Universidade de Aveiro Departamento de Química
Ano 2018

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DEVELOPMENT OF PURIFICATION PLATFORMS FOR IMMUNOGLOBULIN Y USING AQUEOUS BIPHASIC SYSTEMS

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Tese apresentada à Universidade de Aveiro para cumprimento dos requisitos necessários à obtenção do grau de Doutor em Bioquímica, realizada sob a orientação científica da Doutora Mara Guadalupe Freire Martins, Investigadora Coordenadora do Departamento de Química, CICECO, da Universidade de Aveiro, e coorientação do Professor Doutor Pedro Miguel Dimas Neves Domingues, Professor Auxiliar com Agregação do Departamento de Química da Universidade de Aveiro.

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Para a minha cara-metade.

o júri

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Professora Doutora Rita Maria Pinho Ferreira
Professora Auxiliar do Departamento de Química da Universidade de Aveiro

Doutora Mara Guadalupe Freire Martins
Investigadora Coordenadora do Departamento de Química, CICECO – Instituto de Materiais de Aveiro, da Universidade de Aveiro

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palavras-chave

Biofármacos, anticorpos, imunoglobulina Y, purificação, extração líquido-líquido, sistemas aquosos bifásicos, cromatografia de partição de força centrífuga.

resumo

Com o aumento de microrganismos resistentes a antibióticos e doenças que não respondem a terapias comuns, torna-se fundamental o desenvolvimento de terapias alternativas, económicas e eficazes. A utilização de biofármacos, e em particular anticorpos/imunoglobulinas, é uma alternativa promissora relativamente aos fármacos sintéticos comuns. Dentro da classe dos anticorpos, os obtidos a partir da gema do ovo de galinha, nomeadamente a imunoglobulina Y (IgY), podem ser obtidos em quantidades elevadas por métodos não invasivos, o que pode resultar na produção de biofármacos mais acessíveis. No entanto, o custo de produção da IgY de excelente qualidade e/ ou elevada pureza é ainda superior às terapias comuns devido à escassez de técnicas de purificação rentáveis. Com o objetivo de produzir IgY que possa ser utilizado como um biofármaco recorrente e comum, este trabalho foca-se no estudo de sistemas bifásicos aquosos (SAB) como plataformas de purificação alternativas. Uma vez que a gema de ovo é uma matriz complexa, é necessário um primeiro passo de precipitação dos lipídios/lipoproteínas e recuperação da fração de proteínas solúveis em água (FPSA). A FPSA foi caracterizada por espectrometria de massa (LC-MS/MS) e as principais proteínas presentes nesta fração foram identificadas (IgY, albumina sérica (α-livetina), ovalbumina, ovotransferrina, vitelogenina 1 e vitelogenina 2).

Primeiramente foram estudados SAB constituídos por líquidos iónicos (LIs) e polímeros ou sais para a purificação da IgY a partir da FPSA, nomeadamente SAB constituídos por LIs biocompatíveis e capacidade tampão e PPG 400 (polipropilenoglicol) ou $K_3C_6H_5O_7$. Este estudo permitiu avaliar o efeito do catião e do anião dos LIs, do polímero e do sal na extração seletiva da IgY para uma das fases dos SAB. Posteriormente, foram investigados SAB constituídos por polímeros e sais, nomeadamente SAB compostos por PEG (polietilenoglicol) e Na_2SO_4 ou tampão fosfato (K_2HPO_4/ KH_2PO_4, pH 5,5 - 8,0). Estes sistemas também foram aplicados em cromatografia de partição de força centrífuga (CPC) visando a sua aplicação em larga escala. Avaliou-se o efeito do peso molecular do PEG, o valor de pH, a composição do SAB, e as condições operacionais da CPC (fluxo da fase móvel, rotação e modo de operação). A estabilidade/atividade da IgY foi também avaliada em várias soluções aquosas de LIs, sais e polímeros.

Os melhores resultados em termos de pureza e rendimento de IgY foram obtidos com o SAB do tipo polímero-sal. Em particular, o SAB constituído por 18% de PEG 1000 + 13% de tampão fosfato a pH 6,0 permite obter IgY com uma pureza de 39% e um rendimento de 100% num único passo. Pela aplicação deste SAB em CPC, obteve-se IgY com uma pureza de 51%.

keywords

Biopharmaceuticals, antibodies, immunoglobulin Y, purification, liquid-liquid extraction, aqueous biphasic systems, centrifugal partition chromatography.

abstract

With the increase of antibiotic-resistant microorganisms and diseases that are unresponsive to conventional drugs, it is imperative the development of alternative, economical and effective therapeutics. The use of biopharmaceuticals, and in particular antibodies/immunoglobulins, is a promising alternative over common synthetic pharmaceuticals. Among these, antibodies obtained from chicken egg yolk, namely immunoglobulin Y (IgY), can be obtained in high titers by non-invasive methods, opening the door to low-cost biopharmaceuticals. Nevertheless, the production cost of high-quality and high-purity IgY remains higher than other drug therapies due to the lack of cost-efficient purification techniques. Aiming at producing IgY that could ultimately be used as a recurrent and widespread biopharmaceutical, this work is focused on the selective extraction of IgY from egg yolk using aqueous biphasic systems (ABS) as alternative purification platforms. Since egg yolk is a complex matrix, a first step including the lipids/lipoproteins precipitation and the water-soluble protein fraction (WSPF) recovery is required. The WSPF was recovered and characterized by mass spectrometry (LC-MS/MS) and the main proteins identified (IgY, serum albumin (α-livetin), ovalbumin, ovotransferrin, vitellogenin 1 and vitellogenin 2).

ABS composed of ionic liquids (ILs) and polymers or salts were initially investigated to purify IgY from the egg yolk WSPF, namely ABS composed of biocompatible and self-buffering ILs and PPG 400 (poly(propylene) glycol) or $K_3C_6H_5O_7$. This study allowed to conclude on the ILs cation and anion, and polymer and salt effects on the selective extraction of IgY to one of the ABS phases. Subsequently, polymer-salt-based ABS were investigated, namely ABS composed of PEG (polyethylene glycol) and Na_2SO_4 or phosphate salt buffer (K_2HPO_4/ KH_2PO_4, pH 5.5 - 8.0). These systems were also applied in centrifugal partitioning chromatography (CPC) while foreseeing the technology scale-up. The effect of the molecular weight of PEG, pH, mixture composition of the ABS, and CPC operating conditions (mobile phase flow rate, rotation, and the operation mode) were evaluated. The IgY stability/activity in various aqueous solutions of ILs, salts, and polymers was assessed.

The best results regarding purity and yield of IgY were obtained with polymer-based ABS. In particular, the ABS composed of 18 wt% PEG 1000 + 13 wt% phosphate buffer at pH 6.0 allows obtaining IgY with a purity of 39% and yield of 100% in a single step. By the application of this ABS in CPC, IgY with a purity of 51% was obtained.

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List of symbols

EE % – extraction efficiency

K – partition coefficient

wt % – weight fraction percentage

List of abbreviations

[Ch][CHES] – cholinium 2-(cyclohexylamino)ethanesulfonate

[Ch][HEPES] – cholinium 2-[4-(2-hydroxyethyl)piperazin-1-yl]ethanesulfonate

[Ch][MES] – cholinium 2-(N-morpholino)ethanesulfonate

[Ch][TES] – cholinium 2-[(2-hydroxy-1,1-bis(hydroxymethyl)ethyl)amino]ethanesulfonate

[Ch][Tricine] – cholinium N-[tris(hydroxymethyl)methyl]glycinate

[N_{4444}][CHES] – tetrabutylammonium 2-(cyclohexylamino)ethanesulfonate

[N_{4444}][HEPES] – tetrabutylammonium 2-[4-(2-hydroxyethyl)piperazin-1-yl]ethanesulfonate

[N_{4444}][MES] – tetrabutylammonium 2-(N-morpholino)ethanesulfonate

[N_{4444}][TES] – tetrabutylammonium 2-[(2-hydroxy-1,1-bis(hydroxymethyl)ethyl)amino]ethanesulfonate

[N_{4444}][Tricine] – tetrabutylammonium N-[tris(hydroxymethyl)methyl]glycinate

[P_{4444}][CHES] – tetrabutylphosphonium 2-(cyclohexylamino)ethanesulfonate

[P_{4444}][HEPES] – tetrabutylphosphonium 2-[4-(2-hydroxyethyl)piperazin-1-yl]ethanesulfonate

[P_{4444}][MES] – tetrabutylphosphonium 2-(N-morpholino)ethanesulfonate

[P_{4444}][TES] – tetrabutylphosphonium 2-[(2-hydroxy-1,1-bis(hydroxymethyl)ethyl)amino]ethanesulfonate

[P_{4444}][Tricine] – tetrabutylphosphonium N-[tris(hydroxymethyl)methyl]glycinate

ABS – aqueous biphasic system

CCC – countercurrent chromatography

CHO – Chinese hamster ovary

CPC – centrifugal partition chromatography

DTT – 1,4-dithiothreitol

ELISA – enzyme-linked immunosorbent assay

FDA – Food and Drug Administration

GB – Good's buffers

GB-ILs – Good's buffers ionic liquids

HCP – host cell proteins

Ig – immunoglobulin

IgG – immunoglobulin G

IgY – immunoglobulin Y

IL – ionic liquid

LDL – low-density lipoproteins

mAb – monoclonal antibody

MS – mass spectrometry

Nano-LC-MS/MS – nano-liquid chromatography-tandem mass spectrometry

Nano-LC-orbitrap MS – nano-liquid chromatography-mass spectrometry using an Orbitrap high-resolution instrument

PEG – polyethylene glycol

PEG 1000 – polyethylene glycol with a molecular weight of 1000 $g \cdot mol^{-1}$

PEG 3000 – polyethylene glycol with a molecular weight of 3000 $g \cdot mol^{-1}$

PEG 3350 – polyethylene glycol with a molecular weight of 3350 $g \cdot mol^{-1}$

PEG 600 – polyethylene glycol with a molecular weight of 600 $g \cdot mol^{-1}$

PEG 6000 – polyethylene glycol with a molecular weight of 6000 $g \cdot mol^{-1}$

PPG 400 – polypropylene glycol with a molecular weight of 400 $g \cdot mol^{-1}$

SDS-PAGE – sodium dodecyl sulfate polyacrylamide gel electrophoresis

SE-HPLC – size exclusion-high performance liquid chromatography

TL – tie-line

TLL – tie-line length

WSPF – water-soluble proteins fraction

1. Brief introduction and main objectives

According to the World Health Organization, the occurrence of antibiotic-resistant microorganisms is nowadays one of the most severe threats to global health, food security, and development. Antibiotic resistance is rising to dangerous levels in all world and can affect anyone, of any age, in any country [1]. In this context, the development of new effective antimicrobial therapeutics, such as biopharmaceuticals, is a crucial goal to be achieved within the next few years [2]. Biopharmaceuticals have significantly improved the treatment of many diseases and sometimes are the only approved therapies available for a specific disease. These biologic-based products, which include antibodies, recombinant proteins, and nucleic-acid-based products, have application in several therapeutic areas, such as immunization and oncology, and in the treatment of autoimmune, cardiovascular, inflammatory and neurological diseases [3]. The first biopharmaceutical approved by the Food and Drug Administration (FDA) was the human recombinant insulin Humulin® produced by Escherichia coli, in 1982, for the treatment of Diabetes Mellitus [4]. In 1986, the first therapeutic protein obtained from mammalian cells reached the market, and in the same year, FDA also approved the first therapeutic monoclonal antibody (mAb), Orthoclone OKT3 (muromonab-CD3), produced *in vivo* by hybridoma cell technology [4]. The global market for biopharmaceutical products is currently over US$ 200 billion (Figure 1.1.) and is projected to achieve US$ 498 billion in 2020 [5].

