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

5

Juan Carlos Sánchez-Rangel, Daniel A. Jacobo-Velázquez, and Jorge Benavides

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

J.C. Sánchez-Rangel
Universidad de Colima, Campus Tecomán,
Autopista Colima-Manzanillo km. 40, Tecomán, Col. 28100, México
e-mail: jsanchez4@ucol.mx

D.A. Jacobo-Velázquez • J. Benavides (✉)
Tecnológico de Monterrey, Escuela de Ingeniería y Ciencias, Centro de Biotecnología-FEMSA,
Ave. Eugenio Garza Sada 2501, Monterrey, NL 64849, México
e-mail: djacobov@itesm.mx; jorben@itesm.mx

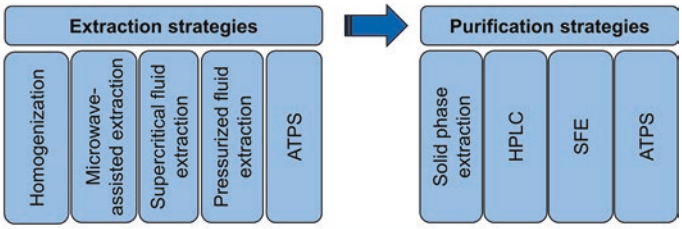
M. Rito-Palomares, J. Benavides (eds.), *Aqueous Two-Phase Systems for Bioprocess Development for the Recovery of Biological Products*, Food Engineering Series, DOI 10.1007/978-3-319-59309-8_5

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



Parameters									
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

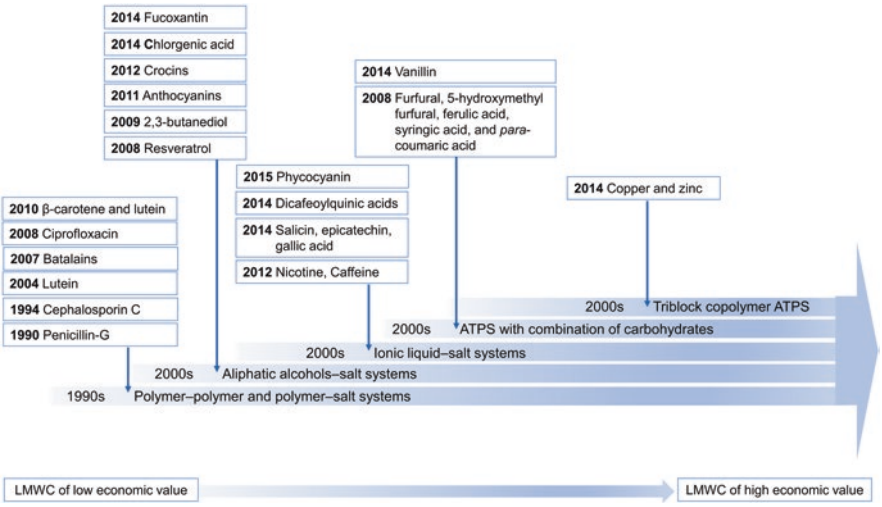


Fig. 5.2 Timeline of the recovery and purification of LMWC in different ATPS. The recovery of LMWC has gone from compounds of low economic value to compounds with significant economic value

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.

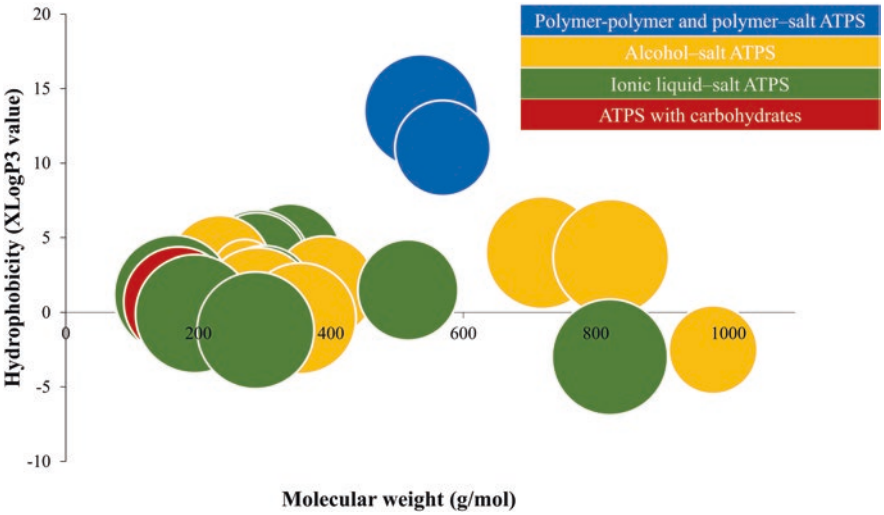


Fig. 5.3 Distribution of LMWC according to their molecular weight and hydrophobicity of the compounds mentioned in Table 5.1. The area of the *circle* represents the recovery yield (%) of the LMWC in specific ATPS strategies

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 pre-treatment 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.