowa: Humana Press Inc; 2000. p. 121–40.

Ling YQ, Nie HL, Su SN, Branford-White C, Zhu LM. Optimization of affinity partitioning conditions of papain in aqueous two-phase system using response surface methodology. Sep Purif Technol. 2010;73(3):343–8. doi:10.1016/j.seppur.2010.04.020.

Lu M, Tjerneld F. Interaction between tryptophan residues and hydrophobically modified dextran. Effect on partitioning of peptides and proteins in aqueous two-phase systems. J Chromatogr A. 1997;766(1–2):99–108.

Mannesse M, Cox R, Koops B, Verheij H, De Haas G, Egmond M, van der Hijden H, de Vlieg J. Cutinase from *Fusarium solani pisi* hydrolyzing triglyceride analogues: effect of acyl chain length and position in the substrate molecule on activity and enantioselectivity. Biochemistry. 1995;34:6400–7.

Oshima T, Oshima C, Baba Y. Selective extraction of histidine derivatives by metal affinity with a copper(II)-chelating ligand complex in an aqueous two-phase system. J Chromatogr B. 2015;990:73–9. doi:10.1016/j.jchromb.2015.03.023.

Park HM, Lee SW, Chang WJ, Koo YM. Affinity separation by protein conjugated IgG in aqueous two-phase systems using horseradish peroxidase as a ligand carrier. J Chromatogr B. 2007;856(1–2):108–12. doi:10.1016/j.jchromb.2007.05.036.

Persson A, Jergil B. The purification of membranes by affinity partitioning. FASEB J Off Publ Fed Am Soc Exp Biol. 1995;9(13):1304–10.

Ramakrishnan V, Goveas LC, Suralikerimath N, Jampani C, Halami PM, Narayan B. Extraction and purification of lipase from *Enterococcus faecium* MTCC5695 by PEG/phosphate aqueous-two

phase system (ATPS) and its biochemical characterization. Biocatal Agric Biotechnol. 2016;6: 19–27. doi:10.1016/j.bcab.2016.02.005.

Rito-Palomares M. Practical application of aqueous two-phase partition to process development for the recovery of biological products. J Chromatogr B. 2004;807(1):3–11. doi:10.1016/j.jchromb.2004.01.008.

Rocha MV, Di Giacomo M, Beltramino S, Loh W, Romanini D, Nerli BB. A sustainable affinity partitioning process to recover papain from *Carica papaya* latex using alginate as macro-ligand. Sep Purif Technol. 2016;168:168–76. doi:10.1016/j.seppur.2016.05.025.

Rosa PAJ, Azevedo AM, Ferreira IF, de Vries J, Korporaal R, Verhoef HJ, Aires-Barros MR. Affinity partitioning of human antibodies in aqueous two-phase systems. J Chromatogr A. 2007;1162(1 SPEC. ISS.):103–113. doi:10.1016/j.chroma.2007.03.067.

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. doi:10.1016/j.chroma.2012.04.077.

Schindler J, Nothwang HG. Aqueous polymer two-phase systems: effective tools for plasma membrane proteomics. Proteomics. 2006;6(20):5409–17. doi:10.1002/pmic.200600243.

Schügerl K, Hubbuch J. Integrated bioprocesses. Curr Opin Microbiol. 2005;8(3):294–300. doi:10.1016/j.mib.2005.01.002.

Silva MFF, Fernandes-Platzgummer A, Aires-Barros MR, Azevedo AM. Integrated purification of monoclonal antibodies directly from cell culture medium with aqueous two-phase systems. Sep Purif Technol. 2014;132:330–5. doi:10.1016/j.seppur.2014.05.041.

Silvério SC, Madeira PP, Rodríguez O, Teixeira JA, Macedo EA. ΔG(CH2) in PEG-salt and Ucon-Salt aqueous two-phase systems. J Chem Eng Data. 2008;53:1622–5.

Sousa AF, Andrade PZ, Pirzgalska RM, Galhoz TM, Azevedo AM, da Silva CL, Cabral JMS. A novel method for human hematopoietic stem/progenitor cell isolation from umbilical cord blood based on immunoaffinity aqueous two-phase partitioning. Biotechnol Lett. 2011;33(12):2373–7. doi:10.1007/s10529-011-0727-0.

Sturesson S, Tjerneld F, Johansson G. Partition of macromolecules and cell particles in aqueous two-phase systems based on hydroxypropyl starch and poly(ethylene glycol). Biotechnol Appl Biochem. 1990;26(3):281–95. doi:10.1007/BF02921507.

Teotia S, Gupta MN. Purification of phospholipase D by two-phase affinity extraction. J Chromatogr A. 2004;1025(2):297–301. doi:10.1016/j.chroma.2003.10.104.

Teotia S, Lata R, Gupta MN. Chitosan as a macroaffinity ligand: purification of chitinases by affinity precipitation and aqueous two-phase extractions. J Chromatogr A. 2004;1052(1–2):85–91. doi:10.1016/j.chroma.2004.08.096.

Wang S, Xiong N, Dong XY, Sun Y. A novel nickel-chelated surfactant for affinity-based aqueous two-phase micellar extraction of histidine-rich protein. J Chromatogr A. 2013;1320:118–24.

Wang S, Bai S, Dong XY, Sun Y. Partitioning behavior of enhanced green fluorescent protein in nickel-chelated affinity-based aqueous two-phase micellar system and its purification from cell lysate. Sep Purif Technol. 2014;133:149–54.

Wu Q, Lin DQ, Yao SJ. Evaluation of poly(ethylene glycol)/hydroxypropyl starch aqueous two-phase system for immunoglobulin G extraction. J Chromatogr B. 2013;928:106–12.

Wu H, Yao S, Qian G, Yao T, Song H. A resolution approach of racemic phenylalanine with aqueous two-phase systems of chiral tropine ionic liquids. J Chromatogr A. 2015;1418:150–7.

Yang Z, Mesiano AJ, Venkatasubramanian S, Gross SH, Harris JM, Russell AJ. Activity and stability of enzymes incorporated into acrylic polymers. J Am Chem Soc. 1995;117(17):4843–50. doi:10.1021/ja00122a014.

Zalipsky S. Functionalized poly(ethylene glycol) for preparation of biologically relevant conjugates. Bioconjug Chem. 1995;6(2):150–65. doi:10.1021/bc00032a002.

Aqueous Two-Phase Systems for the Recovery and Purification of Bioproducts from Plants and Vegetable Tissues

7

Oscar Aguilar

Abbreviations

ATPSs	Aqueous two-phase systems
CRISPR/Cas9	Clustered regularly interspaced short palindromic repeats and CRISPR-associated protein 9 system
DSP	Downstream processing
GRAS	Generally recognized as safe
HPLC	High-performance liquid chromatography
HSA	Human serum albumin
K_P	Partition coefficient
LC/MS	Liquid chromatography with mass spectrometry detector
mAbs	monoclonal antibodies
MW	Molecular weight
PEG	Polyethylene glycol
RuBisCO	Ribulose-1,5-bisphosphate carboxylase/oxygenase
TLL	Tie-line length
V_R	Volume ratio

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7.1 Introduction

During the last decades, several successful commercial recombinant proteins produced in plants have been released and reached the market, from β-glucuronidase, avidin, and laccase in maize to hepatitis B surface antigen in tobacco leaves and glucocerebrosidase from carrot (Pniewski 2013; Aviezer et al. 2009; Hood 2002). It has become patent that novel and efficient strategies for the extraction and purification of bioproducts from plant tissues are needed. One of the reasons why plants are attractive hosts for recombinant proteins is the possibility of glycosylation, an important feature in the case of therapeutics. With the recent outbreaks of deadly or potentially pandemic diseases, pharmaceutical companies and regulatory agencies are focusing its attention on plant-derived pharmaceuticals, given the potential to produce high yield of a recombinant protein in short periods of time and regionally for a fast response. Although the lack of regulatory aspects is still evident, the construction of large manufacturing facilities looks feasible with the use of inexpensive production infrastructure for the rapid production of plant-based pharmaceuticals (Fischer et al. 2015; Holaskova et al. 2015; Sack et al. 2015). This possibility to use green tissues for the constitutive expression of glycosylated therapeutic proteins represents a challenge for downstream processing given the high concentration of contaminant proteins and the presence of the highly abundant protein RuBisCO (Aguilar et al. 2009).

One of the process-related drawbacks for the general acceptance of plants as economically viable production systems is the lack of efficient initial concentration and separation procedures. The biotechnological industry has traditionally evolved around the unit operations derived from the chemical industry, in order to design purification schemes that meet the regulatory aspects for the approval. However, alternative strategies capable to deal with the particularities of plant-derived proteins should be considered. To facilitate the general acceptance of plants as bioreactors, the establishment of efficient downstream operations is critical. Any potential economic benefit obtained from the use of plant-derived bioreactors would be certainly nullified in the absence of an economically efficient downstream processing technology, creating a bottleneck in the production of an affordable commercial product (Buyel et al. 2015; Platis et al. 2008).

The use of aqueous two-phase systems (ATPS) for the primary recovery and purification of plant-derived products has been documented from several decades ago. However, even when in the case of microbial products, the success and advantages have been proved, the number of reports dealing with the actual use of ATPS technology for the recovery and purification of products from plants is still low, when compared with other sources. A clear exponential trend on the use of ATPS can be seen in Fig. 7.1, arising from the documented advantages of liquid-liquid partitioning. The number of articles related with the use of two-phase partitioning is showing a growing interest on the exploration of alternative strategies for downstream processing (DSP); now with the rising of the novel CRISPR/Cas system for more efficient genome editing, the future of recombinant proteins (not only for plants) looks promising. In the present chapter, the particular features of

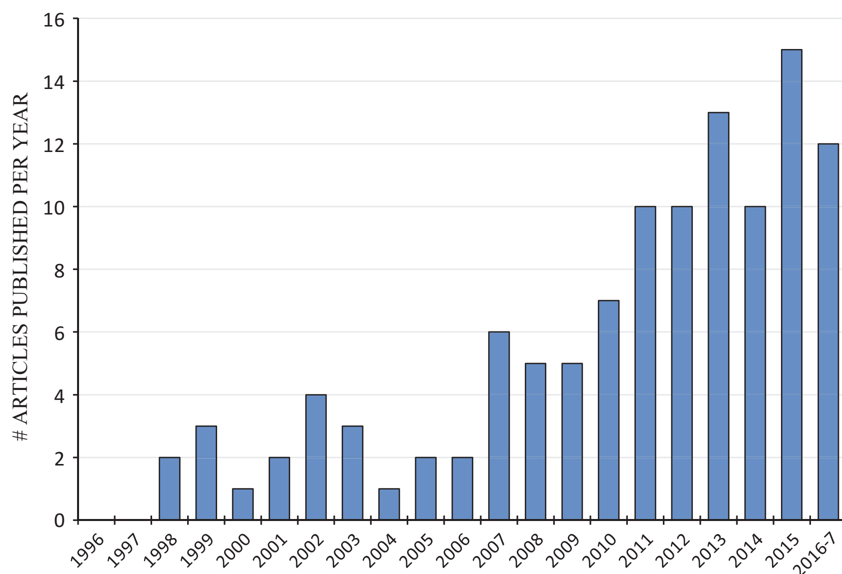

Fig. 7.1 Progress on the use of aqueous two-phase systems for the recovery and purification of plant-derived products within the last 20 years (Source: Scopus database (search criteria: ATPS, plants, extraction, purification))

plant-derived bioproducts will be addressed, as well as some selected examples of the use of this technology for the recovery and purification of proteins and other bioproducts from plants.

7.2 Particular Features of Plant Tissues

In the case of plants, one of the main issues during protein extraction is the presence of chlorophyll and other low molecular compounds related with the photosynthesis. They can act as interference compounds in many of the spectrophotometric and immunoaffinity techniques and for lipid and other pigment quantifications (Archanaa et al. 2012; Chen et al. 2012; Hua et al. 2012; Berges et al. 1993). This is a problem with particular importance when working with proteins expressed in green tissues or in general with leafy crops. Additional operations for removal of chlorophyll have been documented; however, the main disadvantage of them is the considerable loss of the desired bioproduct by the introduction of additional unit operations (Eze and Dumbroff 1982). New extraction techniques have to be developed to recover intact colorless protein fractions or chlorophyll-free extracts. Vázquez-Villegas has recently reported the use of a series of PEG/phosphate systems for the development of a chlorophyll-free protein extract from the aerial parts of alfalfa. Such results could be generalized for tissues of the same characteristics given that the majority of the proteins present in green tissues have similar characteristics (Vázquez-Villegas et al. 2015).

As in most of liquid-liquid partition experiments, the partition behavior of the major contaminants provides information to establish a purification strategy exploiting ATPS for the recovery of target products from a particular mixture. In this case, process conditions under which the product of interest and the contaminants concentrate in opposite phases must be established. Regarding the potential contaminants in green tissues, the subunits of the most abundant enzyme in nature, RuBisCO, represent the main contaminants found in this kind of samples. Several authors have reported the use of different kind of ATPS for the selective partitioning of RuBisCO subunits. An important feature to emphasize is the hydrophilic nature of such subunits, making them particularly susceptible to shifts in partitioning by small changes in ATP-forming components. Ionic liquids, as well as polymer-salt systems, have been reported in literature for the partitioning of proteins where RuBisCO is present as the main contaminant (Desai et al. 2014; Jacquet et al. 2014; Ibarra-Herrera et al. 2011; Aguilar et al. 2010).

In the case of seed-derived proteins, a different story can be told, since the majority of the proteins obtained from massive crops used (or potentially suggested) for recombinant protein production are those related with the storage proteins. These proteins are usually associated to energy storage compartments and, in some cases, comprise up to 70% of the total proteins from seeds. As in the case of soybean, several reports on the use of ATPS demonstrate that overall partitioning is mainly influenced by the partition behavior of the hydrophilic storage proteins. Aguilar demonstrated that the presence of these dominant storage proteins (the subunits of glycinins and β-conglycinin) and its multimers could make a purification strategy complicated given the case that a low-abundance recombinant or a native protein is desired (Aguilar et al. 2010). Similar results have been reported for the case of proteins from corn, a system widely used also as host for several recombinant proteins. The presence of the major storage proteins from corn kernel and endosperm drives partitioning of most of the contaminants (Gu and Glatz 2007). For a successful ATPS strategy, elucidating the partitioning behavior of plant proteins represents the first step to develop a primary recovery process to potentially separate a recombinant or native protein.

7.3 ATPS for Cell Sorting

One of the first uses of ATP partitioning was for the isolation and selection of cell organelles, as described by the original work of Per-Ake Albertsson in which he performed partitioning of bacteria and cell components using ATPS more than 60 years ago (Albertsson 1956). Since then, the use of ATPS has been an important tool in plant research not only for the isolation of cells, organelles, and subcellular fractions but also for understanding intracellular organization (Danielsson and Albertsson 2013; Pielak 2005; Åkerlund 2000).

The basis for separation in two-phase systems is the nature and extent of the interaction between the surface of the organelle or the cell and the polymer in the two-phase system. Typically, polymer/polymer systems are between the most

Table 7.1 Selected applications of polymer/polymer aqueous two-phase systems for isolation of plant subcellular components

Plant species	Use of ATPS	Plant tissue/ organelle	Ref.
Spinach (*Spinacia oleracea* L.)	Isolation and sorting of thylakoid vesicles	Thylakoid membranes	Danielsson and Albertsson (2009)
Strawberry cells	Continuous ATPS for cell isolation	Cultured cell aggregates	Yamada et al. (2004)
Soybean (*Glycine max*, var. L. Merr. Var. Williams)	Isolation of inside-out vesicles	Hypocotyl	Morré et al. (1998)
Tomato (*Lycopersicon esculentum*)	Isolation of plasma membrane fractions	Roots	Muchhal and Raghothama (1999)
Maize (*Zea mays* cv. Mutin)	Isolation of plasma membrane vesicles	Coleoptiles and primary leaves	Zettl et al. (1992)
Suspension-cultured tobacco (*Nicotiana tabacum*) cells	Enrichment and purification of microsomal membrane fraction	Microsomal membranes	Maurel et al. (1997)
Pea (*Pisum sativum* cv. Argona)	Purification of the symbiosomes	Infected root nodules	Hernández et al. (1996)
Oat roots and cauliflower inflorescences	Separation of plasma membrane vesicles	Microsomal fractions	Larsson et al. (1984)
Cultured strawberry cells (*Fragaria ananassa* cv. Shikinari)	Screening of high anthocyanin-producing cells	Cell cultures	Edahiro et al. (2005)

employed for cell sorting, since the mild hydrophobicity and lower ionic force are desired conditions in order to preserve organelles integrity and avoid dehydration or rupture of internal membranes. These partition methods nicely complement more traditional centrifugation techniques such as sucrose and cesium chloride gradients. There have been numerous reports on the use of two-phase partitioning for isolation and purification of membranes and vesicles from cell lysates (see a summary on Table 7.1).

ATPS using whole cell partitioning have been reported for bioconversions where the cells and reaction products concentrate in opposite phases (Liao et al. 1999). Commonly, the use of PEG/dextran systems has been reported as a method for isolation of membranes and vesicles. Several groups have documented the use of aqueous two-phase systems for the selective isolation of vacuolar and plasma membranes from several tissues, and high enrichment factors from crude fractions of greater than four- to fivefold have been reported, with significant reduction of contaminants from other cellular membranes (Maurel et al. 1997). The isolation of plant membranes is a key step in understanding the biochemical events occurring during nutrient absorption and metabolism, and it is the interphase between the cell wall and the cytoskeleton. For example, Hernández proposed a dextran T500/PEG 3350 two-phase partitioning method, for the recovery of the symbiosome, an organelle

involved in N_2 fixation from pea nodules (Hernández et al. 1996). In this particular case, the symbiosomes were recovered from the interface of the system, and the contaminant proteins were partitioned to both phases according to their hydrophobicity and charge; a single-step recovery was proposed as a feasible process for the recovery of this organelle, followed by re-solubilization of the interface as a homogeneous solution. The authors suggested the use of an ATPS strategy for the substitution of time-consuming density gradient methods using sucrose or Percoll. The intact organelles play a crucial part in the understanding of biochemical processes during the in vitro studies.

Selected examples of the use of polymer/polymer ATPS for the isolation of plant cell components involve PEG 3350/dextran including chloride and phosphate ions as modifiers of the ionic force to drive partitioning toward one of the phases (Larsson et al. 1984). Thylakoid membranes for chloroplast function studies require purification and isolation of vesicles and membrane fractions. Some preparations that have proved to be particularly suited for that purpose consist of membrane vesicles that were turned inside out. Åkerlund and Andersson documented the use of mechanical disruption and PEG/dextran ATPS for these purposes. Such procedure has been extensively adopted for the isolation of cell fragments from multiple species. This experimental protocol uses a PEG 3350/dextran T500 ATPS in the presence of sucrose, sodium phosphate, and sodium chloride operating as a countercurrent system, where inside-out vesicles partition mainly toward the lower phase, while the right side out partition toward the top phase (Åkerlund and Andersson 1983). The production of different metabolites in cultures cells has an influence on the surface properties of cell membranes, as demonstrated by Edahiro (2005). These researchers reported the differential partitioning of cultured strawberry cells in ATPS by modifying the system composition exploiting the external changes caused by internal changes in the intracellular secondary metabolism achieving a selective partitioning of high anthocyanin-accumulating cells from the rest of cell population. Further addition of lithium sulfate and potassium phosphate buffer shifted the partitioning behavior of cells from the bottom to the top phase. The negatively charged cells changed the partitioning according to their cell surface by adding lithium sulfate to the system. This caused a decrease in the electric potential of the system balancing the electrochemical and hydrophilic interactions between the cell surface and phase solution, a behavior reported not only for intact cells but also for proteins and other charge-susceptible biomolecules (Edahiro et al. 2005; Meychik and Yermakov 2001).

These reports show the potential use of ATPSs for selective partitioning of plant cells just based on the small differences on cell surface properties caused by a change in the production levels of metabolites. A simple and rapid method could be developed for the selection of transformed cell lines with minimal damage and exploiting differences on the cell membrane properties (Edahiro et al. 2005; Yamada et al. 2004). Recent strategies include the use of free-flow electrophoresis combined with ATPS for the production of a proteomic library of membrane-associated proteins from *Arabidopsis*. This is a crucial species widely used for plant proteome

studies, cell signaling, and protein expression and particularly challenging due to heterogeneity, low abundance, and high dependence on the biotic and abiotic factors of this species (De Michele et al. 2016; Yadeta et al. 2013).

7.4 Primary Recovery and Purification

Since the first use of plant tissue cultures for the production of recombinant proteins, such as human serum albumin (HSA) in potato and tobacco, there has been an increasing interest of the biopharmaceutical industry to exploit plants as economically viable production systems (Sijmons et al. 1990). This interest is demanding the development of new downstream strategies to maximize product recovery.

As mentioned before, the presence of highly abundant contaminants, the RuBisCO subunits, the storage proteins in the case of seeds, or polyphenols and other molecules such as the chlorophylls, makes the recovery and purification of bioproducts from plants particularly challenging. Numerous reports have demonstrated the potential application of ATPS-based strategies to address the major disadvantages of the traditional recovery and purification techniques. The use of ATPS for the recovery and purification from plant tissues follows a similar procedure than previously reported for different hosts (Benavides and Rito-Palomares 2008; Gu and Glatz 2007; Aguilar et al. 2006; Zhang et al. 2001).