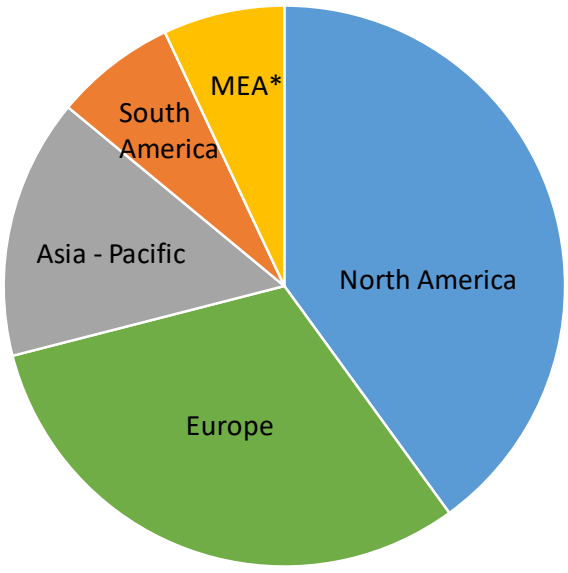

Figure 1.1. Current biopharmaceutical market distribution: global revenue in US$ million by region. Adapted from [5]. *MEA – Middle East and Africa.

Since 1986, with the first therapeutic mAb product approval, an increase in the commercial potential of antibodies as prospective therapeutic drugs was observed [6]. At the current approval rate of around four new products *per* year, seventy mAbs will possibly be in the market by 2020, and combined worldwide sales for these products will be nearly US$ 125 billion [6]. The ability of antibodies to bind to an antigen with a high degree of affinity and specificity is the main reason behind their high performance to act as biopharmaceuticals [7].

Antibodies are glycoproteins found in plasma and extracellular fluids of all vertebrate species, being members of a particular family of molecules - immunoglobulins (Ig), which constitute the humoral branch of the immune system and represent approximately 20 % of the plasma proteins in humans [8, 9]. Antibodies are host proteins produced in response to bacteria, viruses, or other foreign molecules or agents [9], being this response a key mechanism used by the host organism for protection. Each animal can produce millions of different specific antibodies, and each antibody can specifically bind to a particular foreign substance known as an antigen [8]. mAbs are identical antibodies produced by a single B-lymphocyte clone, and all of them can only recognize unique *epitopes*, or binding sites, on a single antigen [8]. In contrast, polyclonal antibodies are frequently produced in animals and recognize independent *epitopes* on the antigen and can bind to different substances [9]. Regarding antibody production, although it is essential that the antibodies be pathogen-specific, the monospecificity of mAbs can be a disadvantage regarding effectiveness [10]. Polyclonal antibodies (immunoglobulin G, IgG) have been conventionally isolated from *sera* of animals, such as rabbits, goats, and sheep which have been superimmunized with an aimed antigen [11]. However, the use of chickens for the production of polyclonal antibodies (immunoglobulin Y, IgY) provides several advantages [12]. IgY is recovered by a non-invasive method which does not cause pain to animals or lead to their death since it is based on the act of collecting eggs [11]. Moreover, few eggs *per* week can provide the same amount of immunoglobulins as repeated bleeding of immunized mammalians. Usually, more than 100 mg of IgY can be isolated *per* egg [11]. IgY is the functional equivalent of mammal IgG formed during egg formation [2]. Regarding their structure, IgY presents two heavy chains with a molecular weight of 65 kDa each and two light chains with a molecular weight of 25 kDa each, and a molecular weight of ~180 kDa (larger than mammalian IgG (~159 kDa)) [13, 14]. The

main difference between IgG and IgY is the number of constant regions in the heavy chains: IgG has three C regions, while IgY has four regions (Figure 1.2.) [13]. The heavy chain of IgY does not present a hinge in contrast with the IgG structure, where two of the three constant domains are separated by a hinge [15]. The absence of this hinge region in IgY reduces de mobility of the antibody containing the variable domains (the portion containing the antigen-binding site), and the intra-molecular forces of IgY are thus weaker than those in IgG [14, 16]. IgY is also more hydrophobic than IgG, and it has a lower isoelectric point [17]. The IgY isoelectric point ranges between 5.7 and 7.6, whereas that of IgG is in the range between 6.1 and 8.5 [13, 14, 17].

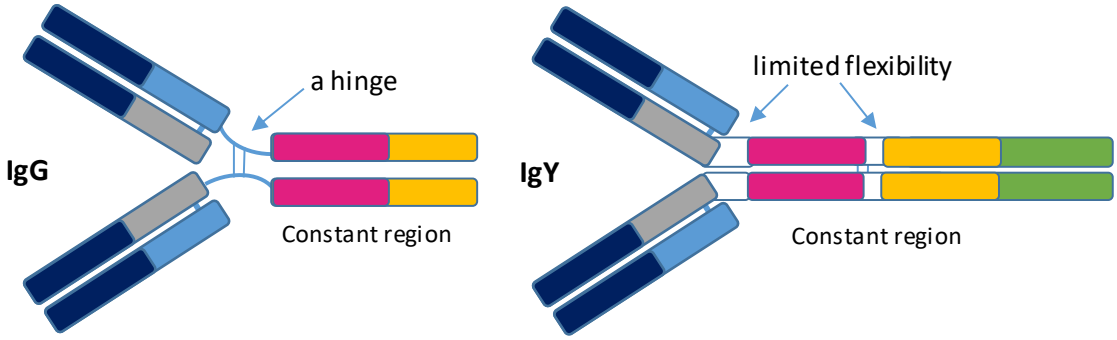

Figure 1.2. Structure of IgG and IgY.

Despite the advantages of IgY over other antibodies, only less than 2% of the total polyclonal antibodies produced worldwide are IgY [18]. This low percentage is due to the high production cost of high-quality IgY, given the difficulties in isolating antibodies from the complex egg yolk matrix [12]. Therefore, several attempts to purify IgY have been described in the literature for the purification of IgY from egg yolk, which can be divided into three main groups, namely: (i) precipitation methods, involving simple water dilution [19], and addition of salts [20], organic solvents [21, 22] or polymers (such as polyethylene glycol (PEG) [23] or natural gums [24, 25]); (ii) chromatographic methods, namely ion exchange [19, 26, 27], gel filtration [19], hydrophobic interaction [28, 29] and affinity chromatographies [30]; and (iii) ultrafiltration [19, 31, 32]. These methods are usually applied in two steps. Given the complex nature of egg yolk, in a first step, the lipids and lipoproteins are removed, allowing to obtain a water-soluble protein fraction (WSPF) from egg yolk. Then, the WSPF is used to purify and recover IgY [33, 34]. It should be however remarked that most methods described in the literature for the purification of IgY are time-consuming, labor intensive, low yielding, have high cost and

cannot easily be scaled-up for industrial applications [35]. Also, in some of these protocols, the solvents used are not innocuous (e.g., chloroform), which can be seen as a drawback when considering the recovery of IgY as a potential biopharmaceutical.

Taking in account the potential of IgY as an alternative biopharmaceutical and the described difficulties in its purification from egg yolk, this PhD thesis focused on the evaluation of aqueous biphasic systems (ABS) as alternative purification platforms for the purification of IgY from the WSPF, that contains the target antibody, obtained from egg yolk. ABS is a type of liquid-liquid extraction process formed by ternary mixtures, *i.e.,* two solutes (polymer-polymer, polymer-salt or salt-salt combinations) and water. Above given concentrations of these solutes, there is phase separation and the creation of two-phase systems in which both phases are aqueous, one enriched in each solute. Based on the advantages of ABS, particularly considering their high water content which could be beneficial for labile macromolecules such as proteins/antibodies, this PhD thesis contemplates the initial recovery of the WSPF from egg yolk, the optimization of ABS at the laboratory scale to purify IgY from the WSPF, and a final step addressing their use in centrifugal partition chromatography (CPC) foreseeing large-scale applications. Furthermore, the stability and activity of IgY were evaluated in several aqueous solutions and in several steps of the developed processes. The results obtained during this Ph.D. thesis development will be presented in 3 main chapters (Figure 1.3.):

(i) recovery of the WSPF from egg yolk and identification of the proteins present in this fraction (Chapter 2.);

(ii) evaluation of ABS composed of ionic liquids (ILs) and salts/polymers and on their selective potential to extract IgY from the WSPF for one of the ABS' phases, and study of the IgY stability/activity in the presence of aqueous solutions of ILs, salts, and polymers (Chapter 3. - subchapter 3.1.);

(iii) evaluation of polymer-salt-based ABS capable of selectively extracting IgY from the WSPF and application of the optimized ABS in centrifugal partition chromatography (CPC) (Chapter 3. - subchapter 3.2.).

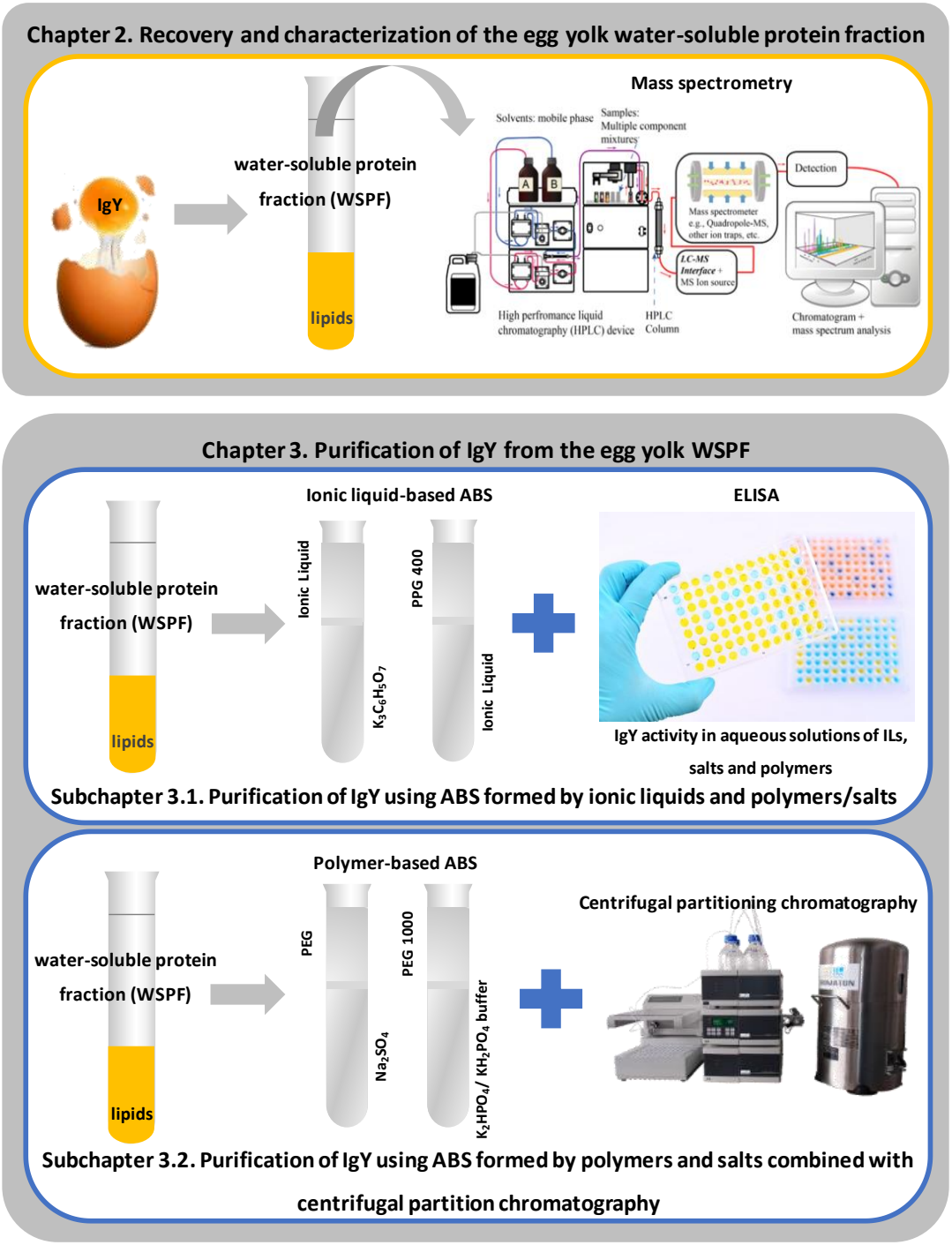

Figure 1.3. Layout of the current thesis.

The first set of results comprise the characterization of the egg yolk WSPF, *i.e.*, the identification of the proteins present in this fraction by mass spectrometry (MS). MS has become a standard method for proteins identification in complex mixtures of biological nature, such as egg yolk [36]. Using a nano-LC-MS/MS approach, Nilsson et al. [37] identified 26 proteins in the WSPF. Later, Mann and Mann [38] identified 119 proteins

in egg yolk also using nano-LC-MS/MS, 86 of which had not been previously identified [37]. In this work, the identification of proteins was performed by nano-LC-orbitrap MS/MS of peptides prepared by in-gel tryptic digestion after separation in sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE), 1-D gel, and slice-by-slice analysis. The WSPF was obtained according to Liu et al. [31]. This set of results allowed us to adequately characterize the WSPF used in the subsequent purification studies by the application of ABS and to infer the IgY purity in this fraction.

After the water-soluble proteins were identified by MS, the capability of ABS composed of ILs and salts or polymers to selectively extract IgY from the egg yolk WSPF was evaluated, and the IgY stability/activity in the presence of aqueous solutions of ILs, salts, and polymers studied. Although the use of ABS composed of two polymers or a polymer and a salt for the purification of mAbs was previously addressed [39-42], these systems display a restricted polarity difference between the two phases, thus compromising their success as purification platforms [42]. Therefore, the addition of salts [43, 44] or specific ligands [41, 42], hybrid processes [45], among others, have been investigated aiming at increasing the purification performance of these platforms. In 2003, Gutowski et al. [46] proposed a novel approach to create ABS by applying ILs, which have shown later to be able to overcome the significant drawback associated to the more traditional polymer-based ABS [47]. ILs are salts that are liquid below a conventional temperature of 100 °C, and they are usually composed of a large asymmetric organic cation and either an organic or inorganic anion. The ability to tailor the polarities of the coexisting phases by the proper manipulation of cation/anion IL chemical structure is the major benefit of IL-based ABS [47, 48]. Moreover, IL-based ABS are substantially less viscous than typically polymer-based ABS and usually display a faster phase separation rate [47, 49]. Due to these advantages, the separation of the most diverse biomolecules using IL-based ABS has been extensively investigated [50-55], including proteins and enzymes [56-59]. However, most of the ILs investigated for ABS formation affect the pH of the aqueous solution, a major drawback when the goal is the extraction of proteins such as antibodies. The pH can be however adjusted to optimum values by the addition of proper ILs with buffer capacity [60]. Good's buffers (GBs), which are zwitterionic amino acid derivatives, can be used as anion or cation radicals of ILs - Good's buffers ionic liquids (GB-ILs) - covering the physiological pH range (6 to 10) [61]. Both GBs and IL-

derived GBs are protein structure stabilizers [60]. Based on these advantages, these ILs were studied in this work. The first set of IL-based ABS studied were composed of polypropylene glycol 400 g·mol^{-1} (PPG 400), and cholinium-based ILs combined with anions derived from GBs. The partition coefficients (*K*) and extraction efficiencies (*EE*%) of the total protein content in the WSPF to the IL-rich phase were determined, allowing to confirm the preferential partitioning of all proteins to this phase. SDS-PAGE results further demonstrated that the corresponding band of the IgY heavy chain does not significantly change, providing evidence for the lack of antibodies' aggregation or fragmentation in the IL-rich phase. Furthermore, a slight difference in the SDS-PAGE bands intensity was observed, meaning that these systems display a slight (but still not sufficient) selectivity for the target antibody. Based on these results, a second set of IL-based ABS was investigated to purify IgY from the WSPF from egg yolk. ABS composed of $K_3C_6H_5O_7$ and GB-ILs synthesized by the combination of the same Good's buffers derived anions with the tetrabutylphosphonium ($[P_{4444}]^+$), and tetrabutylammonium ($[N_{4444}]^+$) cations were investigated. In this set of ILs, a salt was used instead of a polymer since these ILs do not form ABS with PPG 400 (as experimentally confirmed). Despite the already proved high efficiency of GB-ILs in the extraction of proteins [60], the selective extraction of IgY to the IL-rich phase was not adequately achieved with this type of systems, thus hindering it adequate purification. The proteins profile of the coexisting phases obtained by size exclusion-high performance liquid chromatography (SE-HPLC) allowed concluding that all proteins migrate to the top (IL-rich) phase and that a complex between the ILs and the different proteins present in the WSPF is formed. Overall, IL-based ABS display a high extraction ability, but lack in selectivity since all proteins tend to be enriched in the IL-rich phase. Trying to better understand the formation of the IL-IgY complex and induced precipitation of some proteins from the WSPF, the impact of several aqueous solutions of ILs and other ABS phase-forming components on the IgY stability and activity was evaluated. By enzyme-linked immunosorbent assays (ELISA) it was demonstrated that IgY has higher activity in aqueous polymer solutions, followed by salts and ILs aqueous solutions. Furthermore, the IgY activity decreases with the increase of polymer or IL concentration and increases with the salt concentration. Regarding the polymers and salts investigated, it was found that polyethylene glycol 600 g·mol^{-1} (PEG 600) and Na_2SO_4 allow higher preservation of the IgY activity.

After gathering the first set of results with IL-based ABS and the evaluation of the IgY stability/activity in the presence of aqueous solutions of ILs, salts, and polymers, the use of ABS composed of polymers and salts was investigated. This type of systems is also advantageous when foreseeing the technology scale-up given the lower cost of the ABS phase-forming components. Although conventional polymer-based ABS display a small polarity difference between the two phases, as highlighted before, these systems were previously investigated for the purification of mAbs, namely IgG [39-42]. Salt-polymer-type ABS provide advantages over ABS formed by polymer-polymer combinations, such as low interfacial tension, fast and high phase separation rate and low cost, turning them more viable for downstream processing [50]. These ABS are usually chosen for the extraction/purification of biomolecules, such as antibodies, due to their biocompatibility since most of the polymers used have a stabilizing effect on the protein tertiary structure [39-41]. PEG is commonly used since it presents high biodegradability, low toxicity, low volatility, low melting temperature, high water miscibility and low cost [50, 62]. In 2007, Rosa et al. [42] described the partitioning of immunoglobulins in conventional polymer-polymer and polymer-salt ABS, and purification factors of 2.7 and 1.9 were reported. In this work, authors showed the purification of IgG from an artificial mixture of proteins containing human serum albumin and myoglobin, using an ABS formed by PEG 3350 and a K_2HPO_4/ NaH_2PO_4 buffer [42]. The same group of authors later reported the recovery of human IgG from Chinese hamster ovary (CHO) and hybridoma cell culture supernatants using an ABS constituted by PEG 6000 and K_2HPO_4/ NaH_2PO_4 buffer at pH 6 [44]. Recovery yields of 88 and 90% in the polymer-rich phase and purification factors of 4.3 and 4.1 were reported.

Based on the drawbacks associated with the purification of IgY from the WSPF using IL-based ABS, on the evidences of polymer-salt ABS to purify IgG, on the results obtained by ELISA, and on the potential of polymer-salt-based ABS to be scaled-up by CPC, ABS formed by PEG and Na_2SO_4 were then investigated to purify IgY from the egg yolk WSPF. The effect of the molecular weight of PEG (200, 300, 400 and 600 $g \cdot mol^{-1}$) on the purification of IgY was addressed, and the influence of the ternary mixture composition was evaluated. The results obtained with the several polymers revealed the complete migration of IgY and other contaminant proteins in the WSPF to the polymer-rich phase, except the system composed of PEG 600 in which selectivity was identified (IgY

completely partitions to the PEG-rich phase whereas the contaminant proteins partition similarly between the coexisting phases). Furthermore, the change of the PEG and salt concentrations in the ternary mixture composition led to a higher selectivity. Based on these results, this ABS was applied in CPC, a multistage liquid-liquid approach, while also foreseeing the scale-up and industrial implementation of the developed process.

CPC is a type of liquid-liquid chromatography requiring two immiscible liquid phases, one of which acts as the stationary phase and the other acts as the mobile phase (CPC uses centrifugal force to hold the stationary liquid phase). Evidence on the use of CPC for the successful separation/purification of biomolecules was already reported [63]. Schwienheer et al. [64] applied the ABS formed by PEG 3000 and K_2HPO_4/NaH_2PO_4 buffer at pH 7 in CPC to separate laccases, demonstrating the feasibility of ABS and CPC to purify biologically active biomolecules. Oelmeier et al. [65] used CPC as an alternative separation step combined with other precipitation and resolubilization techniques, allowing the purification of mAbs in an easily scalable approach. The authors found that after removing the cells from culture supernatant, either via centrifugation or by liquid-liquid separation approaches by ABS (PEG 400 and $Na_3C_6H_5O_7$/ citric acid buffer pH 6), and performing a CPC run in dual-mode, an upper phase enriched in antibodies and free of host cell proteins could be attained [65]. Based on these literature results and our previous results, ABS composed of PEG 600 and Na_2SO_4 were investigated in CPC to purify IgY. Nevertheless, due to the similarity of the phases' density, it was not possible to retain the liquid stationary phase at all the operating conditions tested (flow-rate, rotation speed, and ascending/descending mode). For a successful application of CPC, there are three main parameters that should be considered for the selection of a proper two-phase liquid-liquid system to be used: easiness of two-phases formation (difference in densities of the phases); retention capacity of the phases in the CPC cells; and separation/purification effectiveness [66]. To develop an adequate system and composition, a literature review was carried out to identify polymer-salt ABS successfully used in CPC, although with different purposes of purification [65, 67-69]. Sutherland et al. [67], for instance, studied the scale-up of the separation process of lysozyme and myoglobin. Moreover, successful results provided by ABS formed by PEG 1000 and K_2HPO_4 combined with CPC on the purification of enzymes [70] and anti-HIV mAbs [71] were reported. Therefore, these ABS phase-forming components were

considered in our subsequent investigations. The systems formed by PEG 1000 and K_2HPO_4 demonstrated sufficient retention to be used in CPC assays, and further optimization was carried out. To this end, K_2HPO_4/ KH_2PO_4 buffer solutions at different pH values were prepared, namely 5.5, 6.0, 6.5, 7.5 and 8.0, to appraise the effect of the pH on the selective extraction of IgY from the WSPF. By the analysis of both phases by SE-HPLC it was revealed that all the proteins in the WSPF have a higher affinity to the top-rich phase (polymer-rich phase), in which high yields were found, yet with low IgY purity levels (≈ 30-40%) in a single ABS step. Nevertheless, SE-HPLC results revealed that this system is highly specific for IgY, which partitions entirely to the top phase, while the remaining proteins partition among both phases, being thus promising to be applied in CPC. The best results regarding purity and yield of IgY were obtained with the ABS composed of 18 wt% PEG 1000 + 13 wt% K_2HPO_4/ KH_2PO_4 buffer at pH 6.0. An IgY purity of 39% and a yield of 100% were achieved in a single-step ABS. By the application of this ABS in CPC, IgY with a purity of 51% was successfully obtained from the egg yolk WSPF. The following chapters comprise a brief introduction to each topic/section and the results obtained during this thesis development.