The practical strategies for the development of primary recovery processes can be divided into four main stages:

(i) Initial physicochemical characterization of the feedstock
(ii) Selection of the type of ATPS (polymer/salt/ionic liquid/alcohol)
(iii) Selection of system parameters (TLL, pH, V_R, sample load)
(iv) Evaluation of the influence of process parameters upon product recovery/purity (Aguilar and Rito-Palomares 2014)

As widely reported for microbial or other bioproducts, system parameters such as molecular weight (MW) of polymer, salt composition, volume ratio (V_R), pH, and sample load have a great impact on the protein distribution (K_p, partition coefficient) and total yield (Gu 2014).

The reported procedures in literature focus on the selection of the type of ATPS to maximize recovery for a particular protein from protein feedstock, providing that certain knowledge exists regarding the nature of the contaminant proteins. Some research groups dedicate an extra set of experiments to characterize the behavior of contaminants; this could allow the generalization of the ATPS strategy for several target products expressed in the same host or hosts with similar proteome (Espitia-Saloma et al. 2016; Ibarra-Herrera et al. 2011; Aguilar et al. 2010; Azevedo et al. 2009; Joensuu et al. 2009).

The integrated extractive partitioning of proteins using ATPS has been demonstrated for a wide variety of hosts using different model recombinant proteins expressed in soybean, maize, and tobacco (Aguilar and Rito-Palomares 2008; Gu and Glatz 2007; Platis and Labrou 2006). These ATPS-based techniques represent

an attractive alternative to facilitate the adoption of bioprocesses using plants as biofactories. Several studies have explored strategies to eliminate the majority of the plant contaminants and prepare the target protein for further refining procedures or by eliminating the main contaminants present, such as in the case of leafy crop processing (Vázquez-Villegas et al. 2015; Babu et al. 2008; Vaidya et al. 2006; De Fátima et al. 2005).

It is generally recognized that ATPS allows processing of samples with high debris content and particulate material; this has been demonstrated by several authors, not only for plant extracts but also from microbial samples. Complex samples, such as potato tuber extracts with high carbohydrate content, as well as potato peels and carrots, demonstrate the robustness of aqueous two-phase extraction for the primary extraction of bioproducts, prior to further purification operations. The before-mentioned reports include very different phase-forming components from PEG/phosphate to alcohol/ammonium sulfate for the recovery of proteins from highly different samples, such as tubers, seeds, and green tissues (Sánchez-Rangel et al. 2014; Duman and Kaya 2013; Ibarra-Herrera et al. 2011; Aguilar and Rito-Palomares 2008).

The proposed easiness and scalability of ATPS-based processes have been documented before and gave place to a large number of scientific reports of the use of this technology for the recovery of plant-derived compounds. Aguilar and Rito-Palomares reported the use of a PEG 600/phosphate system comprising 14.5% (w/w) polyethylene glycol (PEG) and 17.5% (w/w) phosphate, for the potential recovery of up to 80% of recombinant glucuronidase from a complex mixture of soybean proteins. The use of such partitioning system demonstrated the potential of ATPS for processing a highly concentrated protein extract obtained by isoelectric precipitation with loads of up to 40% of the system composition (Aguilar and Rito-Palomares 2008). The in situ extraction offers the possibility of decreasing unit operations, such as in the case suggested by Sánchez-Rangel, where the ionic liquids and phosphate buffers were used to form ATPS and could be integrated to the production system for the extraction of different profiles of phenolic compounds (Sánchez-Rangel et al. 2014). The use of ATPS for the extraction of low molecular weight compounds has been extensively documented, not only from plants but also from native and recombinant microbial sources. A wide variety of ATPS compositions reflect the versatility of this technique for primary extraction, sample cleanup, secondary metabolite profiling, etc. Selected examples are listed in Table 7.2.

In most of the referred methods, the ATP-based extraction method was suggested as a "greener" procedure for bioactive ingredients from plants (Tan et al. 2010), for the cleanup of the major contaminants from green tissues (Vázquez-Villegas et al. 2015), as a rapid and effective pretreatment technique for further analytical characterization methods (Li et al. 2005), or in some other cases as an efficient recovery methodology for large-scale recovery of metabolites for production of industrially relevant compounds (Sánchez-Rangel 2014).

In the case of protein recovery and purification, the use of ATP-based extraction has been widely documented for a large number of proteins and peptides (Table 7.3). One of the main drawbacks on the use of this technology is the mandatory removal

Table 7.2 Primary recovery of low molecular weight products isolated using ATPS from plants

Product	Type of ATPS	Plant source	Ref.
Gallic acid	Alcohol/potassium phosphate PEG/potassium phosphate	Guava	Reis et al. (2014a)
Rutin	Alcohol/salt	Acerola	Reis et al. (2014b)
Crocins	Alcohol/potassium phosphate	Saffron stigmas	Montalvo-Hernández et al. (2012)
Polysaccharides	Ionic liquids/salts	*Aloe vera*	Tan et al. (2012)
Alkaloids	Ionic liquids/salts	*Pericarpium papaveris*	Li et al. (2005)
Phenolic compounds	Ethanol/potassium phosphate	*Ficus carica*	Feng et al. (2015)
Chlorogenic acid	Ionic liquids/potassium phosphate	Wounded carrot (*Daucus carota*)	Sánchez-Rangel et al. (2014)
Chlorophylls	PEG/potassium phosphate	Alfalfa (*Medicago sativa*)	Vázquez-Villegas et al. (2015)
Puerarin	PEG/ammonium sulfate	*Pueraria* sp. extracts	Bi et al. (2010)

of polymer and salts involved in phase formation which in some cases could be difficult due to polymer viscosity and/or the size of the biomolecule of interest compared to the polymer molecular size. However, promising results have been reported with high yields achieved in a single or multistep mode, making this technology quite comparable with traditional chromatographic methods.

Some advantages are evident and widely recognized on the use of ATPS as a purification strategy; fractionation and – in some cases – the elimination of primary extraction are among them, as suggested by the results of Truust where wheat proteins were fractionated by countercurrent distribution (CCD) using a polymer-polymer ATPS. In this case a complex protein extract obtained directly from suspension of flour into an adequate buffer was the only previous step for solubilization of the proteins (Truust and Johansson 1996). In a similar manner, reports from Gu and Glatz and Guimarães and Nascimento employed polymer/salt ATPS for the direct purification of industrially relevant enzymes and proteins directly from suspensions of the seed material with minimal or no previous step besides re-solubilization (Nascimento et al. 2010; Guimarães et al. 2001; Truust and Johansson 1996).

The elimination of important contaminants in the single purification step has been an important issue in the case of purification of proteins from plant materials, particularly for further purification steps or coupling with other molecular techniques. The use of ATPS for the removal of polyphenols and other alkaloids has been recently documented by Platis. A tri-step process includes ATPS as a first step for the purification of two anti-HIV monoclonal antibodies from a clarified tobacco protein extract. Optimal conditions reported included a system with 18.5% w/w PEG 1500, 9% w/w phosphate at pH 5 with a V_R = 2.3, and 7.5% w/w transgenic tobacco extract load. Scaling of these conditions allowed 90% recovery with 2.05-fold purification of monoclonal antibody (mAb) 2G12 (Platis et al. 2008).

Table 7.3 Some uses of ATPS for the recovery and purification of proteins from plants and plant tissues

Plant species	ATPS	Product	Results	Ref.
Nicotiana benthamiana	Saline buffer/Triton X-114 micellar ATPS	Hemagglutinin A(H1N1) H1-HFBI	Low-phase recovery 70% Overall purity 50%	Jacquet et al. (2014)
Pineapple (*Ananas comosus* L. *Merr*)	PEG 1500/phosphate	Bromelain and polyphenol oxidase	228% activity recovery – 4.0-fold purity (bromelain) 90% activity recovery – 2.7-fold purity (PPO)	Babu et al. (2008)
Potato tubers (*Solanum tuberosum*)	Three-phase system ammonium sulfate/*t*-butanol	Catalase	Interphase recovery 262% with 14.1 purification factor	Duman and Kaya (2013)
Potato (*Solanum tuberosum*)	PEG 8000/phosphate	Polyphenol oxidase	97.0% yield – 15.7 purification factor	Vaidya et al. (2006)
Canavalia brasiliensis seeds	PEG 600/phosphate w. 4.7% NaCl	ConBr lectin	Complete recovery with 73% purity	Nascimento et al. (2010)
Corn (endosperm)	PEG 1450/Na_2SO_4/NaCl	Cytochrome c[1]	93% recovery – 4.7-fold purity (endosperm) 100% recovery – 9.1-fold purity (germ)	Gu and Glatz (2007)
Soybean (*Glycine max*, germinated)	PEG 1500/phosphate/NaCl	α-Galactosidases	70% activity recovery – 12.7 purification fold	Guimarães et al. (2001)
Mango peel (*Mangifera indica* Chokanan)	PEG8000/dextran	Serine protease	97.3% yield, 14.37 purification factor	Mehrnoush et al. (2012)
Sandalwood somatic embryo	PEG 6000/$(NH_4)_2SO_4$	Peroxidase and arabinogalactan proteins from spent medium	92% recovery, 2.16-fold purity for POD	Pal et al. (2003)

Tobacco	PEG 3400/ Na_2SO_4/ NaCl	Lysozyme[1]	87% recovery – 4-fold purity predicted by surface response design	Balasubramaniam et al. (2003)
Pericarpium papaveris	PEG 4000/ $(NH_4)_2SO_4$	Papaverin	93.9% recovery – 11.0 enrichment factor	Cao et al. (2007)
Tobacco	PEG 1500/phosphate	mAb 2G12	90% yield – 2.05-fold purity	Platis et al. (2008)
Wheat	PEG 2000/ fructose-1,6-bisphosphate	α-Amylase inhibitors	79% recovery – 3.2-fold purity	Chen et al. (2008)
Soybean (*Glycine max*)	PEG 4000/PEG–IDA–Cu^{2+}/Na_2SO_4; then PEG 4000/PEG–IDA–Cu^{2+}/phosphate	Peroxidase	64% yield – 145-fold purity	Silva and Franco (2000)
Ipomoea palmata leaves	PEG 1550/phosphate/ NaCl	Peroxidase	93% yield – 3.4-fold purity	Srinivas et al. (2002)
Carica papaya latex	PEG 6000/$(NH_4)_2SO_4$	Papain	88% recovery, purification fold not reported	Nitsawang et al. (2006)
Waste potato peels	PEG 1500/potassium phosphate	Polyphenol oxidase	77.8% yield with 4.5 purification factor	Niphadkar et al. (2015)

Purified enzymes as processing aids for the production of animal feedstock and human food require the elimination of certain antinutrients from food material. The trend of the market for non-GMO or minimally processed food has driven the market for processing aids – such as GRAS enzymes – from nonrecombinant sources to be incorporated into production processes (Aguilar and Hernández-Brenes 2015). De Fátima reported the use of aqueous two-phase extraction for the partial purification of α-galactosidase from germinating soybean seeds (De Fátima et al. 2005). These findings suggested an alternative enzymatic process to eliminate raffinose oligosaccharides from soybean-derived products in substitution of non-GRAS microbial or recombinant enzymes in order to facilitate the approval of an enzymatic process for treatment of soymilk or soybean flour. Phytase is an excellent not very well-explored example of a potential enzyme that could be recovered from waste plant materials at a relatively low cost. Given its importance as an additive for the production of feedstock with vegetable protein (with better phosphorous digestibility) for farm animals (Poulsen et al. 2010), in this matter, Mehrnoush suggests the utilization of the industrial waste mango peels (*Mangifera indica*) for the recovery of serine proteases reporting close to 100% recovery and 14.4 purification factor using a PEG/dextran ATPS, although their claim of a fast and scalable process based on ATPS could be debatable without further actual large-scale approaches (Mehrnoush et al. 2012).

7.5 Trends and Challenges

With the advancement on genome editing tools such as CRISPR/Cas9 system (Bortesi and Fischer 2015; Feng et al. 2013; Shan et al. 2013), the future of recombinant technology will require similar advancements in the field of downstream processing. Alternative and integrated technologies with intensification capacities will be necessary to avoid more restrictions in the production pipelines than already are the DSP operations. In this regard, ATPS technology will have to offer fast and reliable methods to be easily integrated into the purification train and capable to fit within a series of traditional unit operations such as HPLC.

Typically, the development of an ATPS-based process, regardless of the origin, implies the careful selection of the operation conditions that favor partition toward one of the phases; this is a major challenge regarding the large amount of experimental data that usually takes to determine those optimal conditions. The design of experiment strategy has been a common approach that has been largely documented not only for plants but for any type of bioproducts from any origin (Zimmermann et al. 2016; Vázquez-Villegas et al. 2015; Mehrnoush et al. 2012; Nascimento et al. 2010). The rational selection of the optimal combinations of pH, sample load, TLL, type of polymer and/or salt, etc. has to be studied following miniaturization and high-throughput methods, in order to speed up the process design. Several research groups worldwide are exploring these strategies, reporting promising results for the recovery and purification of bioproducts. Pioneer groups are proposing the use of

microfluidics for the process design and also for bimodal curve estimation, an exhaustive process necessary to explore new two-phase-forming compounds. Although it may require some additional equipment for the flow control and partition monitoring, considerable time savings have been documented, making this strategy an attractive promising way to speed up the development of plant-derived biopharmaceuticals from plants (Vázquez-Villegas et al. 2016; Soares et al. 2014, 2016; Diederich et al. 2013).

The historical and general use of traditional phase-forming salts and polymers for the recovery of proteins from plants has demonstrated practical for many cases, even in combination with low molecular weight alcohols in alcohol/salt ATPS. However, the generic potential application of this technique demands the evaluation of alternative phase-forming compounds. In this context, earlier efforts explored the use of several polymers, such as starch derivatives, maltodextrins, and cellulose derivatives, as an alternative to dextran or salts as phase-forming phases (Szlag and Guiliano 1988; Venancio et al. 1993; Skuse et al. 1992). From several points of view and economical and practical aspects, the high cost of dextran polymers limits its general application; besides when working with sensitive biological structures and proteins, PEG-salt systems have shown limited use due to the high ionic force of the solutions. A recent approach to address this problem includes the use of alternative polymers and copolymers to substitute dextran or even PEG by using nonionic surfactants (such as Triton or Tween). An extensive amount of reports suggests the use of ionic liquids or alcohol/salt systems, particularly for low molecular weight products, or the use of polyacrylic acid to substitute high salt concentrations (Chávez-Castilla and Aguilar 2016; Dong et al. 2012; Tan et al. 2012; Saravanan et al. 2008).

The precise knowledge of the mechanisms that governs phase formation and ultimately drives biomolecule partitioning still hinders its use at the large scale despite the efforts of several groups on the establishment of continuous processes, in order to make this technology more attractive for the industrial world. The lack of adequate and predictable scalability parameters makes this research area a challenge that still needs to be explored and further documented given the proven ability of continuous ATPS to recover proteins and other biomolecules in higher efficiencies, with higher partition coefficients and short processing times (Espitia-Saloma et al. 2016).

The recent applications of aqueous two-phase partitioning coupled with analytical tools such as 2D electrophoresis, LC/MS, and high-speed countercurrent chromatography for the molecular characterization of complex plant extracts will impact the development of bioprocesses based on plants as production systems as well as a broad application of ATPS (Buyel et al. 2015; Aguilar et al. 2010; Bi et al. 2010). The use of 3D mapping for analyzing protein profiles allows the identification of the molecular properties from the main contaminant proteins. With these novel strategies that combine existing powerful techniques with the ATPS benefits, the predictive design of bioprocess to recover high-value products from plant origin could trigger its industrial application.

7.6 Concluding Remarks

Since the discovery of the extractive properties of two-phase systems, its use and applications have been closely related to plant-derived bioproducts. The first documented uses of ATP technology were for the isolation of cell membranes and organelles, a procedure still with remarkable uses for molecular studies on the biochemical events occurring at the subcellular level. Nowadays a large collection of bioproducts have been isolated and purified from different expression systems employing a myriad of ATPS formed with water-soluble compounds including low molecular weight alcohols, synthetic and natural polymers, salts, ionic liquids, and surfactants. For the particular case of plant-derived products, the use of ATPS resulted attractive for the production of fractionated extracts and as a primary extraction strategy, to deplete the highly abundant products typically present in plant-derived extracts: chlorophyll, RuBisCO subunits, and major storage proteins in the case of seed materials. The versatility of ATPS formulations relies on the fact that minimal changes on the phase-forming component concentration or composition may affect the desired product affinity or preference for any of the phases, allowing its use for a wide variety of bioproducts, from membranes and organelles to peptides and proteins or low molecular weight compounds. Such versatility also benefits the potential uses of ATPS; given the complexity of plant-derived extracts, they can be exploited for the recovery of value-added products from industrial waste or low-abundance recombinant proteins from genetically modified plants. In either case, this ATPS technology could encourage the establishment of greener bioprocesses based on the use of plants or plant materials as biofactories.

References

Aguilar O, Hernández-Brenes C. Use of modified phenolic thyme extracts (*Thymus vulgaris*) with reduced polyphenol oxidase substrates as anthocyanin color and stability enhancing agents. Molecules. 2015;20(12):22422–34.

Aguilar O, Rito-Palomares M. Processing of soybean (*Glycine max*) extracts in aqueous two-phase systems as a first step for the potential recovery of recombinant proteins. J Chem Technol Biotechnol. 2008;83(3):286–93.

Aguilar O, Rito-Palomares M. Aqueous two-phase system strategies for the recovery of proteins from plants. In: Labrou NE, editor. Protein downstream processing, vol. 1129. New York: Humana Press; 2014.

Aguilar O, Albiter V, Serrano-Carreón L, Rito-Palomares M. Direct comparison between ion-exchange chromatography and aqueous two-phase processes for the partial purification of penicillin acylase produced by *E. coli*. J Chromatogr B. 2006;835(1–2):77–83.

Aguilar O, Glatz C, Rito-Palomares M. Characterization of green-tissue protein extract from alfalfa (*Medicago sativa*) exploiting a 3-D technique. J Sep Sci. 2009;32(18):3223–31.

Aguilar O, Rito-Palomares M, Glatz C. Coupled application of aqueous two-phase partitioning and 2D-electrophoresis for characterization of soybean proteins. Sep Sci Technol. 2010; 45(15):2210–25.

Åkerlund HE. Partition by countercurrent distribution (CCD). In: Hatti-Kaul R, editor. Methods in biotechnology. Vol 11: aqueous two-phase systems, methods and protocols. New York: Humana Press; 2000.

Åkerlund H, Andersson B. Quantitative separation of spinach thylakoids into photosystem II-enriched inside-out vesicles and photosystem I-enriched right-side-out vesicles. Biochim Biophys Acta. 1983;725(1):34–40.

Albertsson P. Chromatography and partition of cells and cell fragments. Nature. 1956;177(4513): 771–4.

Archanaa S, Moise S, Suraishkumar G. Chlorophyll interference in microalgal lipid quantification through the Bligh and Dyer method. Biomass Bioenergy. 2012;46:805–8.

Aviezer D, Brill-Almon E, Shaaltiel Y, Hashmueli S, Bartfeld D, Mizrachi S, Liberman Y, Freeman A, Zimran A, Galun E. A plant-derived recombinant human Glucocerebrosidase enzyme – a preclinical and phase I investigation. PLoS One. 2009;4(3):e4792.

Azevedo A, Gomes A, Rosa P, Ferreira I, Pisco A, Aires-Barros M. Partitioning of human antibodies in polyethylene glycol–sodium citrate aqueous two-phase systems. Sep Purif Technol. 2009;65(1):14–21.

Babu B, Rastogi N, Raghavarao K. Liquid–liquid extraction of bromelain and polyphenol oxidase using aqueous two-phase system. Chem Eng Process. 2008;47(1):83–9.

Balasubramaniam D, Wilkinson C, Cott KV, Zhang C. Tobacco protein separation by aqueous two-phase extraction. J Chromatogr A. 2003;989(1):119–29.

Benavides J, Rito-Palomares M. Practical experiences from the development of aqueous two-phase processes for the recovery of high value biological products. J Chem Technol Biotechnol. 2008;83(2):133–42.

Berges J, Fisher A, Harrison P. A comparison of Lowry, Bradford and Smith protein assays using different protein standards and protein isolated from the marine diatom *Thalassiosira pseudonana*. Mar Biol. 1993;115(2):187–93.

Bi P, Dong H, Yuan Y. Application of aqueous two-phase flotation in the separation and concentration of puerarin from Puerariae extract. Sep Purif Technol. 2010;75(3):402–6.

Bortesi L, Fischer R. The CRISPR/Cas9 system for plant genome editing and beyond. Biotechnol Adv. 2015;33(1):41–52.

Buyel J, Twyman R, Fischer R. Extraction and downstream processing of plant-derived recombinant proteins. Biotechnol Adv. 2015;33(6):902–13.

Cao Q, Li S, He C, Li K, Liu F. Extraction and determination of papaverin in *pericarpium papaveris* using aqueous two-phase system of poly(ethylene glycol)–(NH4)2SO4 coupled with high-performance liquid chromatography. Anal Chim Acta. 2007;590(2):187–94.

Chávez-Castilla LR, Aguilar O. An integrated process for thein situ recovery of prodigiosin using micellar ATPS from a culture of *Serratia marcescens*. J Chem Technol Biotechnol. 2016;91(11):2896–903.

Chen X, Xu G, Li X, Li Z, Ying H. Purification of an α-amylase inhibitor in a polyethylene glycol/fructose-1,6-bisphosphate trisodium salt aqueous two-phase system. Process Biochem. 2008;43(7):765–8.

Chen Z, Liu J, Zeng M, Wang Z, Yu D, Yin C, et al. Dot immunobinding assay method with chlorophyll removal for the detection of southern rice black-streaked Dwarf virus. Molecules. 2012;17(12):6886–900.

Danielsson R, Albertsson P. Fragmentation and separation analysis of the photosynthetic membrane from spinach. Biochim Biophys Acta. 2009;1787(1):25–36.