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2. Recovery and characterization of the egg yolk WSPF

Some of the experimental work presented in this section was developed with the collaboration and inputs of Doctor Ricardo Pires and Doctor Sofia Guedes.

Abstract

In order to purify and recover IgY from egg yolk to be used as an alternative biopharmaceutical, several methods have been described in the literature, all of them comprising a first step to remove lipids and lipoproteins to obtain the water-soluble protein fraction (WSPF) that contains the target antibody. This fraction is then used to recover and purify IgY in a second or further/multiple steps. Therefore, the characterization of all proteins present in the egg yolk WSPF is of high relevance given the potential application of IgY as a biopharmaceutical and to appraise the complexity of the matrix from which IgY will be purified. Methods described in the literature were used to obtain the WSPF from egg yolk, and the proteins profile was characterized by one-dimensional gel electrophoresis (SDS-PAGE) and label-free quantitative nano-liquid chromatography-tandem mass spectrometry (nano-LC-MS/MS) using an Orbitrap high-resolution instrument. Using this approach six major proteins were identified in the WSPF: the target antibody IgY, serum albumin (α-livetin), ovalbumin, ovotransferrin, vitellogenin 1, and vitellogenin 2.

Introduction

Hen eggs are the largest biological cells known resulting from one cell division [1]. Since the mid-1970s, many studies on physiological functions of egg components and on the seeking of novel biologically active substances have been conducted [2]. Eggs are a storehouse of variable nutrients, namely proteins, lipids, carbohydrates and other substances with biological functions that extend beyond basic nutrition [1, 3]. Egg yolk is considered as a important source of active principles, including antibodies which may be used in the therapeutic, pharmaceutical, cosmetic and biotechnological areas [3-5]. Egg yolk is mainly composed of proteins and lipids, which can be separated into a granular and a plasma fraction (Table 2.1.) [6]. The plasma fraction is composed of low-density lipoproteins (LDL) and a water-soluble protein fraction (WSPF). The WSPF contains livetins, including α-, β-, and γ-livetin [3]. γ-livetin is predominantly constituted

by immunoglobulin Y (IgY) [3]. IgY is the functional equivalent of mammal immunoglobulin G (IgG) and is formed during egg formation. IgY in the serum is selectively transferred to the yolk via a receptor on the surface of the yolk membrane specific for IgY translocation and to passively protect the chicks development [4, 7]. IgY is composed of two heavy chains, with a molecular weight of 65 kDa each, and two light chains, with a molecular weight of 25 kDa each [8, 9].

Table 2.1. Detailed average composition of fresh egg yolk (adapted from the Handbook of food proteins [10]).

Component	Subgroup	Main Molecules	Content (wt%)
Water			48
Lipids (34%)	Triglycerides	FA (C16:0), PUFA (C18:2), MUFA (C18:1)	22.6
	Phospholipids (9.6%)	Phosphatidylcholine	7.0
		Phosphatidylethanolamine	1.4
		Sphingomyelin	0.6
		Lysophosphatidylethanolamine	0.2
		Plasmogen	0.1
		Inositol phospholipid	0.01
	Sterols	Mainly cholesterol	1.8
Proteins		Phosvitin	1.8
		Livetins (α-, β-, γ-)	5.0
		Lipovitellin (α-, β-) = HDL	5.8
		Lipovitellinin = LDL	3.5
Carbohydrates		Free glucose	0.2
Vitamins		A, D, E, K, B1, B2, B6, B13, …	0.8
Minerals		P, Ca, Na, K, Cl, S, M, Fe	1.0

The interest in the use of IgY over mammal IgG for immunotherapeutic and immunodiagnostic purposes has largely increased in the past years [7]. Few eggs *per* week can provide the same amount of immunoglobulins as repeated bleeding of immunized mammalians, and more than 100 mg of IgY can be isolated *per* egg [1]. Some works providing comparative studies on the production of antibodies by immunized

hens and rabbits can be found in the literature [11, 12]. Moreover, IgY is attractive for immunotherapy because it neither activates the human complement system or reacts with rheumatoid factors, human anti-mouse antibodies, or human Fc receptors, contrary to mammal IgG antibodies, which are all well-known cell activators and mediators of inflammation [13]. Therefore, specific IgY antibodies are an alternative to more common and currently used biopharmaceuticals.

There are several reported works showing the potential of IgY against different pathogens and to effectively treat and prevent infections, both in humans [14-16] and other mammals [17]. For instance, IgY against rotavirus was protective against bovine rotavirus both in calves and in mice, whereas anti-*Escherichia coli* IgY reduced mortality in new-born piglets [17]. Salmonellosis has been prevented both in neonatal calves and in a mice model. In humans, IgY against *Streptococcus mutans* decreased caries when used as a mouth rinse [14], whereas anti-*Helicobacter pylori* IgY reduced *Helicobacter* infections [15]. The longest study with IgY to treat humans has been performed by studying anti-*Pseudomonas* IgY in cystic fibrosis patients, which were treated for up to 13 years [18, 19]. Chronic *Pseudomonas aeruginosa* lung infections are one of the major causes of morbidity and mortality in cystic fibrosis patients [16]. The IgY administration prevented *Pseudomonas aeruginosa* infections and delayed the time to chronic infections [18, 19].

There are several reported methods to purify IgY from egg yolk, which are in general divided into two major steps. These methods and IgY purity and recovery yields are summarized in Table 2.2. First, the fraction of egg yolk containing the water-soluble protein fraction, WSPF, which contains IgY, is isolated, removing lipids and lipoproteins. Then, in a second step, the isolation of immunoglobulins from the WSPF is carried out [20, 21]. Several approaches have been described for the first step of IgY purification, for instance by water dilution followed by centrifugation [22, 23]. This method is based on the aggregation of yolk lipoproteins at low ionic strengths [7]. For these dilution methods, optimization of the operational conditions (pH, temperature, dilution factor, etc.) allowed to increase the IgY recovery up to 90% [24]. However, different authors have reported different results and have thus different perspectives. Nakai et al. [25] reported that the best results were obtained using a six-fold water dilution, at pH 5.0. Luo et al. [26] demonstrated that the best results are obtained with a fifteen-fold water

dilution. Marcet et al. [3] proposed a 1.5 water dilution, at pH 7.0, of the egg yolk. Beyond the water dilution methods, other approaches were developed to separate IgY from the lipoproteins and other impurities, including delipidation by organic solvents (isopropanol and acetone [27] and chloroform [28]), by a mixture with κ-carrageenan, low-methoxyl pectin and $CaCl_2$ [13], and lipoprotein precipitation using polyethylene glycol (PEG) [3, 25, 29], and xanthan gum [25]. Hydrophobic interaction chromatography has also been used to separate the WSPF from lipoproteins [30].

The IgY purification from the WSPF (second or multiple subsequent steps) has been carried out with the addition of ammonium sulfate [23, 31], sodium sulfate [22, 26], sodium alginate [2], cadmium sulfate [31], zinc sulfate [31], sodium chloride [32], dextran sulfate [25], or PEG [25], and by ultrafiltration [22]. Ion exchange chromatography [33] has also been used as a final step in the IgY purification, as well as gel filtration [30]. Because of the failure of IgY to bind to proteins A and G, several other types of affinity chromatography have been investigated, including immobilized metal ion affinity chromatography, thiophilic interaction chromatography, affinity chromatography using alkaline conditions, and synthetic peptide ligands specifically designed for immobilizing antibodies [7, 34].

All the protocols described above demonstrate different results in what concerns the IgY purity, and all of them are not capable to completely purify IgY in one-step. Aiming at better understanding the complexity of the WSPF and given the final aim of using IgY as an alternative biopharmaceutical, in this work, it was carried out the characterization and identification of all proteins present in the WSPF. Based on the procedures reported to remove the lipidic part of egg yolk and to isolate the WSPF, and considering some experimental studies that were initially conducted, the protocol to extract WSPF from egg yolk reported by Liu et al. [22] was chosen. This protocol comprises the water-dilution of egg yolk, freeze/thawing, and centrifugation. The proteins profile in the WSPF was assessed by one-dimensional gel electrophoresis (SDS-PAGE) and label-free quantitative nano-liquid chromatography-tandem mass spectrometry (nano-LC-MS/MS) using an Orbitrap high-resolution instrument.

Table 2.2. Comparison of the literature methods for the WSPF isolation and IgY purification.

Step 1 - Isolation of WSPF from egg yolk	Step 2 – IgY purification from WSPF				
Purification method	**Purification method**	**IgY Recovery (%)**	**IgY (mg/ml)**	**Purity of IgY (%)**	**Ref.**
Water-dilution	Sodium sulfate	--	--	--	[22]
	Ammonium sulfate	69.07	--	--	[23]
		--	--	72.7	[26]
	Ultrafiltration	91/87	--	85/93	[24]
		--	--	--	[22]
	Sodium alginate	--	--	75	[3]
		--	--	--	[2]
	Cadmium sulfate	--	1.9	31.8	[31]
	Zinc sulfate	--	2.4	41.2	
	Sodium chloride	24	--	--	[32]
	Dextran sulfate	71	--	--	[25]
	Affinity chromatography	--	--	--	[34]
Hydrophobic interaction chromatography	Gel filtration	--	--	--	[30]
Polyethylene glycol	Ion exchange chromatography	--	--	--	[33]
	Polyethylene glycol	--	--	96	[3]
Isopropanol/ acetone	Isopropanol	69/58**	160/120	1.0/1.1*	[27]
Chloroform	Polyethylene glycol	--	--	--	[28]
κ-carrageenan, low-methoxyl pectin and $CaCl_2$	Ammonium sulfate	--	--	80	[13]
Xanthan gum	Sodium sulfate	81	--	83	[25]
Caprylic acid	Ammonium sulfate	97/90**	160/120	4.0/4.3*	[27]

*Purification (fold) - Final volume of the sample divided by the initial volume of the egg yolk used.

**IgY content in the purified sample divided by the original IgY content in the egg yolk.

Experimental Section

Materials

Fresh eggs were periodically provided by Dr. Ricardo Pires from Biocant, located in Cantanhede, Portugal. The reagents used in the preparation of the gels for the SDS-PAGE analysis were supplied by Bio-Rad. Laemmli buffer, Coomassie Brilliant Blue G-250 were purchased from Sigma- Sigma-Aldrich. Trypsin was purchased from Promega.

Water-soluble proteins fractionation

The WSPF of egg yolk was prepared from fresh eggs, following the protocol described by Liu et al. [22]. This method was selected based on preliminary experimental studies carried out by us and results described in the literature. This protocol includes a dilution of egg yolk with distilled water (1:6, v:v) followed by a pH adjustment (pH 5.0), a freeze (overnight)/thaw and a final step of centrifugation.

Size exclusion-high performance liquid chromatography (SE-HPLC) was applied to separate the proteins present in the WSPF by size. An ÄKTA pure instrument was used for this purpose, with a GE Healthcare High-Performance column Superdex^{TM} 200 10/300 GL. A 100 mM phosphate buffer with NaCl 0.3 M was run isocratically with a flow rate of 0.5 mL·min^{-1}. The injection volume was 25 µL. The protein content on collected samples was quantified at 280 nm using a NanoDrop ND-100 Spectrophotometer.