Danielsson R, Albertsson P. Aqueous polymer two-phase systems and their use in fragmentation and separation of biological membranes for the purpose of mapping the membrane structure. Prep Biochem Biotechnol. 2013;43(5):512–25.

De Fátima S, Monteze V, Chamel I, de Almeida M, Brunoro NM, Gonçalves E, Alves M, Tavares S. Hydrolysis of oligosaccharides in soybean flour by soybean α-galactosidase. Food Chem. 2005;93(4):665–70.

De Michele R, McFarlane H, Parsons H, Meents M, Lao J, González Fernández-Niño S, Petzold CJ, Frommer WB, Samuels AL, Heazlewood JL. Free-flow electrophoresis of plasma membrane vesicles enriched by two-phase partitioning enhances the quality of the proteome from Arabidopsis seedlings. J Proteome Res. 2016;15(3):900–13.

Desai R, Streefland M, Wijffels RH, Eppink MHM. Extraction and stability of selected proteins in ionic liquid based aqueous two phase systems. Green Chem. 2014;16(5):2670–9.

Diederich P, Amrhein S, Hämmerling F, Hubbuch J. Evaluation of PEG/phosphate aqueous two-phase systems for the purification of the chicken egg white protein avidin by using high-throughput techniques. Chem Eng Sci. 2013;104:945–56.

Dong Y, Zhang F, Wang Z, Du L, Hao A, Jiang B, Tian M, Li Q, Jia Q, Wang S, Xiu Z. Extraction and purification of recombinant human serum albumin from *Pichia pastoris* broths using aqueous two-phase system combined with hydrophobic interaction chromatography. J Chromatogr A. 2012;1245:143–9.

Duman YA, Kaya E. Three-phase partitioning as a rapid and easy method for the purification and recovery of catalase from sweet potato tubers (*Solanum tuberosum*). Appl Biochem Biotechnol. 2013;170:1119–26.

Edahiro J, Yamada M, Seike S, Kakigi Y, Miyanaga K, Nakamura M, et al. Separation of cultured strawberry cells producing anthocyanins in aqueous two-phase system. J Biosci Bioeng. 2005;100(4):449–54.

Espitia-Saloma E, Vâzquez-Villegas P, Rito-Palomares M, Aguilar O. An integrated practical implementation of continuous aqueous two-phase systems for the recovery of human IgG: from the microdevice to a multistage bench-scale mixer-settler device. Biotechnol J. 2016;11(5):708–16.

Eze J, Dumbroff E. A comparison of the Bradford and Lowry methods for the analysis of protein in chlorophyllous tissue. Can J Bot. 1982;60(7):1046–9.

Feng Z, Zhang B, Ding W, Liu X, Yang D-L, Wei P, et al. Efficient genome editing in plants using a CRISPR/Cas system. Cell Res. 2013;23(10):1229–32.

Feng Y, Li W, He F, Kong T, Huang X, Gao Z, Lu N, Li H. Aqueous two-phase system as an effective tool for purification of phenolic compounds from fig fruits (*Ficus carica* L.). Sep Sci Technol. 2015;50(12):1785–93.

Fischer R, Vasilev N, Twyman RM, Schillberg S. High-value products from plants: the challenges of process optimization. Curr Opin Biotechnol. 2015;32:156–62.

Gu Z. Recovery of recombinant proteins from plants using aqueous two-phase partitioning systems: an outline. In: Labrou NE, editor. Protein downstream processing, vol. 1129. New York: Humana Press; 2014.

Gu Z, Glatz C. Aqueous two-phase extraction for protein recovery from corn extracts. J Chromatogr B. 2007;845(1):38–50.

Guimarães VM, Rezende STD, Moreira MA, Barros EGD, Felix CR. Characterization of α-galactosidases from germinating soybean seed and their use for hydrolysis of oligosaccharides. Phytochemistry. 2001;58(1):67–73.

Hernández L, Rojas-Ojeda P, Cooke D, Carpena-Ruiz R. Purification of pea nodule symbiosomes using an aqueous polymer two-phase system. J Chromatogr B. 1996;680(1–2):171–81.

Holaskova E, Galuszka P, Frebort I, Oz MT. Antimicrobial peptide production and plant-based expression systems for medical and agricultural biotechnology. Biotechnol Adv. 2015;33(6):1005–23.

Hood E. From green plants to industrial enzymes. Enzym Microb Technol. 2002;30(3):279–83.

Hua X, Yang J, Wang L, Fang Q, Zhang G, Liu F. Development of an enzyme linked immunosorbent assay and an immunochromatographic assay for detection of organophosphorus pesticides in different agricultural products. PLoS One. 2012;7(12):e53099.

Ibarra-Herrera C, Aguilar O, Rito-Palomares M. Application of an aqueous two-phase systems strategy for the potential recovery of a recombinant protein from alfalfa (*Medicago sativa*). Sep Purif Technol. 2011;77(1):94–8.

Jacquet N, Navarre C, Desmecht D, Boutry M. Hydrophobin fusion of an influenza virus hemagglutinin allows high transient expression in *Nicotiana benthamiana*, easy purification and immune response with neutralizing activity. PLoS One. 2014;9(12):e115944.

Joensuu J, Conley A, Lienemann M, Brandle J, Linder M, Menassa R. Hydrophobin fusions for high-level transient protein expression and purification in Nicotiana benthamiana. Plant Physiol. 2009;152(2):622–33.

Larsson C, Kjellbom P, Widell S, Lundborg T. Sidedness of plant plasma membrane vesicles purified by partitioning in aqueous two-phase systems. FEBS Lett. 1984;171(2):271–6.

Li S, He C, Liu H, Li K, Liu F. Ionic liquid-based aqueous two-phase system, a sample pre-treatment procedure prior to high-performance liquid chromatography of opium alkaloids. J Chromatogr B. 2005;826(1–2):58–62.

Liao L, Ho C, Wu W. Bioconversion with whole cell penicillin acylase in aqueous two-phase systems. Process Biochem. 1999;34(5):417–20.

Maurel C, Tacnet F, Guclu J, Guern J, Ripoche P. Purified vesicles of tobacco cell vacuolar and plasma membranes exhibit dramatically different water permeability and water channel activity. Proc Natl Acad Sci U S A. 1997;94(13):7103–8.

Mehrnoush A, Mustafa S, Sarker MZI, Yazid AMM. Optimization of serine protease purification from mango (*Mangifera indica* cv. Chokanan) peel in polyethylene glycol/dextran aqueous two phase system. Intl J Mol Sci. 2012;13(12):3636–49.

Meychik NR, Yermakov IP. Ion-exchange properties of cell walls isolated from lupine roots. Biochem Mosc. 2001;66:556–63.

Montalvo-Hernández B, Rito-Palomares M, Benavides J. Recovery of crocins from saffron stigmas (*Crocus sativus*) in aqueous two-phase systems. J Chromatogr A. 2012;1236:7–15.

Morré D, Peter A, Morré D, Van Alstine J. Effect of centrifugation on separation by aqueous two-phase partition of an early and late endosome model using inside-out plasma membrane vesicles from plants. J Chromatogr B. 1998;711(1–2):195–201.

Muchhal U, Raghothama K. Transcriptional regulation of plant phosphate transporters. Proc Natl Acad Sci U S A. 1999;96(10):5868–72.

Nascimento K, Rosa P, Nascimento K, Cavada B, Azevedo A, Aires-Barros M. Partitioning and recovery of *Canavalia brasiliensis* lectin by aqueous two-phase systems using design of experiments methodology. Sep Purif Technol. 2010;75(1):48–54.

Niphadkar SS, Vetal MD, Rathod VK. Purification and characterization of polyphenol oxidase from waste potato peel by aqueous two-phase extraction. Prep Biochem Biotechnol. 2015;45(7):632–49.

Nitsawang S, Hatti-Kaul R, Kanasawud P. Purification of papain from *Carica papaya* latex: aqueous two-phase extraction versus two-step salt precipitation. Enzym Microb Technol. 2006;39(5):1103–7.

Pal S, Das S, Dey S. Peroxidase and arabinogalactan protein as by-products during somatic embryo cultivation in air-lift bioreactor. Process Biochem. 2003;38(10):1471–7.

Pielak G. A model of intracellular organization. Proc Natl Acad Sci U S A. 2005;102(17):5901–2.

Platis D, Labrou N. Development of an aqueous two-phase partitioning system for fractionating therapeutic proteins from tobacco extract. J Chromatogr A. 2006;1128(1–2):114–24.

Platis D, Drossard J, Fischer R, Ma J-C, Labrou N. New downstream processing strategy for the purification of monoclonal antibodies from transgenic tobacco plants. J Chromatogr A. 2008;1211(1–2):80–9.

Pniewski T. The twenty-year story of a plant-based vaccine against hepatitis B: stagnation or promising prospects? Int J Mol Sci. 2013;14(1):1978–98.

Poulsen H, Carlson D, Nørgaard J, Blaabjerg K. Phosphorus digestibility is highly influenced by phytase but slightly by calcium in growing pigs. Livest Sci. 2010;134(1–3):100–2.

Reis I, Campos A, Santos P, Santos S, Soares C, Lima Á. Potassium phosphate salts-based aqueous two-phase systems applied in the extraction of Gallic acid from guava. Sep Sci Technol. 2014a;50(4):520–8.

Reis I, Santos S, Pereira F, Sobral C, Freire M, Freitas L, Soares CMF, Lima A. Extraction and recovery of Rutin from acerola waste using alcohol-salt-based aqueous two-phase systems. Sep Sci Technol. 2014b;49(5):656–63.

Sack M, Hofbauer A, Fischer R, Stoger E. The increasing value of plant-made proteins. Curr Opin Biotechnol. 2015;32:163–70.

Sánchez-Rangel J, Jacobo-Velázquez D, Cisneros-Zevallos L, Benavides J. Primary recovery of bioactive compounds from stressed carrot tissue using aqueous two-phase systems strategies. J Chem Technol Biotechnol. 2014;91(1):144–54.

Saravanan S, Rao JR, Nair BU, Ramasami T. Aqueous two-phase poly(ethylene glycol)–poly (acrylic acid) system for protein partitioning: influence of molecular weight, pH and temperature. Process Biochem. 2008;43(9):905–11.

Shan Q, Wang Y, Li J, Zhang Y, Chen K, Liang Z, et al. Targeted genome modification of crop plants using a CRISPR-Cas system. Nat Biotechnol. 2013;31(8):686–8.

Sijmons P, Dekker B, Schrammeijer B, Verwoerd T, van den Elzen P, Hoekema A. Production of correctly processed human serum albumin in transgenic plants. Nat Biotechnol. 1990;8(3):217–21.

Silva MED, Franco TT. Purification of soybean peroxidase (*Glycine max*) by metal affinity partitioning in aqueous two-phase systems. J Chromatogr B. 2000;743(1–2):287–94.

Skuse D, Norris-Jones R, Yalpani M, Brooks D. Hydroxypropyl cellulose/poly(ethylene glycol)-co-poly(propylene glycol) aqueous two-phase systems: system characterization and partition of cells and proteins. Enzym Microb Technol. 1992;14(10):785–90.

Soares RRG, Novo P, Azevedo AM, Fernandes P, Aires-Barros MR, Chu V, et al. On-chip sample preparation and analyte quantification using a microfluidic aqueous two-phase extraction coupled with an immunoassay. Lab Chip. 2014;14(21):4284–94.

Soares RRG, Silva DFC, Fernandes P, Azevedo AM, Chu V, Conde JP, et al. Miniaturization of aqueous two-phase extraction for biological applications: from micro-tubes to microchannels. Biotechnol J. 2016;11(12):1498–512.

Srinivas N, Barhate R, Raghavarao K. Aqueous two-phase extraction in combination with ultrafiltration for downstream processing of Ipomoea peroxidase. J Food Eng. 2002;54(1):1–6.

Szlag DC, Guiliano KA. A low-cost aqueous two phase system for enzyme extraction. Biotechnol Tech. 1988;2(4):277–82.

Tan Z, Li F, Xu X, Xing J. Simultaneous extraction and purification of aloe polysaccharides and proteins using ionic liquid based aqueous two-phase system coupled with dialysis membrane. Desalination. 2012;286:389–93.

Truust H, Johansson G. Fractionation of wheat proteins by counter-current distribution using aqueous two-phase systems containing propionic acid. J Chromatogr B. 1996;680(1–2):71–80.

Vaidya B, Suthar H, Kasture S, Nene S. Purification of potato polyphenol oxidase (PPO) by partitioning in aqueous two-phase system. Biochem Eng J. 2006;28(2):161–6.

Vázquez-Villegas P, Acuña-González E, Mejía-Manzano LA, Rito-Palomares M, Aguilar O. Production and optimization of a chlorophyll-free leaf protein concentrate from alfalfa (*Medicago sativa*) through aqueous two-phase system. Rev Mex Ing Quím. 2015;14(2):383–92.

Vázquez-Villegas P, Ouellet E, González C, Ruiz-Ruiz F, Rito-Palomares M, Haynes CA, et al. A microdevice assisted approach for the preparation, characterization and selection of continuous aqueous two-phase systems: from micro to bench-scale. Lab Chip. 2016;16(14):2662–72.

Venancio A, Teixeira JA, Mota M. Evaluation of crude hydroxypropyl starch as a bioseparation aqueous-phase-forming polymer. Biotechnol Prog. 1993;9(6):635–9.

Yadeta K, Elmore J, Coaker G. Advancements in the analysis of the Arabidopsis plasma membrane proteome. Front Plant Sci. 2013;4(86):147–56.

Yamada M, Kasim V, Nakashima M, Edahiro J, Seki M. Continuous cell partitioning using an aqueous two-phase flow system in microfluidic devices. Biotechnol Bioeng. 2004;88(4):489–94.

Zettl R, Feldwisch J, Boland W, Schell J, Palme K. 5′-azido-[3,6-3H2]-1-napthylphthalamic acid, a photoactivatable probe for naphthylphthalamic acid receptor proteins from higher plants: identification of a 23-kDa protein from maize coleoptile plasma membranes. Proc Natl Acad Sci U S A. 1992;89(2):480–4.

Zhang C, Love R, Jilka J, Glatz C. Genetic engineering strategies for purification of recombinant proteins from canola by anion exchange chromatography: an example of β-Glucuronidase. Biotechnol Prog. 2001;17(1):161–7.

Zimmermann S, Gretzinger S, Schwab M-L, Scheeder C, Zimmermann PK, Oelmeier SA, Gottwalde E, Bogsnes A, Hanssong M, Staby A, Hubbuch J. High-throughput downstream process development for cell-based products using aqueous two-phase systems. J Chromatogr A. 2016;1464:1–11.

Continuous Aqueous Two-Phase System Processes

8

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Abbreviations

ATPE	Aqueous two-phase extraction
ATPS	Aqueous two-phase system
DOE	Design of experiments
IgG	Immunoglobuline G
LLE	Liquid-liquid extraction
PDA	Photodiode array detector
PEG	Polyethylene glycol
PO_4	Phosphates
RE	Recovery efficiency

8.1 Introduction

Aqueous two-phase systems (ATPSs) are a clean alternative for traditional organic water solvent extraction systems (Gupta et al. 1999) that have been used for partitioning a great variety of biomolecules of interest from their contaminants (Haraguchi et al. 2004; Kamei et al. 2002; Andrews et al. 1996; Xu et al. 2001; Rosa et al. 2013; Breydo et al. 2013), exploiting their differences in molecular weight, hydrophobicity, isoelectric point, and affinity among other features. To employ this technique in downstream processing, the information about new systems and new applications is gaining spread (Nan et al. 2013; Liu et al. 2013). Although ATPSs

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are known to be a biocompatible, integrative, easily upscaling system, some of the drawbacks of this technology are related to limitations of phase separation of highly viscous systems, high cost of polymer components, limited predictive design, and lack of know-how in terms of installation, validation, and operation (Haraguchi et al. 2004; Soares et al. 2015). Furthermore, most of their applications have been developed at batch and bench scale.

Nowadays, the industry is moving from the large fed-batch/batch systems to produce biopharmaceuticals to more versatile continuous systems (Zydney 2016; Jungbauer 2013; Croughan et al. 2015; Jungbauer and Walch 2015). This involves efforts in the process integration in order to reduce buffer consumption, diminishing process time and costs, and increasing process yields (Jungbauer and Walch 2015; Igarashi et al. 2004a). Thus, if the drawbacks of this technology were fulfilled, ATPS as a continuous or semi-continuous operation would have clear competitive advantages in the biotechnology market. This chapter is dedicated to revise the development of ATPS implemented in continuous processes. First, the equipment devices that have been employed for this purpose are described. Then, the strategy for characterization of those continuous systems, including typical hydrodynamic parameters, is presented. The chapter also discussed selected examples of continuous ATPS processes, from micro- to pilot plant scale.

8.2 Devices Employed for Continuous ATPS Processes

There is not specialized equipment to perform continuous ATPS processes. Devices that have been employed for this purpose are in the classification of column contactors, mixer-settler units, and other contactors that have been also employed in traditional liquid-liquid extractions (Espitia-Saloma et al. 2014). The geometry, the kinetics and the dynamics of the ATPS inside these prototypes can vary, but the same unit operations that are carried out in batch systems should be performed by any of those equipment (Fig. 8.1). The minimum number of operations to perform an ATPS extraction of biomolecules is described below:

- Prepare the stocks of phase components. This step can be performed manually or as part of the continuous system. Stocks of single phase components can be stored in different containers or mixed to give various continuous phases in order to equal the viscosity and density of all the flows, regarding equipment restrains, such as power input in the pumping system. The sample can be pumped individually or diluted in one of the components streams.
- Mix at the right phase composition. Put together the components and sample at their final dilution and mix thoroughly to let the molecule of interest to be in contact with the phase where it has preference. In batch systems, this stage is performed manually by using mechanical agitators. In continuous systems, pump-driven encountered flows can be used to perform this operation. Static mixers also are employed.

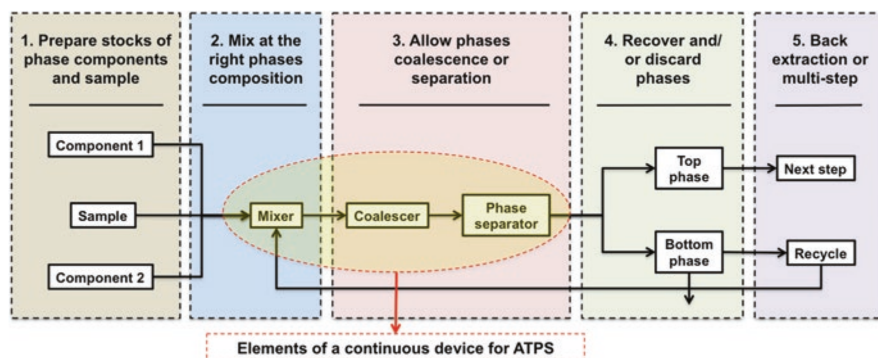

Fig. 8.1 Steps for batch ATPS processes that would be accomplished with a continuous system

- Phase coalescence or separation. It is important to allow phase separation after the phase emulsion formation, so the phase, where the product of interest is contained, could be recovered for further processing. This stage is accomplished by stopping the mixing in batch systems or by transference of the emulsion toward a separation tank or decanter. In continuous processes carried out in columns, top and bottom phases are continuously formed in the top and bottom sides of the column, respectively, because of gravity and density differences.
- Recover the phase where the molecule of interest is present. After phases are separated, the phase with the contaminants should be removed. Whether it is top or bottom phase which contains the product of interest, the easiest way is to open a valve in the bottom part of the decanter to allow the bottom phase be pumped out from the vessel followed by the top phase. In continuous systems, the overflow of the top phase can drive this phase toward an opening in the respective column top part.
- Discard the opposite phase or recycle it. By means of pumping, the phase that contains the contaminants can be discarded or conducted back to the mixer tank or to another mixer tank in order to partition residual product of interest. However, since the amount and characteristic of the contaminants can differ from the original sample, this can imply a change in the product of interest partitioning behavior that should be previously considered.
- Multistep or back extraction. Alternatively, it would be possible to require a second round of ATPS extraction, using a different composition to allow back extraction or increase purity or recovery of the molecule of interest.

In sum the adequate equipment to perform all these tasks would be a closed system with the ability to change the flows of all the entrance streams, with three or more inlets, sampling ports at different stages of the extraction procedure, and final recovery of phases.

In this sense, the most common devices employed for continuous ATPS processes are columns (Table 8.1). Some designs in the set of column contactors employed for continuous ATPS are spray columns, perforated rotating disk contactors, pulsed cap columns, and other columns (packed, sieve plate and vane-agitated columns). The main variable among them is the mechanism by which the mass transfer between the phases is promoted (pulsed caps, rotating disks, rotating vanes, spray mechanism, static packing, and static mixer). Whether the dispersed phase is delivered as spray through a nozzle located in the bottom of the column or mixing elements located all around the column to mix the heavier and lighter phases as well as the product of interest which commonly is diluted in one of both phases, these columns commonly share two inlets and two outlets, while the mass transfer and phase coalescence occur inside the column. Phases are prepared in a previous step and let overnight to separate in order to pump them to their respective inlets.

Mixer settlers/mixer-settler columns are also widely employed. Among their advantages are cleaning and control easiness, multistep assembly, batch to continuous flow, extreme to unlimited phase ratio, and small footprint, co-countercurrent, or countercurrent fashion. Mixer-settler units were one of the first devices employed for continuous ATPS (Veide et al. 1984) and nowadays are being exploited at pilot plant scale to downstream recovery of antibodies. They pose as an important advantage to their inherent assembling easiness, suitable for individual stage screening (mixing, coalescence, and separation). However, a noteworthy gap is the hydrodynamics characterization, needed for a practical design platform implementation (Mistry et al. 1996; Salamanca et al. 1998). The two different modes of operation (static and dynamic) basically consist of a mixing stage in tanks or columns, coupled to a series of settling and separating units. Steady state is reached when the partition coefficient obtained in the continuous mode is similar to the one in the batch mode.