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE)

The protein profile of the coexisting phases was investigated by SDS-PAGE using a Mini-PROTEAN® Tetra Vertical Electrophoresis Cell from Bio-Rad. The protein concentration was quantified at 280 nm using a NanoDrop ND-100 Spectrophotometer, diluted in order to achieve a total protein content of 0.003 mg, and further mixed with the Laemmli buffer (2:1, v:v). The samples were then subjected to SDS-PAGE in 15% polyacrylamide gels. The proteins were stained with Coomassie Brilliant Blue G-250 overnight and then distained at room temperature. All gels were analyzed using the Image Lab 3.0 (BIO-RAD) analysis tool.

Tryptic digestion, mass spectrometry analysis, and protein identification

Tryptic digestion was performed according to [35], with a few modifications. Protein bands were manually excised from the gel and transferred to eppendorf tubes. Replicate bands were excised and also identified. The gel pieces were washed three times with 25 mM ammonium bicarbonate/ 50% acetonitrile and one time with acetonitrile. The protein's cysteine residues were reduced with 6.5 mM 1,4-dithiothreitol (DTT) and alkylated with 54 mM iodoacetamide. Gel pieces were dried in a SpeedVac (Thermo Savant) and rehydrated in digestion buffer containing 12.5 $\mu g \cdot mL^{-1}$ sequence grade modified porcine trypsin in 25 mM ammonium bicarbonate. After 90 min, the supernatant was removed and discarded, 100 µL of 25 mM ammonium bicarbonate was added, and the samples were incubated overnight at 37 °C. Extraction of tryptic peptides was performed by the addition of 10% formic acid/ 50% acetonitrile three times and finally with acetonitrile. Tryptic peptides were lyophilized in a SpeedVac (Thermo Savant) and resuspended in 5% acetonitrile/ 0.1% formic acid solution. The samples were analyzed with a QExactive Orbitrap (Thermo Fisher Scientific) that was coupled to an Ultimate 3000 (Dionex, Sunnyvale) HPLC system. The trap (5 mm × 300 µm I.D.) and analytical (150 mm × 75 µm I.D.) columns used were C18 Pepmap100 (Dionex, LC Packings), the latter having a particle size of 3 µm. Peptides were trapped at 30 µl/ min in 95% solvent A (0.1% formic acid: 5% acetonitrile, v:v). Elution was achieved with the solvent B (0.1% formic acid: 100% acetonitrile, v:v) at 300 nL/ min. The 50 min gradient used was as follows: 0 - 3 min, 95% solvent A; 3 - 35 min, 5 - 45% solvent B; 35 - 38 min, 45 - 80% solvent B; 38 - 39 min, 80% solvent B; 39 - 40 min, 20 - 95% solvent A; 40 - 50 min, 95% solvent A. Nanospray was achieved using an uncoated fused silica emitter (New Objective) (o.d. 360 µm; i.d. 50 µm, tip i.d. 15 µm) biased to 1.8 kV. The mass spectrometer was operated in the data-dependent acquisition mode. An MS2 method was used with an FT survey scan from 375 to 1600 m/z (resolution 35,000; AGC target 3E6). The 10 most intense peaks were subjected to HCD fragmentation (resolution 17,500; AGC target 5E4, NCE 25%, max. injection time 120 ms, dynamic exclusion 35 s). Spectra were processed and analyzed using Proteome Discoverer (version 2.0, Thermo), with the MS Amanda search engine (version 2.1.4.3751, University of Applied Sciences Upper Austria, Research Institute of Molecular Pathology). Uniprot (TrEMBL and Swiss-

Prot) protein sequence database (version of January 2016) was used for all searches under Gallus gallus. Database search parameters were as follows: carbamidomethylation and carboxymethyl of cysteine as a variable modification as well as oxidation of methionine, and the allowance for up to two missed tryptic cleavages. The peptide mass tolerance was 10 ppm, and fragment ion mass tolerance was 0.05 Da. To achieve a 1% false discovery rate, the Percolator (version 2.0, Thermo) node was implemented for a decoy database search strategy and peptides were filtered for high confidence and a minimum length of 6 amino acids, and proteins were filtered for a minimum number of peptide sequences of 2 and only rank 1 peptides.

Results and discussion

The WSPF of egg yolk was obtained according to the protocol described by Liu et al. [22], where the lipids and lipoproteins are removed as an isolated and precipitated fraction. Figure 2.1. shows the SE-HPLC chromatogram of the egg yolk WSPF, which includes 3 major peaks (A, B and C). There is a small peak before peak A (higher molecular weight proteins) that was not considered in the analysis and discussion of the results regarding the identification of the several proteins present since it is related with an aggregation phenomenon [36]. According to the literature, there are three classes of livetins in egg yolk: γ-livetins (180 kDa), α-livetins (80 kDa) and β-livetins (45 kDa) [37]. The ratio between them in egg yolk is 3:2:5, being in close agreement with the SE-HPLC results and the identified peaks A, B, and C in Figure 2.1. The γ-livetin fraction is predominantly constituted by IgY as described in the literature [3, 38]; however, only a few reports describe the specific proteins that constitute α-livetins and β-livetins, namely serum albumin and α2-glycoprotein, respectively [38]. In order to identify the several proteins in the WSPF, this matrix was initially fractionated by SE-HPLC, where the proteins are separated according to their molecular weight. For each peak (A, B and C), two fractions were collected, with a total of 6 collected samples (A, A', B, B', and C, C'). These samples were then analyzed by SDS-PAGE, with SDS-gels of 15%, for better separation and refinement of individual proteins separated by molecular weight.

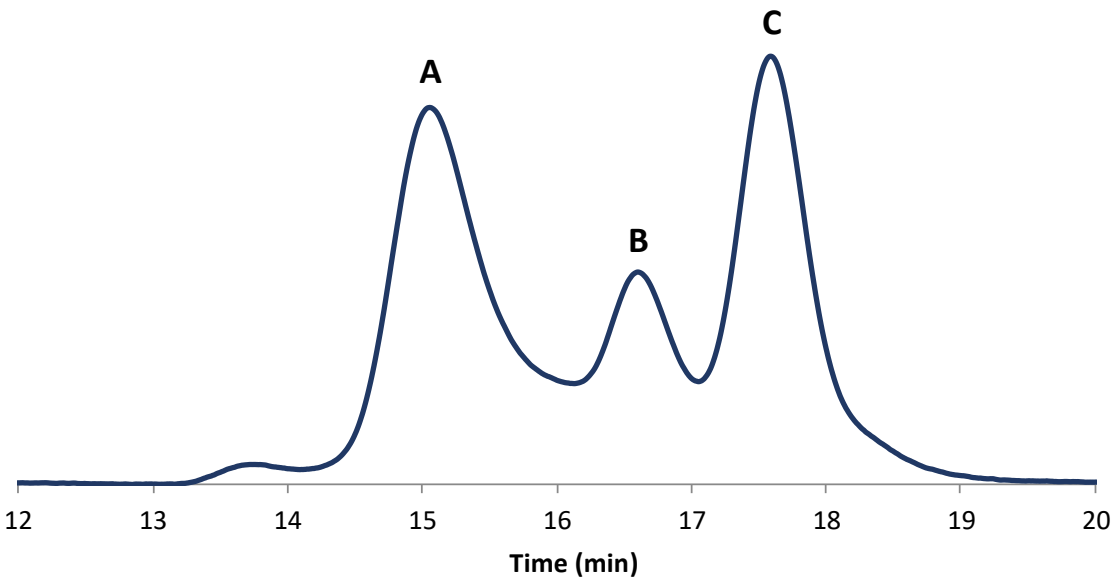

Figure 2.1. SE-HPLC chromatogram of the WSPF from egg yolk.

Figure 2.2. shows the proteins profile of each sample collected from the initial gel filtration by SE-HPLC. These SDS-PAGE results (obtained under reducing conditions), show that there are two major bands corresponding to peak A (samples A and A'), and which correspond to the heavy (≈70 kDa) and light (≈25 kDa) chains of IgY. These results agree with the information previously reported in the literature describing IgY as the major constituent of γ-livetins [38]. Although the main protein of peak A was identified, also based on literature data [3, 38], peaks B and C are composed of several non-identified small size proteins (< 48 kDa). In order to identify them by mass spectrometry (MS), each band/spot was excised (identified by the orange squares in Figure 2.2.). A total of 27 bands/spots were subjected to trypsin digestion and peptide extraction, and the digested peptides were analyzed by nano-LC-M/MS. In this setup, peptides were first separated in a nano-HPLC system, and tandem mass spectrometric analysis was carried out in a QExactive instrument (Thermo Scientific).

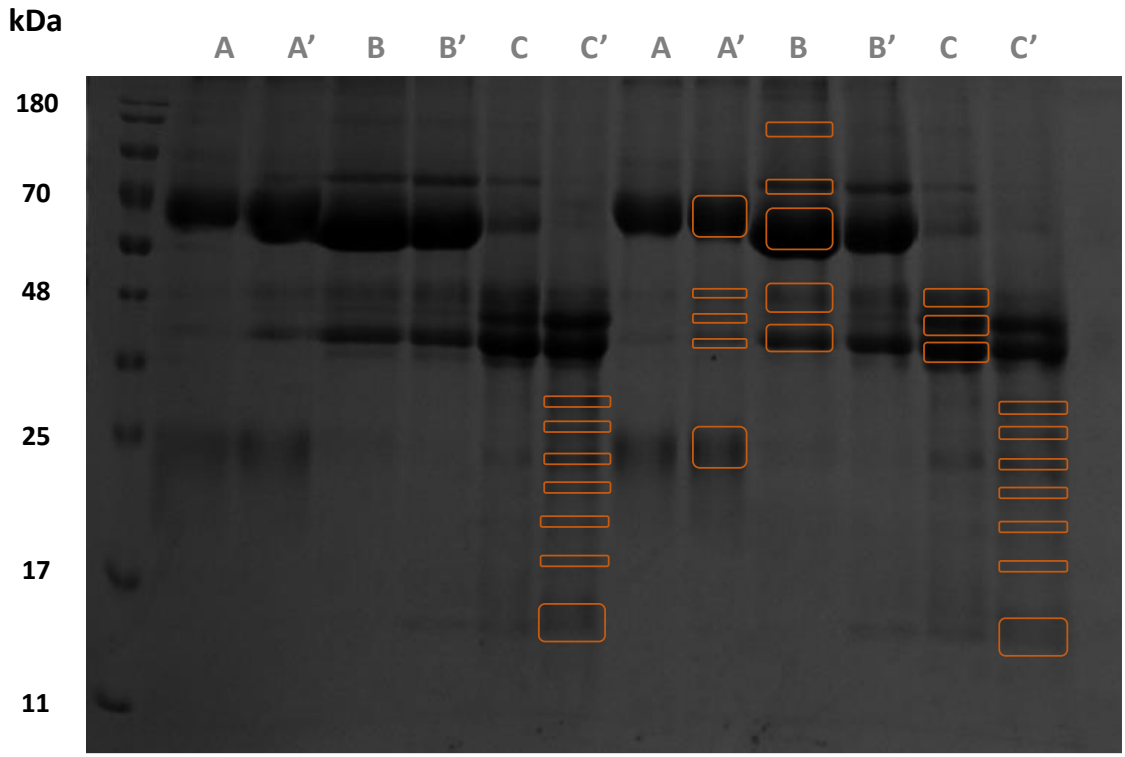

Figure 2.2. SDS-PAGE stained with Coomassie blue for each fraction of the WSPF collected from gel filtration chromatographic separation.