8.3 Problems Faced by Continuous ATPS Process Equipment

Having overviewed ATPS continuous devices, the main operational problems that can be identified are flooding, backmixing, emulsification, and poor phase separation degree (Fig. 8.2). They limit the selection of optimum operational parameters. Flooding-related problems may arise if a drastic agitation or countercurrent operation drives to small droplets of a dispersed phase into a continuous phase with high flow velocity, which leads to phase accumulation and increase in coalescence time (Cuhna and Aires-Barros 2002). However, it should be kept in mind that in some kind of contactors, optimal operation is near the flooding point, since the dispersion area is maximized and the mass transfer rate is the highest (Wachs et al. 1997).

Whereas backmixing can be an advantage at the beginning of the ATPS extraction process, the two-phase behavior should exclude it at the end of the process. Most contactors have an agitation device that accelerates the interaction of phases allowing mass transfer among phases. While the physical presence of these devices

Table 8.1 Column contactors employed in continuous ATPS processes

Equipment	Advantages	Disadvantages	References
Spray column	Construction simplicity, easy cleaning, and low operational and maintenance costs	High degree of continuous backmixing, large time to reach a steady state, used only with systems with low interfacial tension and biomolecules with high diffusivity coefficients	Venancio and Teixeira (1995), Pawar et al. (1993), Igarashi et al. (2004a), Rostami and Alamshahi (2002), Srinivas et al. (2002)
Perforated rotating disk contactor	High throughput and efficiency, operational flexibility and easiness of continuous mode of operation, multistage redispersion	Flooding; separation efficiency seems to be inhibited by resistance of the flow toward the rotating disks at higher dispersed flow rates	Cavalcanti et al. (2008), Figuereido et al. (2004), Porto et al. (2010)
Pulsed cap columns	Low time to reach steady state due to a lower turbulence generation	Has not shown an appreciable advantage over batch operation; low flow rates as a condition for an optimal performance limit the device throughput	Bim and Teixeira (2000), Rabelo and Tambourgi (2003)
Packed, sieve plate and vane-agitated columns	Less space requirement, easier to stabilize, short time to reach steady state, reduces backmixing, and provides rapid coalescence and drops redispersion	Have only been employed with polymer-salt systems	Igarashi et al. (2004a, b), Biazus et al. (2007).
Other columns (Kühni, Karr-reciprocating, Scheibel and Oldshue-Rushton extractors)	Rapid coalescence	Works only with ATPS with low difference in phase density and when redispersion is needed to avoid emulsification	Cuhna and Aires-Barros (2002)
Raining bucket or Graesser contactor	Operation flexibility, interfacial renewal, and good mass	Its mechanical complexity can be a characteristic that limits its adoption; the absence of a unique dispersed or continuous phase increases the time needed to achieve steady state	Coleby (1983), Jarudilokkul et al. (2000), Dos-Reis et al. (1994), Giraldo-Zuniga et al. (2006)

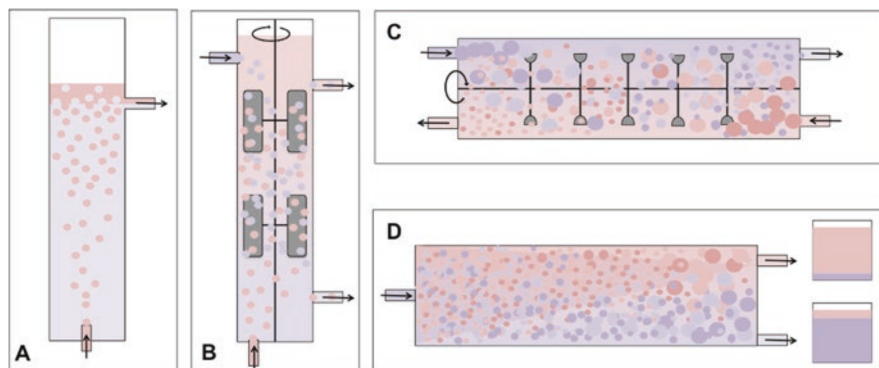

Fig. 8.2 Problems faced by continuous ATPS processes. (**a**) Flooding (flow rates are higher than should be, coalescence is not allowed, and contaminant phase level is above the limit). (**b**) Backmixing (coalesced phases are in contact with fresh feedings). (**c**) Emulsification (very small droplets are formed, increasing coalescence time beyond the needed, and contaminant phase invades the phase of interest at the outlets). (**d**) Separation efficiency of the device or holdup (short-length separation device does not allow phase coalescence, and both phases are obtained at the same outlet)

decreases the contact of coalesced phases with the unreacted fresh feedings (backmixing), simultaneously the agitation stimulated by these devices increases it (Cuhna and Aires-Barros 2002; Martin 2000; Lounes and Thibault 1996; Stella and Clive 2006).

Emulsification is an issue that has not been extensively discussed when working with continuous ATPS. This may be due to the scarce analysis of ATPS other than polymer-salt systems and also for the larger-scale trials that would be needed for this purpose (Leng 2004; Selber et al. 2004). A high emulsification degree reduces mixing efficiency and increases coalescing time. Aggressive agitation should be avoided in order to restrain the problem, especially with systems that pose low interfacial tension. The mass transfer rate is inversely proportional to drop size, and, thus, an analysis of cost-benefit on these two parameters should be performed before deciding the mixing rate.

Separation efficiency can be measured by the holdup phenomena, which is the volume of the continuous phase divided by the total volume at the dispersed-phase outlet, and vice versa (Cavalcanti et al. 2008). It is directly proportional to the dispersed-phase flow rate and the drop inherent coalescence time and inversely proportional to the purification factor and the recovery efficiency (RE) (Igarashi et al. 2004b; Rostami and Alamshahi 2002). For any continuous ATPS process, a phase's separation stage is fundamental. Even if the device promotes an excellent mixing and if the separation efficiency at the end of the process is reduced, the extraction performance in general is greatly diminished.

8.4 Characterization of Continuous Equipment

Because of the problems exposed previously, it is important to characterize the equipment that will be used for a continuous ATPS process. The evaluation of a continuous device to perform aqueous two-phase partitioning of biomolecules, with the aim to replicate the partition coefficients obtained from batch experiments, is often a hard work to do. One operational parameters studied in these devices is the physicochemical characteristics governed by phase composition and flow rates. ATPS composition and its related physical properties (viscosity, density, and interfacial tension) may limit the extraction and act as a resistance to mass transfer (Arsalani et al. 2005; Cuhna and Aires-Barros 2002; Igarashi et al. 2004a; Srinivas et al. 2002; Venancio and Teixeira 1995; Pawar et al. 1993, 1997).

Phase's flow rate is another critical operational parameter in any contactor performance, since it plays a key role in mass transfer and in the column process related to operational issues as backmixing and flooding. In most of continuous ATPS processes, an increase in the dispersed-phase flow produces minor drop sizes that cause higher areas for mass transfer (Figuereido et al. 2004). However, flow rates cannot be increased unlimitedly, since they can hamper the separation efficiency.

Observing the partition behavior of any dye can help in the characterization of continuous equipment. The concentration of such dye can be measured at the outlet of each phase through time. The amount of colorant in each phase can be plotted and show residence time, which can be related to the total operation volume, given a determined total flow rate. If the distribution coefficient of the dye at the system equilibrium remains more or less close to unity, it means that the mixing time and intensity were not enough to mix thoroughly the phases, and thus the mixer efficiency should be improved. Intimate phase dispersion is necessary to improve mass transfer, and such solute interchange occurs immediately during dispersion. Different mixer configurations ensure the creation of an interfacial surface area that allow bulk homogenization ensuring that all flow components are distributed uniformly and exposed to similar levels of turbulence.

A high top-phase affine dye can be employed to characterize the length or height of the coalescer, since immediate transfer of color to the top phase is allowed using mixers. A compound that highly prefers one or other phase allows a considerable tubular settler length reduction and a significantly shorter residence time resulting in lower solvent and energy requirements. It is important to point that with a batch ATPS, every unit operation is realized in a discontinuous independent manner, with consequent loss of sample and time for analysis. With a continuous device, the manipulation of samples is diminished, and the partition time is decreased without a significant effect on partition coefficients.

When using model proteins in batch systems, larger partition times are observed. This reflects the need for an increased processing time and an improved mass transfer and, at the same time, the need to increase the contact area between phases. With the use of a continuous system, protein partitioning could be significantly improved, allowing the continuous harvesting of product from both phases. A minor disadvantage of

the use of in-line static mixers is the flow restriction and power adjustments needed to surpass the packing of the mixers; however, such restriction is necessary to maximize transfer of the desired product.

When implementing continuous devices to recover different protein fractions from a complex feedstock, it is reasonable to suggest that different protein profiles could be found in each phase. The use of constant and controllable flows enabled simple and reproducible generation of stable interfaces. Variability will be observed mainly due to manual sampling of the phases or pulses generated by the pumping system; however, once the system evolved, a quasi-steady state is reached with partition coefficient values similar to batch systems. An additional clear advantage of a continuous device is a significantly lower accumulation of protein at the system interface, due mainly to the dynamic nature of the interface of two moving liquids.

In general, any continuous device can be suggested and tailor-made for different industrial purposes, from the continuous extraction of dyes from textile wastewater effluents to treatment of whey from dairy plants and further processing and commercialization of crude fermentation broths. However, to have a full knowledge of the hydrodynamic parameters needed to carry out the process, the aforementioned simple steps can be conducted.

8.5 Platform for Continuous ATPS Processes: From Microdevices to Pilot Plant

Most of the continuous ATPS processes that have been carried out start with a known system for a given particle. This means that previously there should have been a complex design of experiments that was accomplished through many batch small systems, in order to obtain the best composition for a maximum partition coefficient or purification factor. And only when the batch system is corroborated to maximize yield that it is employed to run the continuous process, either with columns or mixer-settler equipment. Thus, a complete continuous platform in order to avoid such waste of materials, reagents, and time should allow selection and characterization of the system previous to a scaling-up.

This platform must include a versatile and easy to manage set of continuous systems that can be categorized in three main scales: micro, bench, and pilot plant (Fig. 8.3). The microscale devices have been designed in order to observe phase formation of many mixtures of different phase components in a relatively short amount of time. This information can be used to prepare binodal curves for new systems or even for known devices but whose composition or properties may vary in different laboratories because of reagent or environmental conditions. Furthermore, given that phase composition may be varied by fine-tuning the flow rates of the stocks of phase components, there is a whole new and bigger range of phase compositions that can be studied to select an appropriate system for a given biomolecule (Silva et al. 2017).

Fig. 8.3 Recommended platform to develop a continuous process

Bench-scale systems can be employed after the ATPS is selected by direct scaling-up of the modular device. Studies have demonstrated that even when other kind of equipment is employed, the partitioning behavior of that given molecule is maintained at this scale. At this stage, other parameters can be varied in order to improve yields of certain particle that want to be recovered in higher amounts. Most of the model proteins that have been recovered in these kinds of systems come from fermentation products that can be also scaled up to several liters. An important advantage in this scale is that different modalities of the equipment can be studied, such as multistage arrangements or phase recycling, or can be adapted to other phases of the biomolecule recovery process, directly, because of the flow streams that are constantly supplied.

Continuous systems have been scaled up to pilot plants. The most common application is the recovery of antibodies from cell cultures. The advantage of pilot plant ATPS continuous processes is that the operational variables of the system can be studied in order to further apply them at an industrial level, whether if the phases are recycled or if back extraction is being applied. Also, as most of the instruments for flow control and temperature and pressure sensing, for example, are more developed for this scale, the system can be better controlled for some of these parameters, as well as agitation and phase behavior given the material of the walls of the system, en route to industrial implementation of the system. In Fig. 8.4, there is a summary of the characteristics of each of these stages of the proposed platform and the data

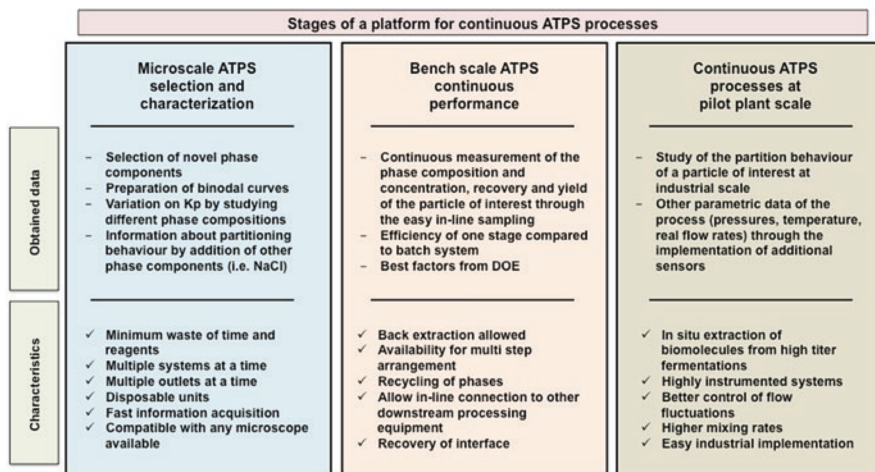

Fig. 8.4 Characteristics and obtained data of the stages of scaling platforms for continuous ATPS processes

that can be obtained at each stage. The selection of the starting point in either case will depend on the information available from the DOE or previous literature regarding the recovery for a specific bioproduct. In general, the first two stages could allow the implementation of different factors (back extraction, recycling, multistage, etc.) aiming to improve performance at pilot plant scale shortening times and resources.

8.6 Microdevice-Assisted Approach for ATPS Characterization

The miniaturization of continuous ATPS processes highlights some characteristics of the ATPS previously unknown while studying the partition behavior of certain high valuable pharmaceutical biomolecules (Rosa et al. 2013; Hardt and Hahn 2012; Ingram et al. 2013). This allows the shortening of data acquisition for the optimal partition parameters and makes more efficient the use of resources.

Under certain flow conditions, ATPS could be formed continuously inside a microchannel, starting from stocks of phase components. Micromixers included within the device, sequentially and rapidly, prepare two-phase systems across an entire range of useful phase compositions. Two-phase diagrams (binodal curves) can easily be prepared using the cloud-point method for systems of different components. In-line agitation system gives the possibility of increasing the diffusion of molecules through the chaotic mixing generated on a laminar flow of adjacent streams. This may allow the collection of preliminary data to work with continuous devices at bench scale. It is important to work with laminar flows and avoid

Rayleigh-Plateau instabilities (perturbation of a jet of fluid of a dispersed phase with lower viscosity, into droplets inside a continuous phase) when working with flows lower than 100 μL/min. This is because the laminar behavior allows for higher reproducibility and no axial dependence on partitioning.

In batch systems, the difference in the density of the phases is responsible of the time needed for two phases, coalescence and sedimentation (Kim et al. 2010); however, at microscopic level, this difference seems to be insignificant, compared to other properties of the phases such as viscosity and interfacial tension (Geschiere et al. 2012). This may be one possible reason for the instantaneous phase formation after the micromixer; thus, the microchannel length is not a limitation.

However, an important restriction in microdevice operation to create the binodal curves was that the total flow inside the microchannel should be enough in order to avoid Rayleigh-Plateau instabilities (Moon et al. 2015) but not so high to generate backflow in the inlet channels of the microdevice. These operational limits will vary according to the nature of the components used (i.e., component concentrations or polymer molecular weight). As explained by Kim et al. (2010), this behavior is owed to viscosity and hydrophilicity (contact angle and superficial tension) and can be overcome by decreasing the contact angle among flows and adding mechanisms of passive control such as internal valves or by capillary action.

By connecting tubing at the outlet streams, the design can be used to recover samples of the phases and to obtain the partition coefficients. The time frame needed to collect samples will depend on the flow rate of the phase component streams. The time needed to recover the necessary amount of sample could be decreased if a direct technique to measure proteins is implemented (i.e., NanoDrop, PDA flow cell, etc.) or in-line systems for biomolecule detection are employed.

The employment of two highly viscous fluids to form an ATPS, such as polyethylene glycol and dextran, implies higher times to reach equilibrium. With the use of a micromixer such as the one proposed in this work, that issue could be minimized, and given polymer concentrations can lead to phase formation.

In using microdevices to run ATPS, different biomolecules have been recovered, for example, cells, bovine serum albumin, proteins such as invertase from yeast, antibodies, etc. However, the differences observed may be explained due to a more efficient mixing at the microscale and the effect of the gravity that drives phase separation at the bench scale and not at the microscale, as pointed by Tsukamoto et al. (2009). However, the microdevice can be employed to select a given kind of phase components for further scale-up. The advantage of this approach dwells in the employment of a micrometric amount of phase components and sample to select and optimize the ATPS in minutes, previous to a scaled application to meet larger productivities. The partition coefficient will be consistent between the bench-scale device and the microscale, as long as the geometry of equipment is preserved. This could boost the use of ATPS at larger scales, in order to work with new systems and increase the database that nowadays exists in the field, which will contribute to integrate ATPS as a viable easy-to-setup unit operation for the primary recovery of biomolecules at pilot and industrial scale.

8.7 Multistage, Bench-Scale Systems

Multistage systems are typically more efficient in terms of selectivity, enrichment, and throughput than a single stage (Rosa et al. 2009a; Luo et al. 2013). With this mode of operation, more solute is transferred from raffinate to the extractive phase when raffinate enters into contact with fresh new solvent, increasing impurities removal. Additionally, with repeated equilibrations using a small amount of solvent more material is removed than when using a single extraction with large volume, avoiding the waste of solvent and dilution of the extract (Geankoplis 1993). There are substantial examples of multistage applications on ATPS. However, all of them are best suited for the commonly liquid-liquid extraction procedures carried out with typical solvents in the chemical industry. Mixing and settling can occur in the same vessel, allowing higher throughputs and smaller footprint.

In polymer-salt systems, it is preferred that enzyme and impurities partition to opposite phases and enrichment of the final product preferred in the bottom-phase outlet, since removal of the phase-forming components would be easier. To calculate the number of stages of countercurrent approach, some methods, such as the mass transfer unit method, have been employed. The calculated number of transfer units is not exactly the number of stages in most of the cases (using this would be costly), but is a useful parameter to have an idea of the efficiency of the system.

8.7.1 Continuous ATPS at Bench Scale for Antibody Purification

It has been demonstrated that immunoglobulin G (IgG) can be successfully extracted with ATPS (Silva et al. 2012). Some continuous operation has been studied and documented in literature (Vazquez-Villegas et al. 2011; Cavalcanti et al. 2008; Porto et al. 2010; Rosa et al. 2013; Espitia-Saloma et al. 2014). The growing market for the monoclonal antibodies and the constant need for more economically attractive large-scale production processes make the studies of novel efficient continuous recovery processes more appealing (Rosa et al. 2013).

Silva and collaborators (2012) have shown that IgG can be effectively partitioned within microfluidic platforms. Microfluidic approaches allow the use of minimal reagent quantities and a quick evaluation of a larger number of ATPS compositions, just adjusting the phase component's flow ratios (Hardt and Hahn 2012). The microfluidic approach proposed here effectively used to perform a quick and general screening of the main partition behavior of a molecule of interest could be a trustworthy tool to accelerate the bioprocess design.

Examples of multistage, bench-scale approach can be found in the literature. The main studied devices include multiplate column contactors and mixer-settler configurations demonstrating potential for an industrial scale process (Rosa et al. 2009a, b; Vazquez-Villegas et al. 2015; Prinz et al. 2014; Eggersgluess et al. 2014). The recovery of monoclonal antibodies has been carried out by using 3.45 L/h for outlet top phase and 2.01 L/h for outlet bottom phase in a countercurrent column extraction (2043 mm^2 of expanded cross-sectional area) using a polyethylene

glycol-phosphate system (Rosa et al. 2012). A steady state was reached in 4–5 h according to the authors. Eggersgluess et al. (2014) suggested a mixer-settler battery (40 mL mixers and 80 mL settlers) of ten stages for a polyethylene glycol-phosphate system (12% PEG, 18% PO_4), using 6 and 18 g/min of light and heavy phases, respectively, with 4 h of residence time. Prinz and collaborators (2014) recovered laccase from fermented broths using a mixer-settler unit with three stages (pump mixer of 65 mL and settler of 135 mL) reaching a steady state after 7 h with 0.3 L/h. Rosa et al. (2009b) simulated a five-stage multistage batch process to recover IgG in a polyethylene glycol-phosphate ATPS with 10% w/w of sodium chloride, predicting an 89% recovery. Diffusive mass transfer has been demonstrated to be superior in countercurrent flow (Eggersgluess et al. 2014) and with the increment of mixing stages resulting in more IgG transferred to the bottom phase (Espitia-Saloma et al. 2016).

Rito-Palomares and Lydiatt (1996) carried out a partial recycling of top phase demonstrating a useful approach to reduce raw material consumption with minor effects on the extraction performance of enzymes. Espitia-Saloma and coworkers (2016) investigated the effect of recycling in a continuous, multistage ATPS on the yield and purity of IgG and observed improved performance in recovery.

There are still concerns on the application of ATPS at industrial scale related to the high consumption of phosphate and polyethylene glycol (PEG) and, consequently, to their impact on water treatment. Although PEG is biodegradable and nontoxic, phosphate disposal is problematic. These bottlenecks may however be minimized if the recycling of both PEG and phosphate is considered (Mündges et al. 2015). In general, ATPSs have demonstrated to be a potential industrially suited primary recovery operation for human IgG; furthermore, it has the versatility to be adapted to several platforms and products in order to achieve a generic and practical usage.