The number of peptides and identification of the correspondent proteins of samples A, B and C are provided in Table 2.3. Two proteins were identified in the sample corresponding to peak A: the target protein IgY (identified as Ig lambda chain C region) and chicken serum albumin usually denominated as α-livetin [38, 39]. By the analysis of Table 2.3., it is notable that the identification of IgY occurs only in peak A, which is accordance with Figure 2.2. (SDS-PAGE), where the bands corresponding to IgY are not present in samples B and C. The proteins predominantly identified in peaks B and C are also present in peak A. Two main proteins were identified in the sample corresponding to peak B, namely serum albumin and ovotransferrin (a glycoprotein of egg white that can be transferred to the yolk). In peak C a high number of peptides identifying vitellogenin 1 and 2 (precursors from which are derived the smaller and well-characterized yolk proteins lipovitellin 1 and 2 and phosvitin) were observed. Serum Albumin was also identified in peak C.

Table 2.3. Identified proteins in the egg yolk WSPF using the *Gallus gallus* proteome in the UniProt databank [40].

	Accession number	Protein	Number of peptides
Peak A	P20763	Serum albumin OS=Gallus gallus GN=ALB PE=1 SV=2	10
	P19121	Ig lambda chain C region OS=Gallus gallus PE=4 SV=1	10
	P01012	Ovalbumin OS=Gallus gallus GN=SERPINB14 PE=1 SV=2	7
	P02845	Vitellogenin-1 OS=Gallus gallus GN=VTG1 PE=1 SV=1	5
	P87498	Vitellogenin-2 OS=Gallus gallus GN=VTG2 PE=1 SV=1	4
Peak B	P19121	Serum albumin OS=Gallus gallus GN=ALB PE=1 SV=2	81
	P02789	Ovotransferrin OS=Gallus gallus PE=1 SV=2	41
	P02845	Vitellogenin-2 OS=Gallus gallus GN=VTG2 PE=1 SV=1	8
	P87498	Vitellogenin-1 OS=Gallus gallus GN=VTG1 PE=1 SV=1	4
	P01012	Ovalbumin OS=Gallus gallus GN=SERPINB14 PE=1 SV=2	4
Peak C	P87498	Vitellogenin-1 OS=Gallus gallus GN=VTG1 PE=1 SV=1	34
	P19121	Serum albumin OS=Gallus gallus GN=ALB PE=1 SV=2	29
	P02845	Vitellogenin-2 OS=Gallus gallus GN=VTG2 PE=1 SV=1	23
	P01012	Ovalbumin OS=Gallus gallus GN=SERPINB14 PE=1 SV=2	12
	P02789	Ovotransferrin OS=Gallus gallus PE=1 SV=2	10

The proteins identified in this work agree with those previously identified by other authors, such as Mann and Mann [41] and Nilsson et al. [6]. The first group of authors [41] identified some plasma proteins in egg yolk, in which our results agree with the identification of serum albumin. Furthermore, egg white proteins (ovalbumin, ovomucoid, ovotransferrin, cystatin, and ovoinhibitor) were identified by Man and Man [41] and by us (ovalbumin and ovotransferrin) in the WSPF obtained from egg yolk. Some of these proteins display relevant biological activities. For instance, ovotransferrin and cystatin have antibacterial activity, whereas ovomucoid and ovoinhibitor have antiviral activity [38]. Nilsson et al. [6] also reported other egg white proteins in the WSPF of egg yolks, such as fibrinogen, hemopexin, and coagulation factor IX. In addition, lipoproteins of the egg yolk have also been suggested to have an antibacterial effect [38], and several apolipoproteins were detected in the WSPF: apolipoprotein A1, apolipoprotein B,

vitellogenins 1 and 2, and apovitellenin 1 by Nilson *et al.* [6], and vitellogenins 1 and 2 by us.

Based on the MS results and literature data [42, 43], Figure 2.3. depicts the identification of each major protein present in each spot resolved by SDS-PAGE. In summary, by using different techniques we are able to identify the main proteins presents in the WSPF obtained from egg yolk, namely the target IgY in the sample corresponding to peak A, serum albumin (α-livetin), ovotransferrin and vitellogenin 2 in the sample corresponding to peak B, and vitellogenin 1, vitellogenin 2 and ovalbumin in the sample corresponding to peak C.

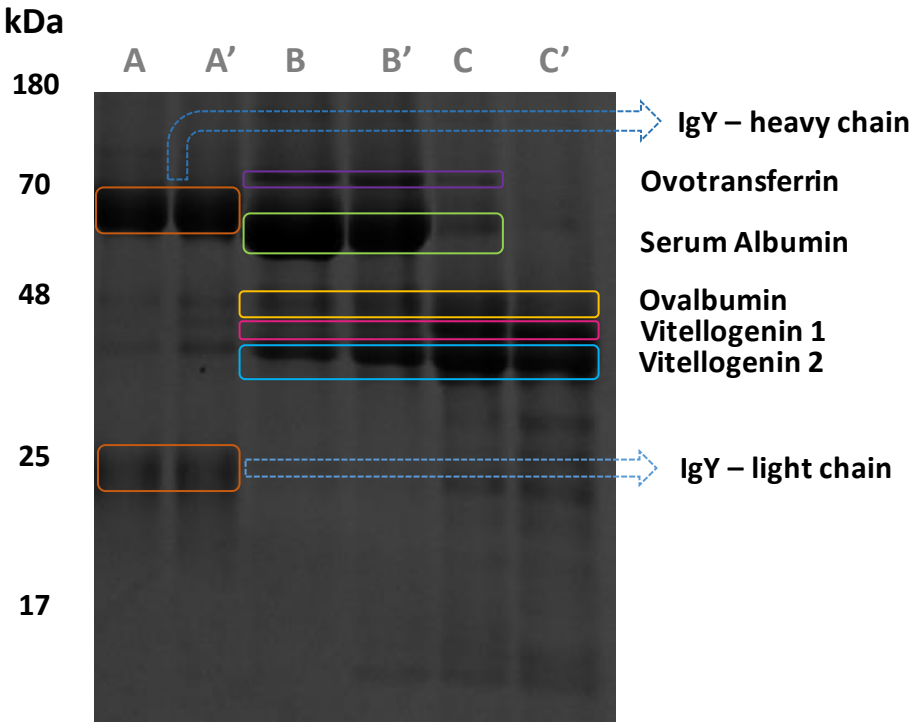

Figure 2.3. SDS-PAGE stained with Coomassie blue for each fraction collected from the WSPF by gel filtration chromatographic separation.

In summary, in this work, six of the most abundant proteins in the egg yolk WSPF were identified. All the proteins identified are in agreement with the results reported by Nilson et al. [6] and Man and Man [41]. However, a lower number of proteins were identified by us. This is due to the fact that only the most intense bands, corresponding to the most abundant proteins were selected for proteomic analysis. Thus, less abundant proteins, observed in the gel were not identified. Also, although other proteins were observed in these intense bands, due to the suppression effect, the

identification was not robust enough to be reported. If the identification of these proteins were necessary, more extended HPLC programs should have been used. Finally, in this study, we have only analyzed the proteins from 3 bands obtained by SE-HPLC of a WSPF fraction.

The WSPF obtained and characterized in this chapter is composed of six main proteins: IgY, serum albumin, ovotransferrin, ovalbumin, vitellogenin 1, and vitellogenin 2. This WSPF was used in the following studies presented in the next chapters, with the main goal of purifying IgY for potential application as a biopharmaceutical. It should also be remarked that some of the proteins identified, namely ovotransferrin, vitellogenin 1, and vitellogenin 2, also display an antibacterial effect, which might be seen as beneficial when considering the use of IgY formulations as therapeutic agents [38].

Conclusions

Both non-specific and antigen-specific avian IgY antibodies have been used in immunotherapy and as an alternative or complement to antibiotics. Taking in account the potential of IgY as a therapeutic and the described difficulties in its purification from egg yolk, this thesis intends to develop alternative purification platforms for the purification of IgY from the WSPF, which contains the target antibody, from egg yolk. Since the WSPF will be the source of IgY, it is of high importance to better understand the complexity of the WSPF and to identify the proteins present. In this chapter, by SDS-PAGE and nano-LC-MS/MS, the WSPF obtained from egg yolk was characterized, and the main proteins present identified. The WSPF analyzed was obtained according to the procedure reported by Liu et al. [22], with comprises a water-dilution step of the egg yolk, freeze/thawing, and centrifugation. Six main proteins were identified in the WSPF: IgY, ovotransferrin, serum albumin(α-livetin), ovalbumin, vitellogenin 1, and vitellogenin 2. Although a lower number of proteins was identified in comparison with previous works reported, it should be remarked that the WSPF obtained by the described method was never characterized. The WSPF characterized in this chapter was applied in the next chapters with the aim of purifying IgY.

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3. Purification of IgY from the egg yolk WSPF

3.1. Purification of IgY using ABS formed by ionic liquids and polymers/salts

Some of the experimental work presented in this section was developed with the collaboration and inputs of Joana Gomes and Juliana Calheiros.

Abstract

The production cost of high-quality immunoglobulin (IgY) for large-scale applications remains higher than other drug therapies due to the lack of cost-efficient purification methods. Thus, the search for new and useful purification platforms is urgent. In the previous chapter, it was described the recovery and characterization of the water-soluble proteins fraction (WSPF) that contains IgY (plus serum albumin, ovalbumin, ovotransferrin, vitellogenin 1, and vitellogenin 2). This fraction was further used in the investigation of alternative purification platforms for IgY, namely aqueous biphasic systems (ABS). In this section, the WSPF was used as the source of IgY in the formation of ABS composed by biocompatible and self-buffering ionic liquids (ILs) and polypropylene glycol 400 $g \cdot mol^{-1}$ (PPG 400) or $K_3C_6H_5O_7$. In particular, ABS was explored as liquid-liquid strategies, where the main goal was to achieve the selective migration of IgY for one of the ABS' phase while the remaining proteins should partition to the opposite phase. Moreover, the IgY stability and activity in various aqueous solutions of ILs, salts, and polymers were evaluated to ascertain which type of ABS phase-forming components can compromise the antibodies functionality. The complete extraction for one of the phases and complete purification of IgY were not achieved in one-step in the investigated ABS. Furthermore, with some ILs it was observed the proteins precipitation and the formation of IL-IgY complexes. The antibody activity is higher in aqueous polymer solutions, followed by salts and ILs aqueous solutions.

Introduction

The increase of drug-resistant microorganisms and diseases that are unresponsive to conventional drug therapies make the search for effective alternative therapeutics, such as biopharmaceuticals, a significant demand [1]. Biopharmaceuticals have significantly improved the treatment of many diseases and sometimes are the only approved therapies available for a specific disease. These biologic-based products, which include antibodies, recombinant proteins, and nucleic-acid-based products, have

application in several therapeutic areas, such as immunization and oncology, and in the treatment of autoimmune, cardiovascular, inflammatory and neurological diseases [2]. The ability of antibodies to bind to an antigen with a high degree of affinity and specificity makes of antibodies great candidates as biopharmaceuticals [3]. Since 1986, with the first therapeutic monoclonal antibody (mAb) product approval, an increase in the commercial potential of antibodies as prospective therapeutic drugs was observed [4]. However, the production cost of high-quality antibodies, and in particular of immunoglobulin Y (IgY), still remains higher than other drug therapies due to the absence of a cost-effective purification method [5]. Thus, several methods have been proposed for the purification of IgY from egg yolk, which can be divided into three main groups, namely: (i) precipitation methods, involving simple water dilution, the addition of salts [6], organic solvents [7], or polymers such as polyethylene glycol (PEG) [8]; (ii) chromatographic methods, by means of ion exchange [9], gel-filtration [10], hydrophobic interaction [10] and affinity chromatographies [11]; and (iii) ultrafiltration [12]. Nevertheless, these methods are multi-step, time-consuming, labor-intensive, low yielding, high cost and cannot easily be scaled-up for industrial applications [11-13]. In this sense, this chapter describes our attempts to develop an alternative purification platform for IgY by the application of aqueous biphasic systems (ABS).

ABS´s were introduced by Albertson in the 1950s [14]. Albertson discovered that a concentrated solution of an organic polymer, such as PEG, and an inorganic salt, such as K_2HPO_4, formed two liquid phases: the upper phase contains most of the PEG, and the lower phase contains most of the inorganic salt, being both phase aqueous-rich [14]. In general, ABS are ternary systems formed when two hydrophilic solutes are dissolved in water above specific concentrations [15, 16]. These solutes can either be two polymers, a polymer and a salt, or two salts [2, 15]. At fixed conditions of temperature and pressure, these ternary mixtures have a characteristic phase diagram. Phase diagrams provide information about the concentration of the phase-forming components required to form a biphasic system, the concentration of the phase components in the top and bottom phases, and the ratio of phase volume or weight [17]. The binodal curve of a phase diagram divides two regions: the monophasic region (below the curve) and the biphasic region (above the curve). Figure 3.1.1. shows an example of a phase diagram, with a binodal curve (yellow curve), and three mixture

compositions in the biphasic region, X, Y, and Z. All systems have a different initial composition and different volume ratios. The example provided by the mixtures X, Y and Z are along the same tie-line (TL) meaning that all the initial mixtures present the same top phase composition ($T_{component\ 1}$, $T_{component\ 2}$) and the same bottom phase composition ($B_{component\ 1}$, $B_{component\ 2}$) [17].

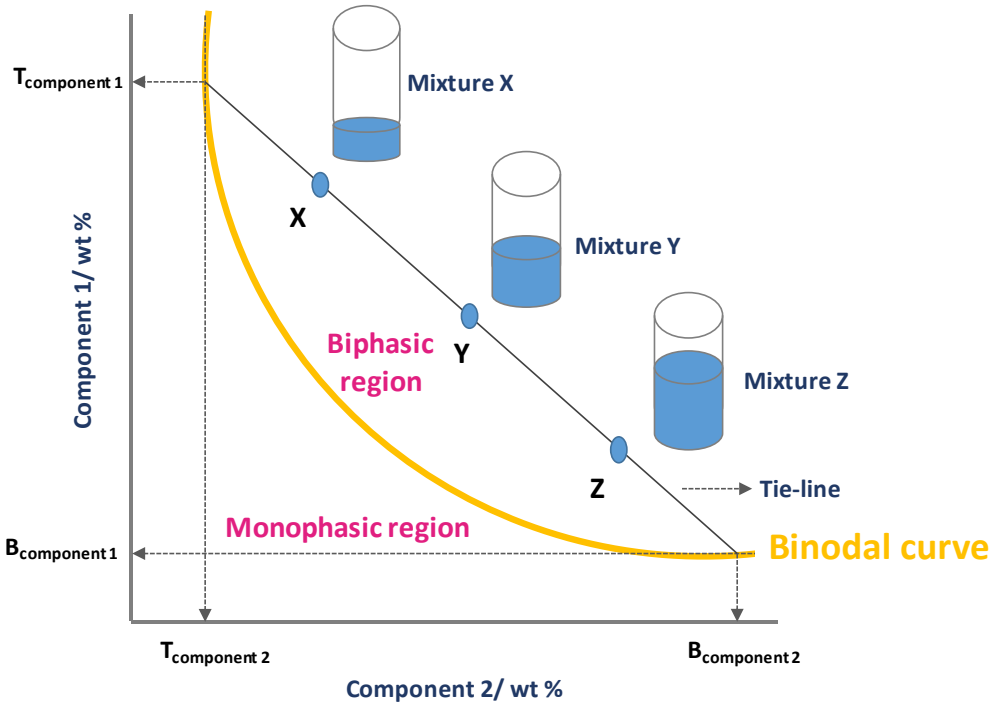

Figure 3.1.1. Phase diagram: binodal curve (yellow line), T = composition of the top phase; B = composition of the bottom phase; X, Y and Z = mixture compositions at the biphasic region.

The partition of a target product in ABS depends on a number of parameters related to the liquid-liquid system properties [2, 18]. The extrinsic properties concern the type, molecular weight, and concentration of phase-forming components, ionic strength, pH, temperature, among others [18]. The intrinsic properties include size and charge of the solute to be partitioned, surface hydrophobicity/ hydrophilicity of the coexisting phases and favorable non-covalent interactions between the target solute and the phase-forming components [18].

Since ABS are mainly composed of water, relatively simple and inexpensive, these systems can be easily operated and scaled-up, allowing the purification and concentration stages to be integrated into one step [15, 16, 19]. By being liquid-liquid systems, ABS have been described as powerful non-chromatographic units for the

separation/purification of cells, virus, organelles, nucleic acids, lipids, amino acids, proteins, enzymes and antibodies [15, 16, 20]. The pioneering work on the use of ABS in the downstream processing of mAbs was reported in 1996, by Zijlstra and co-workers [21], who used a functionalised PEG + dextran ABS to recover IgG from hybridoma cell supernatants. In the following years, other research works have been published on the use of ABS formed by polymers and salts to recover IgG from hybridoma cell supernatants [22]. Still, the hydrophilic-hydrophobic range of the polymers commonly used is restricted, which may condition enhanced extraction efficiencies when used as phase-forming components of ABS [23]. In 2003, Gutowski et al. [24] proposed the creation of ABS by applying ionic liquids (ILs), which have shown later to be able to overcome some drawbacks associated with traditional polymer-based ABS [19]. ILs are salts that are liquid below a conventional temperature of 100 °C, and they are usually composed of a large asymmetric organic cation and either an organic or inorganic anion. The main advantage of ILs relays on their tailored solvation/extraction ability [25-27] which is transposable to IL-based ABS [19, 28, 29]. Moreover, IL-based ABS are substantially less viscous than typical polymer-based ABS and usually display faster phase separation rates, which is also promoted by higher differences in the phases' densities [19, 29]. These advantages led to intensive research on ILs as alternative phase-forming components of ABS [23].

The extraction of biomolecules using IL-based ABS has been extensively investigated [23, 28, 30-33], and several reports are found concerning the extraction of proteins and enzymes [34-37]. However, most of the ILs investigated for ABS formation affect the pH of the aqueous solution - a significant drawback when the goal is the extraction of proteins such as antibodies. Previous reports focused mainly on the use of imidazolium-based ILs with anions that have a strong alkaline or acidic character [19]. Hence, phosphate-based buffered solutions were used to maintain the pH of the aqueous medium, aiming at avoiding the denaturation of proteins [19]. However, adding a buffer into aqueous IL solutions, will not provide an adequate pH control since the ILs acidity or basicity could swamp the buffer effect. The pH can be however adjusted to optimum values by using proper ILs with self-buffering capacity [38]. Good's buffers (GBs), which are zwitterionic amino acid derivatives, can be used as anion or cation radicals of ILs leading to the creation of a new class of ILs - Good's buffers ionic liquids (GB-ILs) [39] -

covering the physiological pH range (6 to 10). Both GBs and GB-ILs have demonstrated to be protein structure stabilizers [38].

Based on the described advantages, GB-ILs were here studied as phase-forming components of ABS to purify IgY from the egg yolk water-soluble proteins fraction (WSPF). Particularly, ABS composed of GB-ILs and polypropylene glycol 400 $g \cdot mol^{-1}$ (PPG 400) or $K_3C_6H_5O_7$ were investigated as alternative purification platforms for IgY, where the goal is to selectively extract IgY for one of the ABS' phases whereas the remaining proteins (identified in the previous section of this work) should migrate to the opposite phase. GB-ILs with anions derived from Good's buffers [39-41], namely N-[tris(hydroxymethyl)methyl]glycine (Tricine), 2-[(2-hydroxy-1,1-bis(hydroxymethyl)ethyl)amino]ethane sulfonic acid (TES), 2-(cyclohexylamino)ethanesulfonic acid (CHES), 2-[4-(2-hydroxyethyl)piperazin-1-yl]ethanesulfonic acid (HEPES), and 2-(N-morpholino)ethanesulfonic acid (MES); combined with cholinium ($[Ch]^+$), tetrabutylammonium ($[N_{4444}]^+$) and tetrabutylphosphonium ($[P_{4444}]^+$) cations were initially synthetized and characterized. The ABS phase diagrams were then determined, and the extraction experiments of IgY from the egg yolk WSPF performed. Trying to better understand the impact of ILs and other ABS phase-forming components on the IgY stability and activity, several aqueous solutions of ILs, salts, and polymers containing IgY were analyzed by enzyme-linked immunosorbent assays (ELISA).

Experimental section

Materials

Fresh eggs were periodically provided by Dr. Ricardo Pires from Biocant, located in Cantanhede, Portugal. Pure IgY, required for comparison purposes and to establish calibration curves by size exclusion-high performance liquid chromatography (SE-HPLC), was obtained using the Pierce® Chicken IgY Purification Kit (Thermo Scientific).

The reagents required for the GB-ILs synthesis were commercially acquired: the buffers 2-(cyclohexylamino)ethanesulfonic acid (CHES, purity > 99 wt%), 2-[4-(2-hydroxyethyl)piperazin-1-yl]ethanesulfonic acid (HEPES, purity > 99.5 wt%), 2-(N-morpholino)ethanesulfonic acid (MES, purity > 99 wt%), N-

[tris(hydroxymethyl)methyl]glycine (Tricine, purity > 99 wt%), and 2-[(2-hydroxy- 1,1-[bis(hydroxymethyl)ethyl)amino]ethane sulfonic acid (TES, purity > 99 wt%), as well as the hydroxide-based compounds, [Ch][OH] (45 wt% in methanol), [P_{4444}][OH] (40 wt% in H_2O) and [N_{4444}][OH] (40 wt% in H_2O), were purchased from Sigma-Aldrich.

Poly(propylene)glycol with a molecular weight of 400 g·mol^{-1} (PPG 400), citrate tribasic monohydrate ($K_3C_6H_5O_7 \cdot H_2O$, purity ≥ 99 wt%), sodium phosphate monobasic (NaH_2PO_4, purity: 99 – 100.5%), sodium phosphate dibasic heptahydrate ($Na_2HPO_4 \cdot 7H_2O$, purity: 98.2 – 102.0%) and sodium chloride (NaCl) were supplied by Sigma-Aldrich.

The reagents used in the SDS-PAGE analysis were: tris(hydroxymethyl)aminomethane, PA from Pronalab; sodium dodecyl sulfate, SDS (> 98.5 wt% pure) and glycerol, 99.5 wt% pure, from Sigma-Aldrich; bromophenol blue and acid acetic, 99.8 wt% pure, from Merck; dithiothreitol, DTT (99 wt% pure), from Acros; and methanol, HPLC grade, from Fisher Scientific. The Amersham ECLGel Box, the Amersham ECL Running Buffer (10X), the Amersham ECL Gel 4-20%, 10 wells, and the Full-Range Rainbow Molecular Weight Marker were acquired from GE Healthcare. The Coomassie Brilliant Blue G-250 was purchased from Sigma-Aldrich.