8.8 Processes at Pilot Plant Scale

The first description of a pilot-scale continuous process of protein purification using ATPS was carried out for the extraction of enzymes from animal tissue. It employed a system with 20% (w/w) of biomass in a PEG-salt system and was successful using a disk separator rather than a decanter. In this study, a computer control was employed for the mixing step, and the obtained protein (fourfold purification factor, 83% recovery yield) was contained in a clarified solution suitable for further chromatographic methods (Boland et al. 1991). The disadvantage of this source of proteins is that it is expensive and animal proteins are increasingly being replaced by recombinant proteins produced in microbes (Boland 2002).

Containers with mechanically rotated stirrer for mixing of phase components and disk stack centrifuge for phase separation at pilot plant scale have been employed to recover enzymes from fermentation broths. The disadvantage of such equipment is that the centrifuge should be regularly desludged. The frequency of this will be a function of the biomass loading, the fineness of the homogenate, and the flow rate.

The interval can only be found by trial and error (Boland 2002). As an example, membrane-bound cholesterol oxidase from unclarified culture broth of *N. rhodochrous* has been separated using an ethoxylated nonionic detergent-based ATPS in a stack centrifugal separator. Phase purity (i.e., only the studied phase, without contaminant phase) was close to 100%, and enzyme recovery yielded 87–93% with fourfold product concentration (Minuth et al. 1997). This method, as stated by the authors, is time-consuming (aprox. 20 h) but not labor-intensive and requires little investment (Minuth et al. 1997).

A 6.25 L pilot-scale centrifugal chromatographic column has also been widely employed for protein separation in ATPS using polymer-salt systems. Rotor speed, mobile-phase flow rate, and sample loading are optimizable parameters that have been studied at lab scale in order to scale up the process. Potential throughputs using this alternative have been over the range of 40 g of products per day (Sutherland et al. 2008). The most important advances in centrifugal partition chromatography technology have come from research into the hydrodynamics and kinetics of mixing (Sutherland et al. 2008).

A pilot-scale packed differential contactor was evaluated for the continuous countercurrent aqueous two-phase extraction (ATPE) of human IgG from a Chinese hamster ovary cell supernatant enriched with pure protein by Rosa et al. (2012). An experimental setup combining the packed column with a pump mixer-settler stage showed to have the best performance and to be advantageous when compared to the IgG batch extraction. An IgG recovery yield of 85% could be obtained with about 50% of total contaminants and more than 85% of contaminant protein removal. Mass transfer studies have revealed that the mass transfer was controlled by the PEG-rich phase. A higher efficiency could be obtained when using an extra pump mixer-settler stage and higher flow rates. Cunha and Aires-Barros (2002), from the same group, have written an extensive review of equipment employed for ATPS in continuous fashion.

In fact, the poor understanding of the responsible mechanisms for the partitioning of biomolecules in ATPS and the usually used batch equipment assembly (agitated vessel + centrifuge) leads to a certain reluctance from industry to embrace this unit operation as part of their own processes (Rito-Palomares 2004).

8.9 Current Challenges and Future Trends

The recovery of the bioproduct from fermentation broths and biological feedstock is one of the major bottlenecks in the bioprocessing industries. ATPS has shown a meaningful potential to be an alternative solution for contributing to downstream bottlenecks solution. Compared to batch systems, the continuous process involves shorter stabilization times and generally avoids centrifugation steps suggested in batch processes, allowing process integration. Only a few companies in the world, such as Genentech, have reported to use batch-mode ATPS for product recovery (Builder et al. 1993; Asenjo and Andrews 2012). Thus, there are still some issues

that should be addressed in order to make continuous ATPS better adopted for other companies.

For instance, the lack of practical rules for an effective implementation has limited their generic application at commercial scale. Column contactors and mixer-settler devices have been the common choice for continuous ATPS processes, but some problems such as flooding, backmixing, emulsification, and efficiency of phase separation are found when working with those designs. This has raised some areas of opportunity identified for the practical implementation of continuous ATPS.

Mathematic modeling of scaling procedures that correlate the recovery of biomolecules with respect to flows and their characteristics is an opportunity area that has not been considered yet and that is expected to be characterized in the future. Different construction materials can be explored, as in the case of stainless steel for the static mixers or cheap acrylic configurations in order to lower initial inversion costs. Computational fluid dynamic simulation programs can be a valuable tool in order to select the best configuration. These can be further translated into theories about phase formation kinetics, taking in consideration the mixing energy and the droplet sizes (depending on the liquid characteristics).

The kind of system and their hydrodynamic characteristics are another opportunity area. Although traditional polymer-salt systems are yet expected to dominate applications, new kinds of ATPS, such as ionic liquids, are the tracking trend. These systems are considered a great option for the replacement of volatile organic solvents in LLE (Novak et al. 2012) and are more easily reusable and recycled (Li et al. 2010). New compounds such as carbohydrates and thermoseparating polymers are also under study (De Brito Cardoso et al. 2013; Show et al. 2012; Li et al. 2002).

One of the great challenges of ATPS is to surpass the apparent unattractive economical image. Studies comparing, in detail, the costs involved in ATPS implementation with alternative technologies are not common (Aguilar et al. 2006; Huenupi et al. 1999; Torres-Acosta et al. 2016). A pre-evaluation of ATPS recuperation costs for each biomolecule of interest should be made in order to determine the viability of an ATPS process.

Another important challenge is the determination of an equipment design platform. The tendency toward the miniaturization of continuous ATPS partition may shorten data acquisition for the optimal partition parameters of valuable pharmaceutical biomolecules and makes more efficient the use of resources (Rosa et al. 2013; Hardt and Hahn 2012; Ingram et al. 2013). Meanwhile, column contactors and mixer-settler devices have the more solid design guidelines, including its hydrodynamic characterization and verification of its performance capacity. Phase recycling is a missing gap that should be considered for continuous operation in column contactors as well as in static mixer units, given the environmental impact of phase-forming compounds. Studies about phase recycling and the employing of new phase components is a work than can be accomplished as part of an integral view of the process before and after ATPS, including the recovery of the product of interest from the phase-forming components. To date ion-exchange chromatography, precipitation, ultrafiltration, dialysis, and supercritical CO2 extraction have been considered (Li et al. 2010).

Finally, a method to work with ATPS as a primary recovery strategy, in a fast and secure way for scaling up a continuous process, should be developed. In this way, the batch application, with its correspondent waste of material and costs, can be neglected and the user-related experimental variance in ATPS formulation minimized. The demonstrated potential of ATPS-based continuous systems places them as a promising liquid-liquid extraction technology that can be successfully implemented at large scales and with great potential to solve needs of biotechnological industry of an economical, efficient, predictable, and reliable downstream operation for bench- and large-scale applications.

8.10 Concluding Remarks

ATPS strategies have been typically performed in continuous mode using equipment adapted from traditional LLE processes of the chemical industry. However, most of these apparatus present challenges for their application using highly viscous and dense phases from the different existing two-phase systems in biomolecules extraction. These challenges are mainly related to hydrodynamic problems such as flooding, backmixing, emulsification, and poor separation efficiency at the end of the continuous process. Thus, new platforms for continuous ATPS should be developed. Regarding this, microscale systems provide an excellent opportunity to change the actual practice of this purification technique from bench to continuous systems. Although this approach is already in early stages and further modeling and bottlenecks have to be solved, a substantial work is being done around the world in order to develop useful guidelines, so this liquid-liquid extraction technology could be successfully implemented at larger scales.

References

Aguilar O, Albiter V, Serrano-Carreón L, Rito-Palomares M. Direct comparison between ion-exchange chromatography and aqueous two-phase processes for the partial purification of penicillin acylase produced by *E. coli*. J Chromatogr B. 2006;835:77–83.

Andrews BA, Nielsen S, Asenjo JA. Partitioning and purification of monoclonal antibodies in aqueous two-phase systems. Bioseparation. 1996;6:303–13.

Arsalani V, Rostami K, Kheirolomoom A. Lipoxygenase-1 mass-transfer coefficient in aqueous two-phase system using spray extraction column. Ind Eng Chem Res. 2005;44:7469–73.

Asenjo JA, Andrews BA. Aqueous two-phase systems for protein separation: phase separation and applications. J Chromatogr A. 2012;1238:1–10.

Biazus JP, Santana JC, Souza RR, Jordao E, Tambourgi EB. Continuous extraction of α- and β-amylases from *Zea mays* malt in a PEG4000/CaCl2 ATPS. J Chromatogr B. 2007;85:227–33.

Bim MA, Teixeira FT. Extraction in aqueous two-phase systems of alkaline xylanase produced by *Bacillus pumilus* and its application in kraft pulp bleaching. J Chromatogr B. 2000;743:349–56.

Boland MJ. Aqueous two-phase extraction and purification of animal proteins. Mol Biotechnol. 2002;20:85–93.

Boland MJ, Heddelink PGM, Papamichael N, Hustedt H. Extractive purification of enzymes from animal tissue using aqueous two phase systems: pilot scale studies. J Biotechnol. 1991;19:19–33.

Breydo L, Mikheeva LM, Madeira PP, Zaslavsky BY, Uversky VN. Solvent interaction analysis of intrinsically disordered proteins in aqueous two-phase systems. Mol BioSyst. 2013;9:3068–79.

Builder S, Hart R, Lester P, Ogez J, Reifsnyder D. USA Patent no. 407,810; 1993.

Cavalcanti MTH, Carneiro-Da-Cuhna MG, Brandi IV, Porto TS, Converti A, Filho JLL, Porto ALF, Pessoa A. Continuous extraction of α toxin from a fermented broth of *Clostridium perfringens* Type A in perforated rotating disc contactor using aqueous two-phase PEG-phosphate system. Chem Eng Process. 2008;47:1771–6.

Coleby J. The RTL (formerly Graesser Raining-Bucket) contactor. In: Lo TC, Baird M, Hanson C, editors. Handbook of solvent extraction. New York: Wiley; 1983.

Croughan MS, Konstantinov KB, Cooney CL. The future of industrial bioprocessing: batch or continuous? Biotechnol Bioeng. 2015;112:648–51.

Cuhna T, Aires-Barros R. Large scale extraction of proteins. Mol Biotechnol. 2002;11:29–40.

De Brito Cardoso G, Mourao T, Menezes-Pereira F, Freire MG, Tinoco Fricks A, Faria Soares CM, Silva LA. Aqueous two-phase systems based on acetonitrile and carbohydrates and their application to the extraction of vanillin. Sep Purif Technol. 2013;140:106–13.

Dos-Reis CJ, Thömmes J, Kula MR. Continuous separation of whey proteins with aqueous two-phase systems in a Greasser contactor. J Chromatogr A. 1994;668:85–94.

Eggersgluess JK, Richet M, Dieterle M, Strube J. Multi stage aqueous two phase extraction for the purification of monoclonal antibodies. Chem Eng Technol. 2014;37:1–9.

Espitia-Saloma E, Vazquez-Villegas P, Aguilar O, Rito-Palomares M. Continuous aqueous two-phase systems devices for the recovery of biological products. Food Bioprod Process. 2014;92:101–12.

Espitia Saloma E, Vazquez-Villegas P, Rito-Palomares M, Aguilar O. An integrated practical implementation of continuous aqueous two-phase systems for the recovery of human IgG: from the microdevice to a multistage bench-scale mixer-settler device. Biotechnol J. 2016;11:708–16.

Figuereido PAL, Asfora SL, Aparecida MK, Farias MHJ, Lima-Filho JL, Campos-Takaki GM, Basile TE. Recovery of ascorbic oxidoreductase from crude extract with an aqueous two-phase system in a perforated rotating disc contactor. Braz Arch Biol Technol. 2004;47:821–6.

Geankoplis CJ. Transport process and unit operation. London: Prentice Hall; 1993.

Geschiere AD, Ziemecka I, van Steijn V, Koper GJM, van Esch JH, Kreutzer MT. Slow growth of the Rayleigh-Plateau instability in aqueous two phase systems. Biomicrofluidics. 2012;6:1–11.

Giraldo-Zuniga AD, Coimbra JSR, Minim LA, Garcia REE. Dispersed phase hold-up in a Graesser raining bucket contactor using aqueous two-phase systems. J Food Eng. 2006;72:302–9.

Gupta R, Bradoo S, Saxena RK. Aqueous two-phase systems: an attractive technology for downstream processing of biomolecules. Curr Sci. 1999;77:520–3.

Haraguchi LH, Mohamed RS, Loh W, Pessoa Filho PA. Phase equilibrium and insulin partitioning in aqueous two-phase systems containing block copolymers and potassium phosphate. Fluid Phase Equilibr. 2004;215:1–15.

Hardt S, Hahn T. Microfluidics with aqueous two-phase systems. Lab Chip. 2012;12:434–42.

Huenupi E, Gomez A, Andrews BA, Asenjo JA. Optimization and design considerations of two-phase continuous protein separation. J Chem Technol Biot. 1999;74:256–63.

Igarashi L, Kieckbusch TG, Franco TT. Xylanase mass transfer studies in aqueous two-phase systems using spray and sieve plate columns. Bioprocess Biosyst Eng. 2004a;26:151–7.

Igarashi L, Kieckbusch TG, Franco TT. Mass transfer in aqueous two-phases system packed column. J Chromatogr B. 2004b;807:75–80.

Ingram T, Mehling T, Smirnova I. Partition coefficients of ionizable solutes in aqueous micellar two-phase systems. Chem Eng J. 2013;218:204–13.

Jarudilokkul S, Paulsen E, Stuckey DC. Lysozyme extraction from egg white using reverse micelles in a Graesser contactor: mass transfer characterization. Biotechnol Bioeng. 2000;69:618–26.

Jungbauer A. Continuous downstream processing of biopharmaceuticals. Trends Biotechnol. 2013;31:479–92.

Jungbauer A, Walch N. Buffer recycling in downstream processing of biologics. Curr Opin Chem Eng. 2015;10:1–7.

Kamei DT, King JA, Wang DI, Blankschtein D. Separating lysozyme from bacteriophage P22 in two-phase aqueous micellar systems. Biotechnol Bioeng. 2002;78:203–16.
Kim SJ, Lim YT, Yang H, Kim K, Kim YT. Passive regulation of volume-flow ratio for microfluidic streams with different hydrophilicity and viscosity. Electrophoresis. 2010;31:709–13.
Leng RB. From bench to plant: scale up specialty chemical processes directly. Chem Eng Prog. 2004;100:37–44.
Li M, Kim JW, Peeples TL. Amylase partitioning and extractive bioconversion of starch using thermoseparating aqueous two-phase systems. J Biotechnol. 2002;93:15–26.
Li Z, Pei Y, Wang H, Fan J, Wang J. Ionic liquid-based aqueous two-phase systems and their applications in green separation processes. TrAC. 2010;11:1336–46.
Liu Y, Feng YQ, Zhao Y. Liquid–liquid equilibrium of various aqueous two-phase systems: experiment and correlation. J Chem Eng Data. 2013;58:2775–84.
Lounes M, Thibault J. Axial dispersion in a reciprocating plate column. Can J Chem Eng. 1996;74:187–94.
Luo Q, Li S, Jing S. The study of fluid dynamics in countercurrent multi-stage micro-extraction system. Energy Procedia. 2013;39:275–82.
Martin AD. Interpretation of residence time distribution data. Chem Eng Sci. 2000;55:5907–17.
Minuth T, Pape GU, Raths HC, Thommes J, Kula MR. Pilot scale processing of detergent-based aqueous two-phase systems. Biotechnol Bioeng. 1997;55:339–47.
Mistry SL, Kaul A, Merchuk JC, Asenjo JA. Mathematical modeling and computer simulation of aqueous two-phase continuous protein extraction. J Chromatogr A. 1996;741:151–63.
Moon BU, Jones SG, Hwang DK, Tsai SS. Microfluidic generation of aqueous two-phase system (ATPS) droplets by controlled pulsating inlet pressures. Lab Chip. 2015;15:2437–44.
Mündges J, Zierow J, Zeiner T. Experiment and simulation of an aqueous two-phase extraction process for the purification of a monoclonal antibody. Chem Eng Process. 2015;95:31–42.
Nan E-L, Williams GR, Song H-H, Quan J, Nie H-L, Zhu LM. Liquid-liquid-solid triple-phase data for aqueous two-phase systems comprising ethanol-1-propanol-2-propanol-acetone and salts. J Chem Eng Data. 2013;58:3314–9.
Novak U, Pohar A, Plazl I, Žnidaršič-Plazl P. Ionic liquid-based aqueous two-phase extraction within a microchannel system. Sep Purif Technol. 2012;97:172–8.
Pawar PA, Rostami JK, Sawant SB, Joshi JB. Enzyme mass transfer coefficient in aqueous-two phase systems: spray extraction column. Chem Eng Commun. 1993;122:151–69.
Pawar P, Parasu U, Sawant S, Joshi J. Enzyme mass transfer coefficient in aqueous two-phase systems: modified spray extraction columns. Can J Chem Eng. 1997;75:751–8.
Porto PS, Marques TP, Porto CS, Moreira KA, Lima-Filho JL, Converti A, Pessoa A, Porto ALF. Extraction of ascorbate oxidase from *Cucurbita maxima* by continuous process in perforated rotating disc contactor using aqueous two-phase systems. Appl Biochem Biotechnol. 2010;160:1057–64.
Prinz A, Koch K, Górak A, Zeiner T. Multi-stage laccase extraction and separation using aqueous two-phase systems: experiment and model. Process Biochem. 2014;49:1020–31.
Rabelo APB, Tambourgi EB. Performance of a pulsed-cap microcolumn for protein extraction. Braz J Chem Eng. 2003;20:357–62.
Rito-Palomares M. Practical application of aqueous two-phase partition to process development for the recovery of biological products. J Chromatogr B. 2004;807:3–11.
Rito-Palomares M, Lyddiatt A. Impact of cell disruption and polymer recycling upon aqueous two-phase processes for protein recovery. J Chromatogr B. 1996;17:81–8.
Rosa PAJ, Azevedo AM, Ferreira IF, Sommerfeld S, Bäcker W, Aires-Barros MR. Downstream processing of antibodies: single-stage versus multi-stage aqueous two-phase extraction. J Chromatogr A. 2009a;1216:8741–9.
Rosa PAJ, Azevedo A, Sommerfeld S, Mutter M, et al. Application of aqueous two-phase systems to antibody purification: a multistage approach. J Biotechnol. 2009b;139:306–13.
Rosa PAJ, Azevedo AM, Sommerfield S, Backer W, Aires-Barros MR. Continuous aqueous two-phase extraction of human antibodies using a packed column. J Chromatogr B. 2012;880:148–56.

Rosa PAJ, Azevedo AM, Sommerfeld S, Mutter M, et al. Continuous purification of antibodies from cell culture supernatant with aqueous two-phase systems: from concept to process. Biotechnol J. 2013;8:352–62.

Rostami K, Alamshahi M. Enzyme mass-transfer coefficient in aqueous two-phase systems sing static mixer extraction column. Bioprocess Biosyst Eng. 2002;25:169–78.

Salamanca MH, Merchuk JC, Andrews BA, Asenjo JA. On the kinetics of phase separation in aqueous two phase systems. J Chromatogr B. 1998;711:319–29.

Selber K, Tjerneld F, Collén A, Hyytiä T, Nakari-Setälä T, Bailey M, et al. Large-scale separation and production of engineered proteins, designed for facilitated recovery in detergent-based aqueous two-phase extraction systems. Process Biochem. 2004;39:889–96.

Show LP, Tan CP, Anuar MS, Ariff A, Yusof YA, Chen SK, Ling TC. Extractive fermentation for improved production and recovery of lipase derived from Burkholderia cepacia using a thermoseparating polymer in aqueous two-phase systems. Bioresour Technol. 2012;116:226–33.

Silva DFC, Azevedo AM, Fernandes P, Chu V, et al. Design of a microfluidic platform for monoclonal antibody extraction using an aqueous two-phase system. J Chromatogr A. 2012;1249:1–7.

Silva DFC, Azevedo AM, Fernandes P, Chu V, et al. Determination of partition coefficients for biomolecules in a microfluidic aqueous two phase system platform using fluorescence microscopy. J Chromatogr A. 2017;1487:242–7. doi:10.1016/j.chroma.2016.12.036.

Soares RR, Azevedo AM, Van Alstine JM, Aires-Barros MR. Partitioning in aqueous two-phase systems: analysis of strengths, weaknesses, opportunities and threats. Biotechnol J. 2015; 10:1158–69.

Srinivas ND, Narayan AV, Raghavarao KSMS. Mass transfer in a spray column during two-phase extraction of horse radish peroxidase. Process Biochem. 2002;38:387–91.

Stella A, Clive PHR. Backmixing in Karr reciprocating-plate extraction columns. Ind Eng Chem Res. 2006;45:6555–62.

Sutherland IA, Audo G, Burton E, Couillard F, Fisher D, Garrad I, Hewitson P, Intes O. Rapid linear scale-up of a protein separation by centrifugal partition chromatography. J Chromatogr A. 2008;1190:57–62.

Torres-Acosta MA, Aguilar-Yáñez JM, Rito-Palomares M, Titchener-Hooker NJ. Economic analysis of uricase production under uncertainty: contrast of chromatographic purification and aqueous two-phase extraction (with and without PEG recycle). Biotechnol Prog. 2016; 32:126–33.

Tsukamoto M, Taira S, Yamamura S, Morita Y, Nagatani N, Takamura Y, Tamiya E. Cell separation by an aqueous two-phase system in a microfluidic device. Analyst. 2009;134:1994–8.

Vazquez-Villegas P, Aguilar O, Rito-Palomares M. Study of biomolecules partition coefficients on a novel continuous separator using polymer-salt aqueous two-phase systems. Sep Purif Technol. 2011;78:69–75.

Vazquez-Villegas P, Aguilar O, Rito-Palomares M. Continuous enzyme aqueous two-phase extraction using a novel tubular mixer settler in multi-step counter current arrangement. Sep Purif Technol. 2015;141:263–8.

Veide A, Lindbäck T, Enfors SO. Continuous extraction of β-D-galactosidase form *Escherichia coli* in an aqueous two-phase system: effects of biomass concentration on partitioning and mass transfer. Enzym Microb Technol. 1984;6:325–30.

Venancio A, Teixeira JA. Protein mass transfer studies on a spray column using the PEG-Reppal PES 100 aqueous two-phase system. Bioprocess Eng. 1995;13:251–5.

Wachs A, Benyamin J, Semiat R, Lewin DR. Control of a pilot-scale Karr liquid–liquid extraction column. Comput Chem Eng. 1997;21:601–6.

Xu Y, Souza MA, Ribeiro-Pontes MZ, Vitolo M, Pessoa-Jr A. Liquid-liquid extraction of pharmaceuticals by aqueous two-phase systems. Braz J Pharm Sci. 2001;37:306–20.

Zydney AL. Continuous downstream processing for high value biological products: a review. Biotechnol Bioeng. 2016;113:465–75.

Process Economics: Evaluation of the Potential of ATPS as a Feasible Alternative to Traditional Fractionation Techniques

9

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Abbreviations

ATPSs	Aqueous two-phase systems
CoG/g	Cost of goods per gram
DSP yield	Downstream processing yield
PEG	Polyethylene glycol
UF/DF	Ultrafiltration/diafiltration

9.1 Introduction

During the past six decades, the impact that biotechnology has made on human society has been significant. The development of biologically active products to be used in diverse sectors such as pharmaceutical or environmental divisions, cosmetic brands, or food industries has exponentially increased, and thus new challenges are experienced in the upstream and downstream processing areas of the involved production facilities of these active molecules. Considering that one of the most

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important areas in which biotechnology has a direct impact upon product design, production, and commercialization is therapeutics based on biologic molecules, novel approaches for the production of these compounds should now consider additional dimensions besides the technical feasibility of the processes at hand.

The traditional outline of a bioprocess is composed of two sections, the upstream and downstream processing divisions. The production steps which involve all the molecular biology designs for the expression systems to be used, strain selection, and cultures to generate the inoculums to be further fed to industrial-scale bioreactors compose the upstream part of the bioprocess at hand. The following operations which may include pertinent cell disruption, concentration, primary recovery, purification, and polishing steps give form to the downstream processing train within a newly designed or established bioprocess.

Considering the vast amount of possible combinations of operations needed to give a particular product its desired commercial presentation (based on its final application and thus purity), typical processing costs for the downstream of a biomolecule account for 80% of the total process expenses. The explanation of this phenomenon is simple; during the past 30 years, important advances in molecular biology, genetic engineering, and cell culture areas have been successfully applied in upstream operations, primarily bioreactor optimization procedures, in order to markedly increase the production titers of high added value molecules. With these advances, the production levels for monoclonal antibodies and other biotechnology-derived compounds have doubled or even tripled in the past 10 years, unveiling the current bottleneck in biotechnology processing which is located in the downstream processing sections. Since now large quantities of feedstocks need to be processed, current processes are not optimal for the recovery and purification of compounds immersed in such large working volumes (Gronemeyer et al. 2014). Even though the production titers have been optimized throughout the years, the economic gain through these increases cannot be directly applied to the downstream processing sections, which have complex separation steps, such as chromatographic protocols, which include specific associated factors like buffers required, ancillary vessels, sanitization protocols, and specific infrastructure that cannot always be scaled up, emphasizing even more the bottleneck these sections suppose in the technical and economical dimensions of bioprocess engineering.

In this tenor, research has been published in order to describe the importance of improving bioprocess economics (Balasundaram et al. 2009). As a first approach, the economic dimension of a bioprocess is dependent on four primary variables: the deep interaction between the performances of the technology, its longevity, and the cost/ease of operation. They establish the initial platform for the economics of a bioprocess. In addition, another important aspect that process engineers or laboratory technicians tend to neglect sometimes is maintenance of the involved unit operations. The heaviest burden for actual bioprocesses resides on effective and selective product fractionation, since traditional unit operations in the biotechnology industry (i.e., chromatography steps with ion exchange, hydrophobic interactions, affinity or gel permeation resins, precipitation techniques, solvent extraction steps, among others) tend to have high operational costs due to the raw materials and consumables

(i.e., chromatographic supports, affinity ligands) needed, as well as to the intensive maintenance required to prolong their industrial life span.

Considering the abovementioned issues, challenges, and opportunity areas for bioprocess engineering designs, along with the potential economic burden that could be part of an industrial application, research has sought solutions within alternative technologies in order to implement them and thus provide a positive scenario for future research and commercial processes. With this in mind, the use of aqueous two-phase systems (ATPSs) has been proposed in the past years since this liquid-liquid extraction technology has shown a valuable option for the downstream processing of complete cells, stem cells, cell membranes, organelles, proteins, and low molecular weight active compounds. Even though this technique has been used since the 1950s, its complete acceptance into the biotech industry has not been deeply studied. Actually, only a few successful industrial processes employing ATPS have been described (Asenjo and Andrews 2012). One of the primary advantages ATPS poses in these processes is the reduced economic burden associated to the extraction process with this technology.

In this context, it is critical to test ATPS in order to obtain an insight on its behavior on the economic scale before a process is actually developed or implemented. Bioprocess modeling is a powerful tool that allows the creation of virtual models that can be both technically and economically analyzed. Using computational packages, the model construction can be based on experimental data obtained from direct research or reported by somebody else. Through this tool, critical operation parameters that affect the most the cost of production can be determined. Then it is possible to develop strategies to maintain costs reduced.

The present chapter aims to describe the potential of ATPS as an economical alternative unit operation for the selective and efficient fractionation of biomolecules when compared to traditional recovery and purification techniques, through the use of bioprocess modeling. This will allow to highlight advantages, disadvantages, and the primary variables to be considered to design economically and technically feasible biotechnology-based processes.

9.2 Bioprocess Modeling in Economic Analysis

When a bioprocess is designed, it is important to consider possible scenarios that could occur during the production stage (parameter variations, fluctuations in supplies costs, etc.). Bioprocess modeling is a powerful tool to create virtual bioprocess that are elaborated through the use of experimental or reported data and used to obtain insights in the production costs. Even more, the use of model-based techniques to analyze the cost of production has the potential to reduce the quantity of experiments needed and to determine where attention needs to be focused after determining critical parts of a bioprocess. This will ultimately reduce production costs (Farid 2007).

One of the best aspects of bioprocess modeling to determine production costs is the incorporation of uncertainty. This means that the probabilistic nature of variations in each production parameter can be emulated in computer software, as long

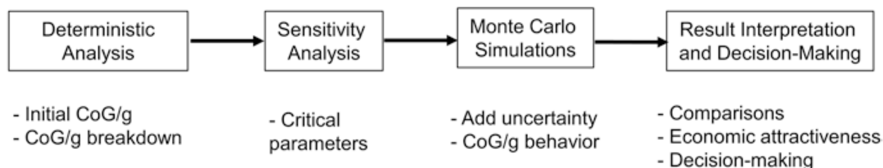

Fig. 9.1 General methodology followed to perform economic evaluations and uncertainty incorporation through bioprocess modeling

as there are reports or experience in how a certain parameter behaves. This allows the bioprocess engineer to quantify the variation of a production cost and therefor to generate multiple strategies to keep this fluctuation to a minimum.

This strategy has been successful to analyze how production cost or cost of goods per gram (CoG/g) behaves. Articles have been published contrasting the effect of using stainless steel or single-use equipment (Farid et al. 2005), different harvest strategies for perfusion reactors (Lim et al. 2005, 2006), strategies for obtaining allogenic cell therapies (Hassan et al. 2015), screening for drugs that are patient specific (Jenkins et al. 2016), and optimization of monoclonal antibody production (Liu et al. 2013, 2015; Rosa et al. 2011). Bioprocess modeling has also been used with positive results to analyze the use of ATPS in potential industrial bioprocesses; with this technique, it has been possible to determine the effect of optimizing production parameters (Torres-Acosta et al. 2015a), to contrast different purification unit operations (chromatography vs. ATPS vs. ATPS with recycling) (Torres-Acosta et al. 2015b), and to understand the behavior of a scaled-up bioprocess (Torres-Acosta et al. 2016).

To perform an economic analysis, there are several reports that are used as outlines, but the most employed methodology is that reported by Farid (2007) (Fig. 9.1). The first step is to construct the model and perform a deterministic analysis; this results in a punctual CoG/g and helps to identify which part of the bioprocess accounts for the largest part of the cost. Then a sensitivity analysis needs to be performed to identify which parameters affect the most the CoG/g behavior. This is performed by systematically increasing and decreasing the values of the model parameters to analyze their effect in the CoG/g, and then the parameters that generated large fluctuations proceed to the next analysis. The Monte Carlo simulations allow the researcher to incorporate uncertainty; this is that the parameters being analyzed can vary under a probabilistic distribution. This analysis provides insights on how a CoG/g can fluctuate during a real bioprocess and helps in the decision-making (risk versus reward). This analysis is performed by a series of simulation runs until a moving average becomes stable; this depends on the model and how strong it is affected by the parameter variations. According to literature, the production parameters that affect the most the CoG/g are the production titer, the downstream processing yield (DSP yield), the changes in materials costs, the target output, the operator wages, the probability of losing a batch, etc. After all the tests have been performed, conclusions are made on how the bioprocess parameters and CoG/g behave; this all allows the researchers and investors to determine the extent of time and resources to be invested on the bioprocess analyzed.

In order to incorporate capabilities of economic analysis into bioprocess modeling, software tools have been developed to facilitate data management. Several computer programs have been created to accomplish this; some of the most used nowadays are Aspen Batch Plus (Aspen Technology, Inc., Bedford, MA, USA), SuperPro Designer (Intelligen, Inc., Scotch Plains, NJ, USA), and BioSolve Process (Biopharm Services Ltd., Chesham, Buckinghamshire, UK). The three software packages have their own strengths and weakness. Aspen Batch Plus and SuperPro Designer have been evaluated before (Shanklin et al. 2001). These software packages have defined unit operations and can display mass and energy balances. They have the potential to generate detailed process diagrams, although their unit operation models are quite simplistic; in the evaluation done (Shanklin et al. 2001), all unit operations for both software were rated poorly. These software have the potential to be more detailed than BioSolve Process, but lack the flexibility that unit operation models have for users to modify them to fit their needs. One additional strength for BioSolve Process is the incorporation of equipment, consumables, and materials directly from suppliers, updated each year. Moreover, BioSolve Process have been developed specifically to calculate production costs, as the whole program is designed around this calculation. Although all software packages will never be 100% accurate, they serve the purpose to estimate either process projection or production costs.

In the next sections, BioSolve Process was used to perform economic analysis to diverse bioprocesses since it was possible to model aqueous two-phase systems and solution construction is easily performed by input of concentrations and not mass values.

9.3 Case Studies

Through the use of bioprocess modeling, it has been possible to perform multiple economic analyses on bioprocesses that involve ATPS. In the next sections, case studies are presented and discussed to demonstrate the economic capabilities of ATPS at pilot or industrial scale and to contrast them with other purification techniques.

9.3.1 Economic Analysis of Scaled-up Production of B-Phycoerythrin

Detailed study presented in Torres-Acosta et al. (2016)

B-Phycoerythrin is a protein related to light absorption in microalgae. It is responsible for giving its red color to the microalgae *Porphyridium cruentum*. Its uses range from food coloring to molecular labeling. The production at the pilot scale using ATPS for its purification has been reported before (Ruiz-Ruiz et al. 2013). That research was used to create a bioprocess model and to determine the economic viability of B-phycoerythrin production. For this process, data required was taken from the literature in its totality.

The model construction was performed in BioSolve Process (Biopharm Services Ltd., Chesham, Buckinghamshire, UK), in which the bioprocess consisted of a photobioreactor for biomass production and then a centrifuge for cell recovery. Protein contents were released through a bead mill and cell debris removed by an additional centrifugal unit operation. For the recovery/purification of B-phycoerythrin, first an isoelectric precipitation was conducted by changing the solution pH and centrifugation to induce pellet formation. Then, an ATPS (PEG1000/PO_4) was applied followed by an ultrafiltration/diafiltration (UF/DF) to remove system components.

The main findings from this research ordered by each analysis are:

1. Deterministic analysis: Materials and consumables are the CoG/g component that has the biggest contribution. This can be explained by two reasons, the first being the size of the ATPS employed, as the sample represents only the 40% of the system, while the second reason is the subsequent step to ATPS, ultrafiltration/diafiltration.
2. Sensitivity analysis: Due to the not-optimized nature of the microalgae culture, the fermentation titer is the most critical parameter for this model; this parameter is responsible for the largest variations in the CoG/g as it affects the protein produced directly. DSP yield has a lower impact as the bioprocess contains a reduced amount of unit operations, compared to other production methodologies (Bermejo et al. 2002, 2007, 2013).
3. Monte Carlo simulations: Fermentation titer, downstream processing yield, and material cost variation are analyzed through Monte Carlo simulations. Using reports in the literature, it is possible to create an optimized scenario for the titer. This new scenario provides insights on how the bioprocess will behave after improvement of a process parameter (titer). Results show that titer optimization causes the potential profits (production cost compared with commercial price) to increase tenfold. The fluctuations in the profits are due, mainly, to the titer and DSP yield variations (standard deviation), and it is important to develop strategies to maintain the CoG/g as constant as possible; the simplest form is to optimize process parameters.

Furthermore, to demonstrate the attractiveness of this bioprocess, an evaluation of the economic viability, through amortization of the capital investment, was performed for a 10-year period for which three possible sales scenarios were created for the process before and after titer optimization. The amortization of the capital is presented in Fig. 9.2, from which it is possible to observe that only the pessimistic scenario before titer optimization does not return the investment, but the improvement of the titer has a deep impact in the economics of a bioprocess, making it profitable and less sensitive to changes in other process parameters.

This study serves to demonstrate the economical capabilities of the scaled-up production of B-phycoerythrin while having ATPS as a central axis. Production of this protein results in an economic viable process that has the potential to attract investors. In order to make it more profitable, the titer requires an optimization, for which strategies to perform this have been published before (Oh et al. 2009; Bermejo et al. 2013; Velea et al. 2011).

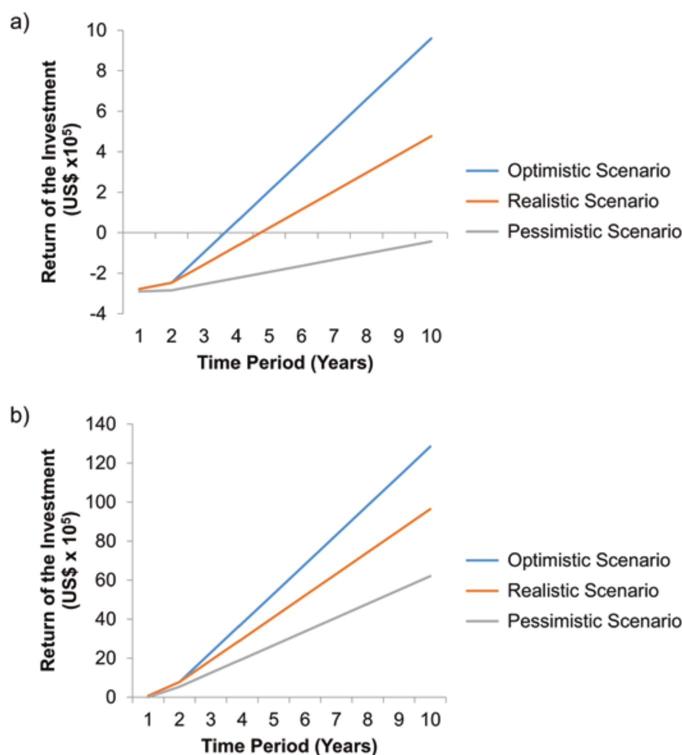

Fig. 9.2 Amortization graphs of the capital invested for the setup of each bioprocess (before and after titer optimization). Each line represents a different sales scenario (pessimistic, realistic, and optimistic scenarios). (**a**) Graph for the bioprocess before titer optimization and (**b**) after titer optimization

9.3.2 Economic Analysis of Royalactin Production

Detailed study presented in Torres-Acosta et al. (2015a)
Royalactin has been found to be the only responsible for the differentiation between worker and queen bees (Kamakura 2011). This protein has several potential applications in different areas, as beneficial effects have been demonstrated in multiple species (Kamakura and Sakaki 2006; Hojo et al. 2010; Kamakura 2011; Detienne et al. 2014). Recently, ATPS was successfully applied to the recovery of royalactin from the fermentation broth of *Pichia pastoris*. (Ibarra-Herrera et al. 2014).

Because of its potential applications and successful recovery, a theoretical bioprocess was scaled up which contained the developed ATPS as its core, and then an economic analysis was performed in order to obtain insights in the CoG/g and the potential of ATPS as an industrial scale.

As a summary from the results of this research:

1. Deterministic analysis: ATPS is known for the use of inexpensive materials, but as many have reported, the amount of materials used for the system construction is larger than the sample input (Ibarra-Herrera et al. 2014; Chen et al. 2010); this

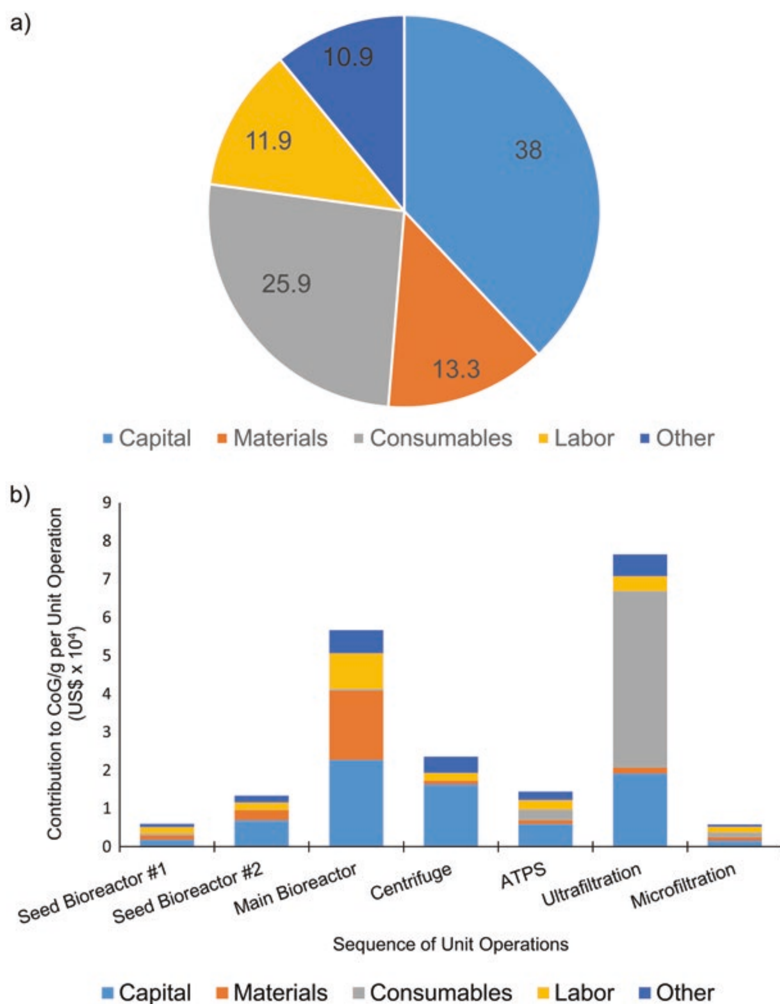

Fig. 9.3 Compositions of the CoG/g as result for the deterministic analysis. (**a**) Composition of CoG/g per cost categories and (**b**) per unit operation per cost category

generates that subsequent operations have the need to handle larger amounts of materials. In this particular case, the unit operation after the ATPS is an ultrafiltration/diafiltration (UF/DF). The costs of UF/DF go up as more membrane filters are needed to process more materials, as it can be seen in Fig. 9.3.

2. Sensitivity analysis: The top three critical parameters for this bioprocess are the fermentation titer, the target output, and the material cost variations, in that order. The three parameters are analyzed then through Monte Carlo simulations.
3. Monte Carlo simulations: From the parameters under this analysis, only the titer can be optimized through research, target output is dependent on the perception

of the product in the market, and material cost variations are controlled by suppliers. An optimized scenario for titer was developed and contrasted with the base scenario for the titer. Results for this analysis are shown in Fig. 9.4.

From this study, along with calculated linear models, it is possible to determine that after optimization of the titer, the key parameter changes from titer to target output, which causes the strategy of a potential company to change from being process oriented to product oriented, meaning that before titer optimization, the focus of a company needs to be in the process to control the parameters and avoid an increase in the CoG/g, but after optimization of process parameters, the attention can shift to the product; this will help to increase the demand and cause an indirect decrease of the CoG/g, as it will be done by increasing attention of the customers and therefore demand of the product.

9.3.3 Comparison Between Chromatography, ATPS, and ATPS with Component Recycle

Detailed study presented in Torres-Acosta et al. (2015b)
Uricase is a biologic pharmaceutical that has application for the treatment of gout, but due to the lack of a human enzyme, its current sources are an *Aspergillus flavus* version expressed in *Saccharomyces cerevisiae* (Perry-Wilson and Berns 2012) for rasburicase and a chimeric version of *Sus scrofa* and baboon (Sherman et al. 2008) expressed in *E. coli*. The first is used as a treatment for tumor lysis syndrome and the latter for treatment-resistant (or refractory) gout, which has been modified through PEGylation to reduce its immunogenicity and to increase its size, in this way to increase its lifetime and action.

The cost of PEGylated uricase has increased to approximately US$5000 per dose, requiring one or two doses per month (Pittman 2011; Helfand 2016). It has become of ultimate importance to develop an economically viable bioprocess in order to capture the most possible market while helping to improve health in society. To solve this, different purification techniques have been developed to reduce the time required to obtain a product, to intensify processes by combining the capabilities of more than one unit operations or by recycling components used in the process in subsequent batches. For uricase production (before PEGylation), usually three chromatographic operations in tandem are required (Li et al. 2006; Liu et al. 2011), but recently an ATPS was developed that could potentially achieve the same level of purity (Chen et al. 2010).

The study from Torres-Acosta et al. (2015b) focused on contrasting the economic capabilities of two unit operations that have been reported to selectively purify uricase, chromatography, and ATPS, but also included two ATPS options: with or without component recycle. This work was done by combining the abilities of bioprocess modeling and economic analysis through the use of BioSolve Process. Two models were constructed which had as a central axis either the chromatographic purification or ATPS recovery. As a base for the bioprocess model size, a reported production in a 25 L bioreactor was used (Li et al. 2006).

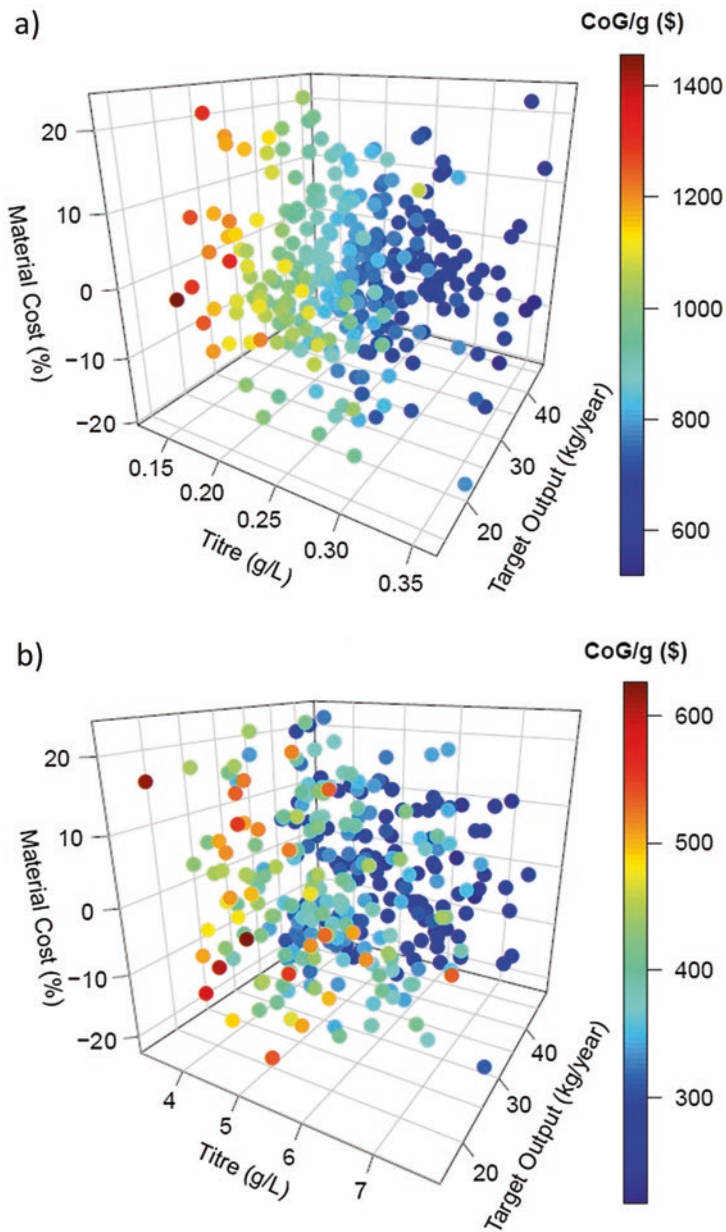

Fig. 9.4 Results for Monte Carlo simulations. Results show behavior of CoG/g under uncertainty for titer, target output, and material cost variation, (**a**) before titer optimization and (**b**) after titer optimization. CoG/g is expressed in US$ (Taken from Torres-Acosta et al. (2015a))

The main results were separated by each type of analysis conducted:

1. Deterministic analysis: This analysis concludes that the CoG/g for each model are US$ 9400 and US$5450 for the chromatographic and ATPS methods, respectively. Chromatographic purification has a larger contribution from the capital charge (equipment) and consumables (resins), while ATPS bioprocess has a larger contribution from materials, which can be explained by the large size of the system constructed (5% sample/95% components).
2. Sensitivity analysis: Results show that DSP yield is the most critical parameter for the bioprocess having as an axis three chromatographic steps, which is to be expected as it has a large number of unit operations, while for ATPS, the titer is the most important process parameter, as it has a reduced number of unit operations and its performance is directly correlated to the amount of uricase produced in the bioreactor.
3. Monte Carlo simulations: The top two parameters from the sensitivity analysis were selected to proceed to the Monte Carlo analysis. Results show that both bioprocesses achieve the lowest CoG/g possible when a combined low titer and DSP yield are achieved. From Fig. 9.5a, b, it can be seen that, as the titer and

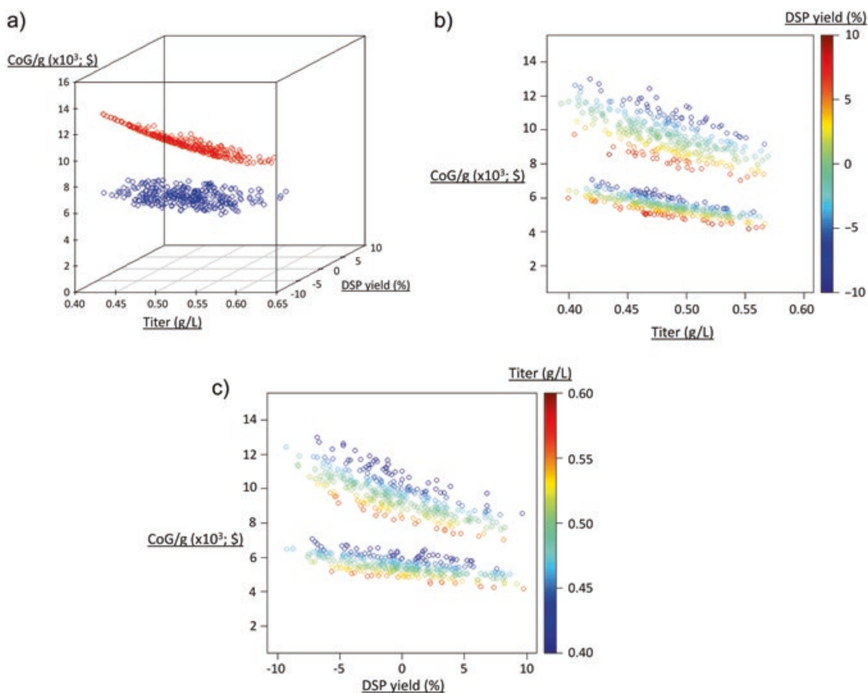

Fig. 9.5 Contrast of results of Monte Carlo simulations for ATPS and chromatography-based bioprocesses. Complete results are showed in (**a**), while (**b**) shows a two-dimensional projection with titer in the x-axis and (**c**) downstream processing yield in the *x*-axis. CoG/g is expressed in US$ (Taken from Torres-Acosta et al. (2015b))

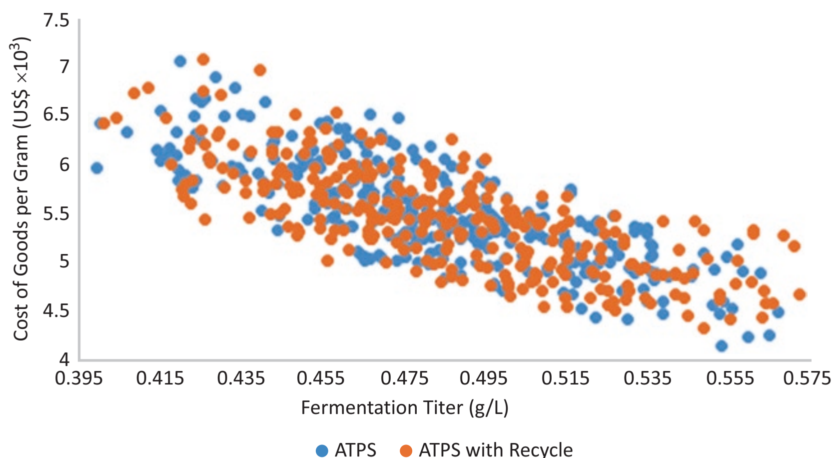

Fig. 9.6 Results for Monte Carlo simulations for ATPS with and without recycling of system components

DSP yield increase, the CoG/g tends to decrease but also to stabilize, therefore suggesting the existence of a region where changes in titer and DSP yield will no longer have a significant impact on the CoG/g. Also, these results represent that for a given product, ATPS provides the lowest cost, and chromatographic purification doesn't have a possible scenario where its costs overlap with ATPS. An additional bioprocess was designed that included recycling of components of the ATPS analyzed here (polyethylene glycol 2000 and phosphates). Results indicates (Fig. 9.6) that ATPS with and without recycle have a comparable CoG/g distribution. These results demonstrate that the use of an additional equipment, time, and resources compensates for the savings achieved by the recycling of phase components; this is a positive result as the use of recycling doesn't contribute to an increase in the CoG/g.

This study served to demonstrates the economic superiority of ATPS against chromatography when both bioprocess are capable of achieving the same purity. More so, it is possible to recycle components of the ATPS to reduce the amount of wastes without having a negative impact in the CoG/g. This serves as a boost for ATPS research, as it will help to develop more accessible therapies by lowering cost of production.

9.3.4 Contrast of ATPS and Protein-A Chromatography for Purification of Monoclonal Antibodies

Detailed study presented in Rosa et al. (2011)

Monoclonal antibodies are one of the current successful biotechnological products. They were estimated to achieve global sales of US$ 75 billion in 2013 (Ecker et al. 2015), but they are also expensive to produce as they require a high purity, several

unit operations, and, in some cases, large doses. As this sector of the biotechnology industry matures, attention has shifted to decrease production costs by reducing the time to market, high quality, and low costs (Farid 2007).

The typical bioprocesses for monoclonal antibody production contain sections for clarification, concentration, selective purification, and virus inactivation. One of the major bottlenecks is the protein-A affinity chromatography located in the selective purification, followed by two additional chromatographic operations (Gottschalk 2008). In order to solve the bottleneck, an ATPS, composed of PEG and phosphate salts, has been recently developed that can selectively isolate the monoclonal antibodies and operate in a continuous mode. More so, it was possible to develop a recycling step for this ATPS to reduce waste disposal and material consumption.

Results indicated that the main contributors to the cost, when utilizing the ATPS, are raw materials (system components), but while operating chromatography, the consumables (protein-A resin) are the largest contributors. Production titer was the most important parameter as it dictates which bioprocess is more economically viable. For low titers, the chromatographic bioprocess is a more viable option as it can capture almost all of the monoclonal antibodies, thanks to the high dynamic binding capacity (DBC) the resins have. After titer increases and exceeds the DBC, ATPS becomes more viable, as it is capable of withstanding larger amounts of sample input.

9.4 Practical Approach for Economic Analyses of ATPS-Based Bioprocesses

The studies presented here follow the methodology mentioned in Fig. 9.1. In order to perform this type of analysis, the key is to be able to mimic a bioprocess in a computer software, in this case BioSolve Process. The procedure followed can be highlighted in clear steps.

1. Data gathering: Each bioprocess has unique requirements for consumables, materials, equipment, process parameters, and operation conditions. These requirements allow the construction of a sequence of unit operations fed with the solutions and consumables that each one requires while operating by a given time.
    - (a) Based on an actual process: When the analysis is based on an existing bioprocess, the data relies on the parameters and materials/consumables employed during production.
    - (b) Based on a theoretical process: If the actual production has not been developed, data to be used is gathered according to reported publications or first-hand experience. One of the parameters that is fundamental for the model creation is the production titer; this value will help to determine the process size, along with the downstream processing yield and production desired (target output).
2. Bioprocess design: Using the data acquired, a bioprocess is designed in its simplest form, with each recipe of solutions and consumables. A bioprocess model can work as a black box where operations can be combined, according to the

capabilities of the modeling software, to make the data input easier, as long as the process engineer knows the implications of doing this and that in reality the practical operability might be different.

3. Data input: The created bioprocess is created now using the software tool and adjusted to fit real production and expenses if it is based on a real process.
4. Deterministic analysis: This analysis collects the results obtained from the model construction. It provides an insight on the composition of the production costs and materials/consumables usage.
5. Sensitivity analysis: The objective of this analysis is to determine parameters that are critical for the production cost. To perform it, two scenarios are created for each potential parameter that is being analyzed, a worst and a best case scenarios. Then each parameter is varied systematically and their respective resultant production cost registered. This gives a range of production cost when each parameter is varied and can be ranked according to their impact in the production cost. A couple of parameters are selected to proceed to the next analysis.
6. Monte Carlo simulations: This step presents the incorporation of uncertainty to the model. The selected parameters from the previous analysis are varied simultaneously under a probabilistic distribution. The less the parameters being analyzed, the shorter the simulation run time and the less data to be analyzed. To perform this, in the studies presented using BioSolve Process, a visual basic program was coded that generated the random values and registered the respective CoG/g obtained.
7. Results interpretation: Results for the Monte Carlo analysis can be analyzed statistically by finding differences of the mean when multiple scenarios or processes are created. Also regression equations can be created to understand the behavior of the data. Tridimensional graphs are helpful to visualize the results.

Through the use of this methodology, insights about the production cost behavior can be obtained. With this, decision-making can have a solid base even before the actual bioprocess is implemented.

9.5 Future Challenges and Trends

ATPSs are constantly being improved by optimizing their efficiency (recovery yield, purification factor, etc.), aspects related to their construction (phase components), and operation (equipment used at small and large scale). This has expanded the possibilities to explore new materials to construct ATPS and to increase the potential to recover/purify with higher selectivity. But it is critical to determine if these new ATPSs are economically viable to be included in bioprocessing. Some of the novel phase components most studied (Ruiz-Ruiz et al. 2012) are ionic liquids, surfactants (aqueous two-phase micellar systems), and alternative polymers (ethylene oxide and propylene oxide), but also protein regions can be used to increase selectivity, for example, carbohydrate-binding molecules.

These novel system components have the potential to be economically viable as they have advantages against traditional ATPS. Ionic liquids can work to form ATPS in conjunction with PEG or salt solutions and have been used to isolated diverse biomolecules, and some of them can be categorized as harmless or practically harmless to the environment (Pereira et al. 2013). Micellar systems have the ability to isolate different types of molecules based on the phase (either organic or aqueous) used to solubilize the surfactants (Roobol-Bóza et al. 2004; Ruiz-Ruiz et al. 2012), most of them are being produced in large quantities and are relatively economic. Alternative polymers are the most promising from a commercial point of view, as they can be manipulated and removed from the product of interest by changes in temperature or by precipitation (Mannesse et al. 1995). The removal of system components is one of the major drawbacks of ATPS, and intensive research can be found in the literature (Persson et al. 2000; Biao et al. 2009; Yan and Cao 2014; Torres-Acosta et al. 2015a, b, 2016; Xu et al. 2016). The removal of system constituents requires at least one additional unit operation, commonly UF/DF, which becomes expensive by the large amount of material to be filtrated.

Additional future challenges for the economic attractiveness of ATPS are operation modes. Case studies presented here operate in batch mode; this means separate steps for system construction, sample loading, and separation. But continuous mode is an option that grants a faster completion of the unit operation process, capable of increasing the recovery yield, and could represent a decrease in costs when combined with system component recycling (Espitia-Saloma et al. 2016).

9.6 Concluding Remarks

Through the content presented in this chapter, it was possible to identify general behaviors that can be generalized among different studies and develop certain considerations that could help the bioprocess engineer to focus research where it is needed in order to maximize profits and capture a larger market.

Three critical concepts have been identified:

1. The fermentation titer is the most critical parameter. This parameter can represent the difference between an economically viable product/process or not.
2. The unit operation in charge of removing ATPS components plays a major role regulating production costs. The presented studies had the largest contribution to the CoG/g in the ultrafiltration/diafiltration operation. Studies in this area are critical to obtain low-cost bioproducts.
3. Recycling of phase-forming components is correlated to ATPS size. It is hypothesized that as the ATPS size increases, the costs for including an additional unit operation will decrease or compensate by the savings from the acquisition of fresh materials. But there should be a critical system size where recycling costs will exceed the saving from new material.

The capabilities of ATPS from an economic point of view have been presented. ATPS are capable of isolating selectively bioproducts making them comparable with traditional unit operations, mainly chromatography. By developing more selective systems with high recovery rates, bioprocesses have the potential to become further economically viable and increase potential profits, like demonstrated here. Although ATPSs have demonstrated economical superiority than other unit operations, they still need to improve certain aspects, for example, the amount of wastes generated. This has been improved by the addition of recycling steps but still needs to be implemented in a more global scale.

Future developments of ATPS need to be evaluated to determine their economic viability. New phase components can be expensive, and regardless of their process capabilities, they can hinder a potential product by increasing its production cost. Therefore, economic evaluations are critical to be performed before any scale-up is done; this will prevent the waste of resources and man power. On the other hand, new phase components might be a cheaper option and become potential alternatives to further reduce production costs and exploit products to maximize profits. ATPS in general represent a viable economical alternative to be used in bioprocesses to produce high value-added products.

References

Asenjo JA, Andrews BA. Aqueous two-phase systems for protein separation: phase separation and applications. J Chromatogr A. 2012;1238:1–10.

Balasundaram B, Harrison S, Bracewell DG. Advances in product release strategies and impact on bioprocess design. Trends Biotechnol. 2009;27(8):477–85.

Bermejo R, Alvárez-Pez JM, Acién FG, Molina E. Recovery of pure B-phycoerythrin from the microalga *Porphyridium cruentum*. J Biotechnol. 2002;93:73–85.

Bermejo R, Ruiz E, Acién FG. Recovery of B-phycoerythrin using expanded bed adsorption chromatography: scale-up of the process. Enzym Microb Technol. 2007;40:927–33.

Bermejo R, Ruiz E, Ramos A, Acién FG. Pilot-scale recovery of phycoerythrin from *Porphyridium cruentum* using expanded bed adsorption chromatography. Separ Sci Technol. 2013;48:1913–22.

Biao N, Junfen W, Xuejun C. Preparation and recycling of aqueous two-phase systems with pH-sensitive amphiphilic terpolymer PADB. Biotechnol Prog. 2009;25(3):820–4.

Chen Y, Meng Y, Tang J, Sun T, Sun F, Liang S. Extraction of uricase from *Candida utilis* by applying polyethylene glycol (PEG)/$(NH_4)SO_4$ aqueous two-phase system. Afr J Biotechnol. 2010;9:4788–95.

Detienne G, De Haes W, Ernst UR, Schoofs L, Temmerman L. Royalactin extends lifespan of *Caenorhabditis elegans* through epidermal growth factor signaling. Exp Gerontol. 2014;60:129–35.

Ecker DM, Jones SD, Levine HL. The therapeutic monoclonal antibody market. mAbs. 2015;7(1):9–14.

Espitia-Saloma E, Vázquez-Villegas P, Rito-Palomares M, Aguilar O. An integrated practical implementation of continuous aqueous two-phase systems for the recovery of human IgG: from the microfluidic device to a multistage bench-scale mixer-settler device. Biotechnol J. 2016;11:708–16.

Farid SS. Process economics of industrial monoclonal antibody manufacture. J Chromatogr B. 2007;848:8–18.

Farid SS, Washbrook J, Titchener-Hooker NJ. Decision-support tool for assessing biomanufacturing strategies under uncertainty: stainless steel versus disposable equipment for clinical trial material preparation. Biotechnol Prog. 2005;21:486–97.

Gottschalk U. Bioseparation in antibody manufacturing: the good, the bad and the ugly. Biotechnol Prog. 2008;24:496–503.

Gronemeyer P, Ditz R, Strube J. Trends in upstream and downstream process development for antibody manufacturing. Bioengineering. 2014;1:188–212.

Hassan S, Simaria AS, Varadaraju H, Gupta S, Warren K, Farid SS. Allogeneic cell therapy bioprocess economics and optimization: downstream processing decisions. Regen Med. 2015;10(5):591–609.

Helfand C. Aiming for $250M sales, Horizon quadruples force behind gout med Krystexxa. FiercePharma [Internet]; 2016 [cited 2017 Jan 19]. Available from: http://www.fiercepharma.com/sales-and-marketing/aiming-for-250 m-sales-horizon-quadruples-force-behind-gout-med-krystexxa.

Hojo M, Kagami T, Sasaki T, Nakamura J, Sasaki M. Reduced expression of major royal jelly protein 1 gene in the mushroom bodies of worker honeybees with reduced learning ability. Apidologie. 2010;41:194–202.

Ibarra-Herrera CC, Torres-Acosta MA, Mendoza-Ochoa GI, Aguilar-Yañez JM, Rito-Palomares M. Recovery of major royal jelly protein 1 expressed in Pichia pastoris in aqueous two-phase systems. J Chem Technol Biotechnol. 2014;89:941–7.

Jenkins M, Bilsland J, Allsopp TE, Ho SA, Farid SS. Patient-specific hiPSC bioprocessing for drug screening: bioprocess economic and optimization. Biochem Eng J. 2016;108:84–97.

Kamakura M. Royalactin induces queen differentiation in honeybees. Nature. 2011;473:478–83.

Kamakura M, Sakaki T. A hypopharyngeal gland protein of the worker honeybee *Apis mellifera* L. enhances proliferation of primary-cultured rat hepatocytes and suppresses apoptosis in the absence of serum. Protein Expr Purif. 2006;45:307–14.

Li J, Chen Z, Hou L, Fan H, Weng S, Xu C, Ren J, Li B, Chen W. High-level expression, purification, and characterization of non-tagged Aspergillus flavus urate oxidase in *Escherichia coli*. Protein Expr Purif. 2006;49:55–9.

Lim AC, Zhou Y, Washbrook J, Sinclair A, Fish B, Francis R, Titchener-Hooker NJ, Farid SS. Application of a decision-support tool to assess pooling strategies in perfusion culture processes under uncertainty. Biotechnol Prog. 2005;21:1231–42.

Lim AC, Washbrook J, Titchener-Hooker NJ, Farid SS. A computer-aided approach to compare the production economics of fed-batch and perfusion culture under uncertainty. Biotechnol Bioeng. 2006;93:687–97.

Liu X, Wen M, Li J, Zhai F, Ruan J, Zhang L, Li S. High-yield expression, purification, characterization, and structure determination of tag-free *Candida utilis* uricase. Appl Microbiol Biotechnol. 2011;92:529–37.

Liu S, Simaria AS, Farid SS, Papageorgiou LG. Designing cost-effective biopharmaceutical facilities using mixed-integer optimization. Biotechnol Prog. 2013;29:1472–83.

Liu S, Simaria AS, Farid SS, Papageorgiou LG. Mathematical programming approaches for downstream processing optimization of biopharmaceuticals. Chem Eng Res Des. 2015;94:18–31.

Mannesse MLM, Cox RC, Koops BC, Verheji HM, de Haas GH, Egmond MR, van der Hijden HTWM, de Vlieg J. Cutinase from Fusarium solani pisi hydrolyzing triglyceride analogues – effect of acyl chain length and position in the substrate molecule on activity and enantioselectivity. Biochemistry. 1995;34:6400–7.

Oh S, Han J, Ha J, Kim S, Jeong M, Jeong H, Kim N, Cho S, Yoon W, Lee S, Kang D, Lee H. Lipid production in *Porphyridium cruentum* grown under different culture conditions. J Biosci Bioeng. 2009;108(5):429–34.

Pereira JFB, Vicente F, Santos-Ebinuma VC, Araújo J, Pessoa A, Freire MG, Coutinho JAP. Extraction of tetracycline from the fermentation broth using aqueous two-phase systems composed of polyethylene glycol and cholinium-based salt. Process Biochem. 2013;48:716–22.

Perry-Wilson D, Berns JS. Onco-nephrology: tumor lysis syndrome. Clin J Am Soc Nephrol. 2012;7(10):1730–9.

Persson J, Kaul A, Tjerneld F. Polymer recycling in aqueous two-phase extractions using thermoseparating ethylene oxide-propylene oxide copolymers. J Chromatogr B Biomed Sci Appl. 2000;743(1–2):115–26.

Pittman G. Gout drug may help some with few treatment options. Reuters [Internet]; 2011 [cited 2017 Jan 19]. Available from: http://www.reuters.com/article/us-gout-drug-idUSTRE77I4RI20110819.

Roobol-Bóza M, Dolby V, Doverskog M, Barrefelt A, Lindqvist F, Oppermann UC, Van Alstine KK, Tjerneld F. Membrane protein isolation by in situ solubilization, partitioning and affinity adsorption in aqueous two-phase systems – purification of the human type 1 11β-hydroxysteroid dehydrogenase. J Chromatogr A. 2004;1043:217–23.

Rosa PAJ, Azevedo AM, Sommerfeld S, Bäcker W, Aires-Barros MR. Aqueous two-phase extraction as a platform in the biomanufacturing industry: economical and environmental sustainability. Biotechnol Adv. 2011;29(6):559–67.

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.

Ruiz-Ruiz F, Benavides J, Rito-Palomares M. Scaling-up of a B-phycoerythrin production and purification bioprocess involving aqueous two-phase systems: practical experiences. Process Biochem. 2013;48:738–45.

Shanklin T, Roper K, Yegneswaran PK, Marten MR. Selection of bioprocess simulation software for industrial applications. Biotechnol Bioeng. 2001;72(4):483–9.

Sherman MR, Saifer MGP, Perez-Ruiz F. PEG-uricase in the management of treatment-resistant gout and hyperuricemia. Adv Drug Deliv Rev. 2008;60:59–68.

Torres-Acosta MA, Aguilar-Yañez JM, Rito-Palomares M, Titchener-Hooker NJ. Economic analysis of royalactin production under uncertainty: evaluating the effect of parameter optimization. Biotechnol Prog. 2015a;31:744–9.

Torres-Acosta MA, Aguilar-Yañez JM, Rito-Palomares M, Titchener-Hooker NJ. Economic analysis of uriciase production under uncertainty: contrast of chromatographic purification and aqueous two-phase extraction. Biotechnol Prog. 2015b;32:126–33.

Torres-Acosta MA, Ruiz-Ruiz F, Aguilar-Yáñez JM, Benavides J, Rito-Palomares M. Economic analysis of pilot-scale production of B-Phycoerythrin. Biotechnol Prog. 2016;32:1472–9.

Velea S, Ilie L, Filipescu L. Optimization of *Porphyridium cruentum* culture growth using two variables experimental design: light and sodium bicarbonate. UPB Sci Bull Series B. 2011;73:1–14.

Xu C, Dong W, Wan J, Cao X. Synthesis of thermo-responsive polymers recycling aqueous two-phase systems and phase formation mechanism with partition of e-polylysine. J Chromatogr A. 2016;1472:44–54.

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.

Perspectives and Future Trends in Aqueous Two-Phase System-Based Technology

10

Jorge Benavides and Marco Rito-Palomares

Abbreviations

ATPS	Aqueous two-phase systems
HGMF	High-gradient magnetic fishing
PEG	Polyethylene glycol

10.1 Introduction

The study, characterization, and application of aqueous two-phase systems (ATPSs) have been conducted since the late 1950s. Nowadays this primary recovery and partial purification technique is regarded as an effective and versatile alternative to more traditional downstream processing approaches. ATPS has been extensively used, particularly at lab scale, for the recovery of a great diversity of biological products, such as proteins, nucleic acids, low molecular weight metabolites, organelles, cells, virus, viruslike particles, nanoparticles, etc. (Benavides and Rito-Palomares 2008; Lima Grilo et al. 2016). However, although much has been

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developed through the last 50 years, there are still developments in the handling, improvement, and alternative uses of ATPS.

One of the major drawbacks that has been associated with ATPS is the lack of selectivity, and even when strategies oriented to the use of affinity ligand (either as co-solutes or covalently attached to one of the constituents of the system) have been developed, there is still a vast field for optimization. Another issue that is frequently discussed when taking about ATPS-related limitations is the technical difficulty of reusing some system constituents, particularly polymers. Besides the technical-related potential drawbacks of ATPS, the studies focused on the economic feasibility of using the technique at industrial scale are scarce. Therefore, although ATPS has proven to be in most cases an effective strategy for the recovery and partial purification of biologicals and overcoming some of the disadvantages of conventional processing approaches (such as solid-phase extraction or liquid chromatography), their use has been primordially limited to lab scale due to the lack of an economic analysis supporting the benefits of their use at industrial level.

Based on these and other needs, the perspectives and future trends related to ATPS-based technology focus on three main topics: (a) ATPS enhancement, (b) novel applications, and (c) economic feasibility. Figure 10.1 depicts in a simplified manner the future trends in ATPS-based technology. In this chapter, the last of the

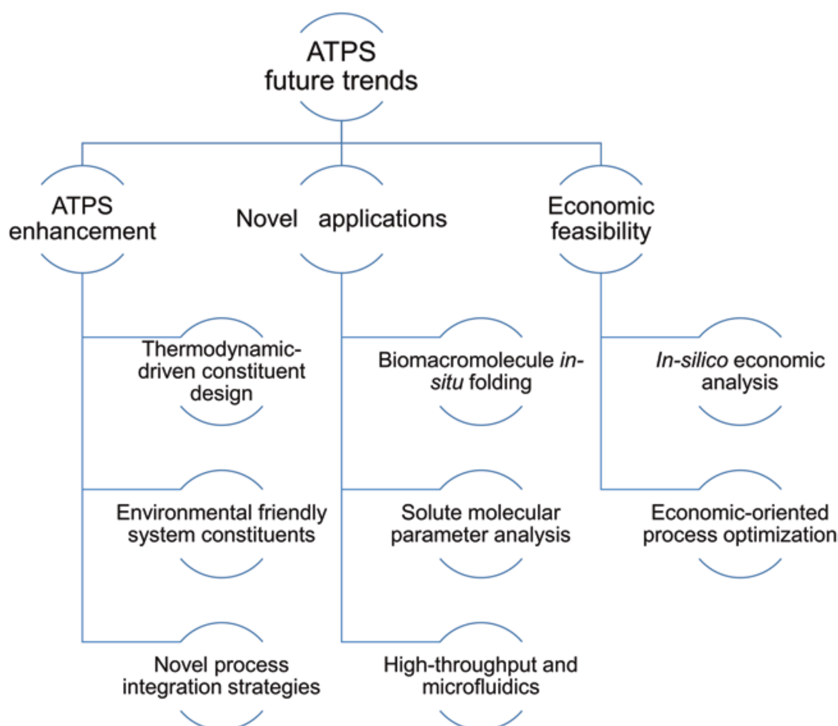

Fig. 10.1 Current and future trends in aqueous two-phase system-based technology

book, we present a brief overview of the trends that are envisioned in the ATPS field for the next years, based on the current developments and processing needs to be addressed.

10.2 Aqueous Two-Phase System Enhancement

Regarding the developments in the ATPS enhancement field, two main research lines are envisioned to strengthen in upcoming years: (a) the development and characterization of novel system constituents and (b) the development of "second-generation process integration strategies."

The development and characterization of novel ATPS constituents has been conducted for the last 20 years, and it is expected to steadily continue in the future. Usually the effectiveness of different novel constituents, either founded in nature (such gums, complex carbohydrates, clays, etc.) or synthetic (chemically produced), is proven following a trial and error approach, in order to correlate the properties of such material with the partition behavior achieved within the system. Although useful, this empirical approach is characterized to be case dependent, costly, and time-consuming. Based on this, in the near future, the development of new constituents is expected to be much more thermodynamic-wise. The use of powerful computational programs oriented to the simulation of thermodynamics on liquid–liquid systems would allow to predict the structure the system constituents should have in order to achieve optimal differential partition between phases. Once the structure of the constituents is determined in silico, the use of multi-scale automated synthesis strategies may reduce cost production at a point in which the systems could be implemented at any production level. Furthermore, the use of this thermodynamic-wise approach may allow the design of constituents serving as affinity ligands, solving the "lack of selectivity" drawback usually attributed to ATPS. If we add to this scenario the use of "smart" polymers that tune their solubility based on the properties of the media, a much more controllable and tunable partition and recovery can be achieved.

Regarding the robustness of the technique, ATPSs have always been considered as a strategy allowing both process integration and intensification. Classic ATPS process integration approaches include extractive fermentation, extractive disruption, and extractive purification, among others. Nowadays, the development of other robust downstream processing techniques allows the design of "second-generation process integration strategies." This involves the use of two or more separation principles for the fractionation of complex biological mixtures with minimal sample preparation. For instance, the study reported by Dhadge et al. (2014), in which the use of ATPS and high-gradient magnetic fishing (HGMF) is combined in one stage, is the perfect example of these "second-generation" process integration strategies. The authors reported that the performance of the integrated stage excels that of the individual operations in terms of recovery yield and purification. In the same way, other robust operations may be integrated to ATPS in order to exploit different separation principles in one stage without the need of removing suspended particles

(such cells, cell debris, etc.) before processing. ATPS are usually regarded as a downstream processing approach for recovery and partial purification. However, it has been demonstrated that they integrated processes involving ATPS that may even be used as a polishing step. Huettmann et al. (2014) reported the use of ATPS for the fractionation and crystallization of a single-chain antibody. Under optimized PEG–sodium sulfate ATPS conditions, the authors achieved a crystallization yield up to 87%, demonstrating that this liquid–liquid biphasic technique can be used not only for recovery and partial purification but also as a polishing. In this regard, it is envisioned that, as part of the development of novel process integration strategies and induced affinity approaches, ATPS could move within the downstream process line to replace purification and polishing stages.

10.3 Aqueous Two-Phase System Alternative Applications

The conventional use of ATPS focuses on the primary recovery and partial purification of biological products. However, due to their biocompatibility characteristics and versatile composition, these liquid–liquid systems can actually be used in diverse biotech-oriented applications.

For instance, as part of the research conducted in our group, it has been confirmed that ATPS can be used as a platform to achieve protein folding while at the same time fractionation and recovery are achieved. In the study reported by Sanchez-Trasviña et al. (2015), it was concluded that the in situ fractionation and folding of invertase in polymer–salt (PEG–potassium phosphate) ATPS was feasible. Furthermore, the authors demonstrate that the system parameters, such as tie-line length and PEG molecular weight, have a significant influence on the protein refolding process. In this sense, it is expected that the folding of other proteins in ATPS can be optimized by using system parameters and constituents promoting electrochemical and hydrophobic interactions prompting the appropriate conformational structure of the product of interest. Although the specific molecular interactions to be exploited may vary significantly from case to case, the vast variety of constituents that can be used for ATPS construction (polymers, salts, ionic liquids, surfactants, etc.) may assure the feasibility of finding adequate folding conditions for most proteins of interest. Moreover, due to their robust nature, ATPS could couple with other techniques based on dilution gradients or surface adsorption to prompt additional molecular mechanisms proven to be involved in protein folding.

Another relatively unexplored application of ATPS is their use as a predictive tool for molecular properties. Once again, based on the recent development of powerful computational tools focused on the prediction of thermodynamic interactions at molecular level, it would be feasible to easily correlate the partition behavior of different solutes within an ATPS and the characteristics of such molecules. Up to now, this approach has been, up to certain level, hampered due to the thermodynamic complexity of the ATPS. An additional approach that has been studied regarding the use of ATPS as an analytical technique is that reported by Aguilar et al. (2009). In such study, ATPS is used along with 2D electrophoresis for the

characterization of complex biological samples. In this way, the liquid–liquid biphasic system may be used in order to fractionate the constituents of the sample by hydrophobicity, while the 2D electrophoresis fractionates by electrochemical charge and molecular weight. Therefore, the combination of these two techniques allows the characterization of hydrophobicity, charge, and size. This constitutes a powerful tool for downstream processing design, as the identity and sequence of stages can be stablished based on the properties and relative abundance of the product of interest and the contaminants. In this same tenor, it was reported by Lima Grilo et al. (2016) that, due to a better understanding of ATPS, the application of these biphasic systems for analytical purposes has increased significantly in the last years. For instance, the authors report that in 2013, approximately 8% of all scientific reports focused on ATPS addressed analytical-oriented applications (Lima Grilo et al. 2016). This upward tendency is expected to continue, particularly with the constant development of robust, thermodynamically oriented, in silico simulation platforms.

The implementation of high-throughput processing technology in the ATPS field also represents an interesting approach from the fractionation and analytical point of view. In this regard, the use of ATPS high-throughput screening approaches may be used to evaluate, in an effective manner, the fractionation behavior of specific solutes under diverse liquid–liquid conditions. These high-throughput approaches overcome the drawbacks that typical ATPS fractionation methodologies follow. For instance, the time needed to characterize the fractionation of a particular solute reduces at least in a scale of magnitude due to the possibility of processing hundreds of samples at a time. Furthermore, high-throughput platforms usually present the flexibility of using volumes on the microliter (μL) scale, reducing the costs related to experimental work. Finally, as the high-throughput platforms are usually completely automated (or at least semiautomated), the experimental variability is usually reduced significantly. This same technology can be implemented not only for characterizing the fractionation behavior of solutes but also for molecular characterization as previously stated. In this regard, high-throughput platforms handling specific ATPS sets, designed with a physicochemical-wise approach, may allow the characterization of molecular properties (hydrophobicity, superficial tension, solubility, etc.) of solutes of interest. In this way, the conjunction of high-throughput platforms and novel microfluidic designs will allow in the near future a complete shift in ATPS experimental strategies.

10.4 Economic Analysis of Aqueous Two-Phase Systems at Large Scale

Aqueous two-phase systems have been in use for a long time since their beginnings in the late 1950s. However, the use of ATPS has focused on the fractionation of solutes at lab and, in some degree, pilot plant scale. However, this liquid–liquid fractionation strategy has not been implemented at larger scale, although demonstrated to be an effective approach for the primary recovery and partial purification

of biologicals. This disparity is believed to be caused, at least partially, due to the lack of experimental data at large scale to support a formal economic analysis for determining the cost feasibility of an industrial ATPS-based process.

In this regard, the development and implementation of robust process simulation software with economic analysis capabilities may, one and for all, demonstrate the technical and economic advantages of using ATPS at large scale, substituting more traditional approaches. Unfortunately, the studies related to the economic analysis of ATPS-based bioprocesses are scarce. Our research group has demonstrated that BioSolve Process (Biopharm Services Ltd., Chesham, Buckinghamshire, UK), a bioprocess simulation software, is robust and effective for the economic analysis of processes involving at least one ATPS stage (Torres-Acosta et al. 2015a, b, 2016). This software is set with experimental data at lab and/or pilot plant scale, allowing the estimation of technical and economic outcomes at larger scale. One relevant characteristic that differentiate BioSolve Process from other specialized bioengineering in silico platforms on the market is its capability of incorporating uncertainty during the analysis of processes. The consideration of uncertainty in key process parameters and responses, based on experimental variability at lab and pilot plant scale, is necessary in order to stablish the economic feasibility in all possible scenarios. This is done by conducting Monte Carlo simulations, allowing the values of the most relevant process parameters to change within a defined range based on experimental variability. This generates a profit/cost analysis of each of the different possible scenarios. Based on such analysis, it is possible to stablish parameters that have a significant effect on process economics and therefore would be of particular interest to optimize once the process is implemented at larger scale. For instance, the simulation of processes reusing ATPS constituents is of great interest due to not only economic but environmental issues. As mention in Chap. 9, it is envisioned that the economic simulation of ATPS-based bioprocesses, either with BioSolve Process or similar programs, will facilitate the change in the reluctance of industry to adopt this fractionation technique at commercial scale.

10.5 Concluding Remarks

Important progress has been done in recent years concerning the application of aqueous two-phase systems for the downstream processing of added value bioproducts. Although originally regarded just as a primary recovery technique, the development of affinity strategies oriented to increasing phase selectivity has allowed the use of ATPS as a purification technique, achieving results similar to those of traditional downstream approaches but reducing processing times and costs. Besides being used in downstream processing, these biphasic systems have also been implemented in alternative applications such as the folding of macromolecules (particularly proteins), the characterization of complex biological samples, and the estimation of molecular properties based on partition behavior between chemically defined phases. Relevant advances have been conducted from the thermodynamic understanding point of view, increasing predictability while studying the partition

behavior of solutes, regardless of the high number of degrees of freedom in the systems.

However, there are still challenges to address before this advantageous technique can be broadly adopted as a commercial-scale processing stage. First systematic approaches are being conducted for the economic analysis of ATPS-based processes. It is expected that the developments in this area will increase the attractiveness of the technique and the willingness of process developers to adopt it at large scale given its technical advantages and cost benefits. Furthermore, the optimization of technology and strategies oriented to recycling ATPS constituents will certainly facilitate their implementation at larger scale, overcoming potential environmental constrains related to waste disposal. In years to come, great advances are envisioned in the study, development, and implementation of ATPS technology at both small and large scale.

References

Aguilar O, Glatz CE, Rito-Palomares M. Characterization of green-tissue protein extract from alfalfa (*Medicago sativa*) exploiting a 3-D technique. J Sep Sci. 2009;32(18):3223–31.

Benavides JA, Rito-Palomares MA. Practical experiences from the development of aqueous two-phase processes for the recovery of high value biological products. J Chem Technol Biotechnol. 2008;83(2):133–42.

Dhadge VL, Rosa SASL, Azevedo A, Aires-Barros R, Roque ACA. Magnetic aqueous two phase fishing: a hybrid process technology for antibody purification. J Chromatogr A. 2014;1339:59–64.

Huettmann H, Berkemeyer M, Buchinger W, Jungbauer A. Preparative crystallization of a single chain antibody using an aqueous two-phase system. Biotechnol Bioeng. 2014;111(11):2192–9.

Lima Grilo A, Aires-Barros MR, Azevedo AM. Partitioning in aqueous two-phase systems: fundamentals, Applications and Trends. Sep Purif Rev. 2016;45:68–80.

Sánchez-Trasviña C, González-Valdez J, Mayolo-Deloisa K, Rito-Palomares M. Impact of aqueous two-phase system design parameters upon the in situ refolding and recovery of invertase. J Chem Technol Biotechnol. 2015;90(10):1765–72.

Torres-Acosta MA, Aguilar-Yañez JM, Rito-Palomares M, Titchener-Hooker NJ. Economic analysis of royalactin production under uncertainty: evaluating the effect of parameter optimization. Biotechnol Prog. 2015a;31:744–9.

Torres-Acosta MA, Aguilar-Yañez JM, Rito-Palomares M, Titchener-Hooker NJ. Economic analysis of uricase production under uncertainty: contrast of chromatographic purification and aqueous two-phase extraction. Biotechnol Prog. 2015b;32:126–33.

Torres-Acosta MA, Ruiz-Ruiz F, Aguilar-Yáñez JM, Benavides J, Rito-Palomares M. Economic analysis of pilot-scale production of B-Phycoerythrin. Biotechnol Prog. 2016;32:1472–9.

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