IgY (Chicken) ELISA Kit, catalog number KA1957, 96 assays, version: 09 from Abnova was used to address the IgY stability/activity. Aqueous solutions of polymers, namely (poly(ethyle)glycol (PEG) with a molecular weight of 200 g·mol^{-1}, 400 g·mol^{-1}, 600 g·mol^{-1} (PEG 200, 400 and 600) and PPG 400, salts (NaCl, Na_2SO_4, Na_2CO_3, $Na_3C_6H_5O_7$, $NaHPO_4$), and aqueous solutions of several ILs, viz. cholinium 2-(N-morpholino)ethanesulfonate ([Ch][MES]), 1-butyl-3-methylimidazolium chloride ([C_4mim]Cl), cholinium acetate ([Ch][Ac]) and tetrabutylammonium chloride ([N_{4444}]Cl), at several concentrations were prepared and used. PEG 200, PEG 400 and PEG 600 were supplied by Fluka and Acros, respectively. PPG 400, Na_2SO_4, Na_2CO_3 and [N_{4444}]Cl were acquired from Sigma-Aldrich. NaCl, $Na_3C_6H_5O_7$, and $NaHPO_4$ were purchased from Prolabo, JMGS and Panreac, respectively. [C_4mim]Cl and [Ch][Ac] were supplied by Iolitec. The water employed was double distilled, passed through a reverse osmosis system and treated with a Milli-Q plus 185 water purification apparatus.

The GB-ILs were synthesized by us through the neutralization of the hydroxide-based compounds with Good's buffers, according to the procedure described by Taha et al. [38]. A slight excess of equimolar buffer aqueous solution was added drop-wise to

[Ch][OH], [P_{4444}][OH] or [N_{4444}][OH] solutions. The mixture was stirred continuously for at least 12 h at ambient conditions. The mixture was then evaporated at 60 °C under reduced pressure by using a rotary evaporator, yielding a viscous liquid. A mixture of acetonitrile and methanol (1:1, v:v) was added to this liquid and then vigorously stirred at room temperature for 1 h to precipitate any excess of the buffer. The solution was then filtrated to remove the precipitated solid, and the filtrate was evaporated to dryness under vacuum (10 Pa) for 3 days at room temperature to yield each GB-IL. The water content in each GB-IL was measured by Karl–Fischer (KF) titration, using a KF coulometer (Metrohm Ltd., model 831) with the Hydranal Coulomat AG reagent (Riedel-de Haën). The water content in each GB-IL was found to be less than 0.05 wt%. The chemical structures of the GB-ILs were confirmed by ^{1}H NMR. The NMR spectra data of each GB-IL are given in Annex A. The ILs synthesized in this work showed high purity level without signs of decomposition. The chemical structures of all ILs investigated in this section are provided in Figures 3.1.2. and 3.1.3.

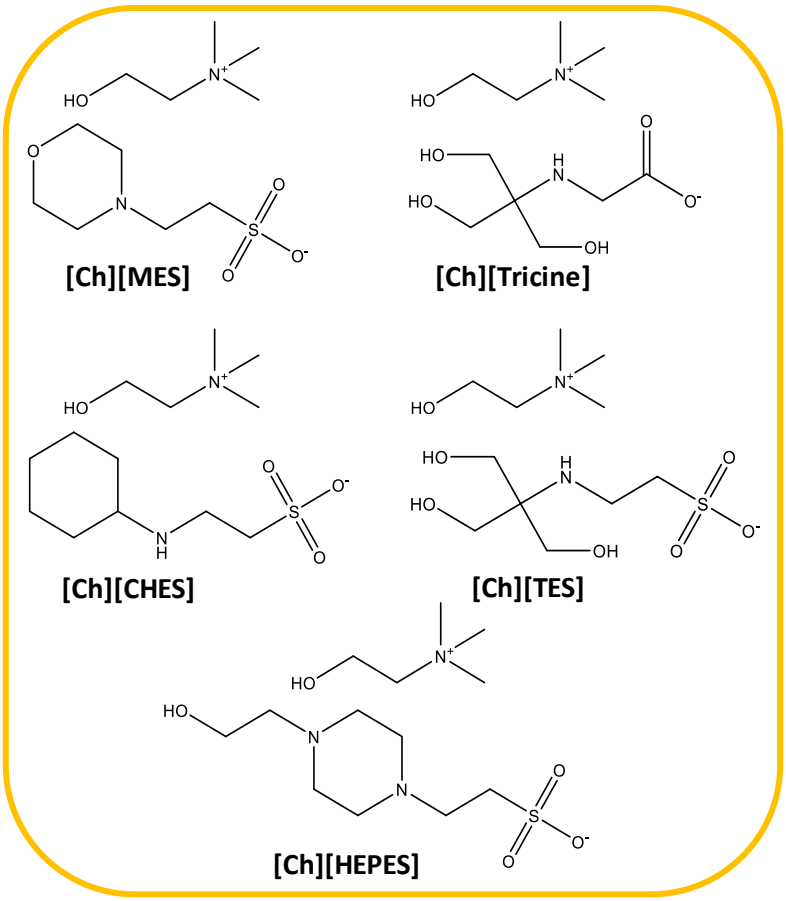

Figure 3.1.2. Chemical structures of the cholinium-based Good's buffers ILs.

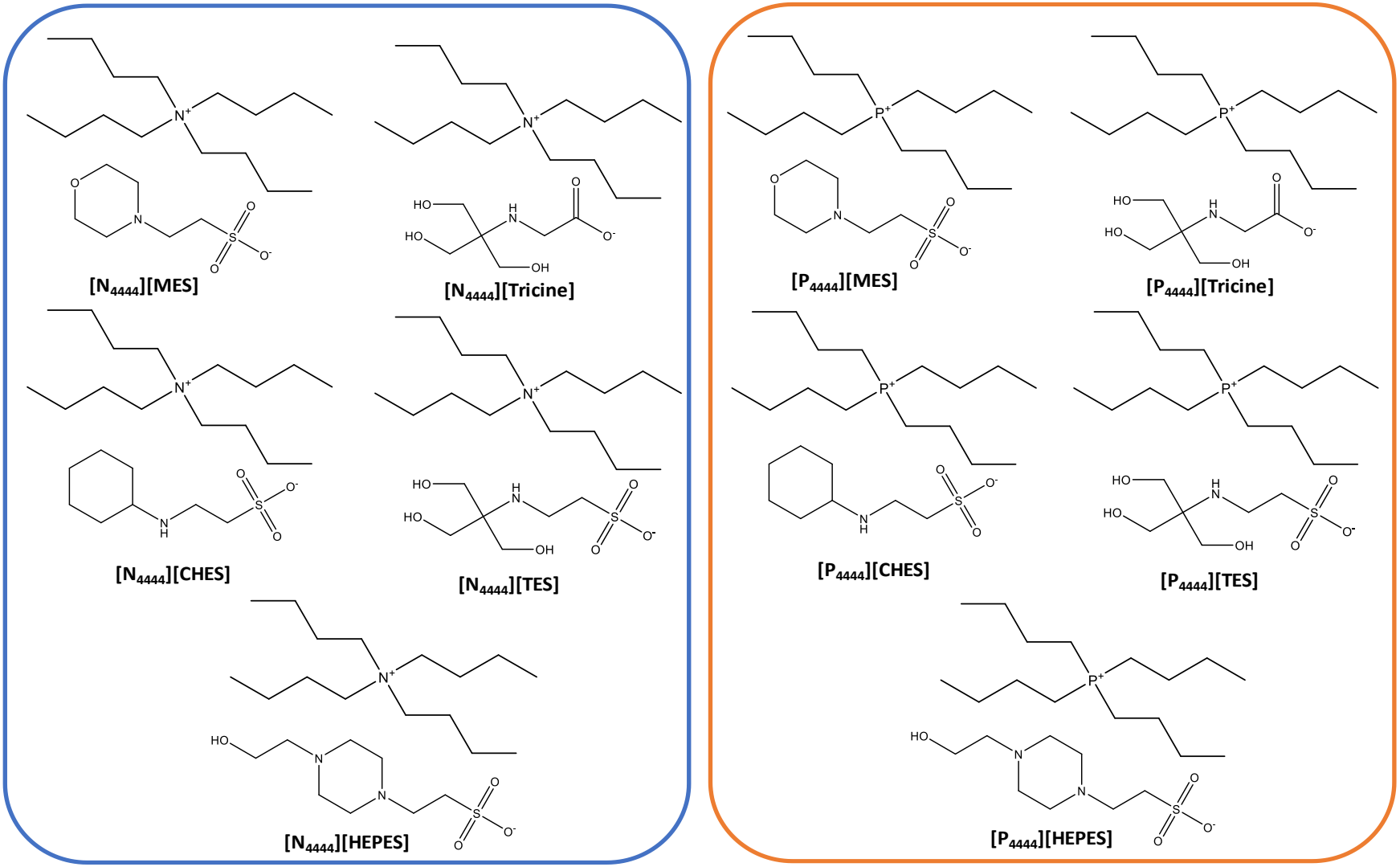

Figure 3.1.3. Chemical structures of the tetrabutylammonium- and tetrabutylphosphonium-based Good's buffers ILs.

ABS phase diagrams and tie-lines

The binodal curve of each phase diagram was determined through the cloud point titration method at (25 ± 1) °C and atmospheric pressure [28]. Aqueous solutions of GB-ILs and $K_3C_6H_5O_7$ at 30-70 wt% and pure PPG 400 were used in the determination of the PPG 400 + GB-IL + water and GB-IL + $K_3C_6H_5O_7$ + water phase diagrams. It should be stressed that cholinium-based ILs are able to form the ABS with PPG 400 but not with $K_3C_6H_5O_7$, while the opposite is observed with tetrabutylammonium- and tetrabutylphosphonium-based ILs, justifying the choice of different phase-forming components for each type of GB-ILs. Repetitive drop-wise addition of the aqueous solution of each GB-IL to PPG 400 or $K_3C_6H_5O_7$ aqueous solution to each GB-IL solution, was carried out until the detection of a cloudy biphasic solution. This was followed by the drop-wise addition of water until the detection of a monophasic region. This procedure was carried out under constant stirring. In particular, for the systems composed of $[N_{4444}]$[HEPES], $[N_{4444}]$[MES] and $[N_{4444}]$[TES] + $K_3C_6H_5O_7$ + H_2O, the turbidimetric method was used [42]. Various mixtures at the biphasic region were initially prepared, and then water was added until the detection of a clear and limpid solution (monophasic region), under constant stirring. In both procedures, the mixtures compositions were determined by weight quantification of all components added (± 10^{-4} g). The TLs of each phase diagram was determined by a gravimetric method originally described by Merchuk et al. [43]. A mixture at the biphasic region was gravimetrically prepared with, vigorously stirred, and allowed to reach equilibrium by separation into the two phases, over at least 12 h at (25 ± 1) °C. The coexisting phases were separated and weighted. Finally, each individual TL was determined by the application of the lever-arm rule to the relationship between the weight of the top and bottom phases and the overall system composition.

For the PPG 400 + GB-IL ABS, the experimental binodal curves were fitted using equation 1 [43]:

$$[\mathrm{PPG}] = A\,exp[(B[\mathrm{GB\text{-}IL}]^{0.5}) - (C[\mathrm{GB\text{-}IL}]^3)] \quad (1)$$

where [PPG] and [GB-IL] are, respectively, the PPG and GB-IL weight percentages and *A*, *B* and *C* are constants obtained by regression of the experimental data.

For the determination of TLs, it was solved the following system of four equations (equations 2 to 5) and four unknown values ($[\mathrm{PPG}]_{\mathrm{PPG}}$, $[\mathrm{PPG}]_{\mathrm{GB\text{-}IL}}$, $[\mathrm{GB\text{-}IL}]_{\mathrm{PPG}}$ and $[\mathrm{GB\text{-}IL}]_{\mathrm{GB\text{-}IL}}$):

$$[\mathrm{PPG}]_{\mathrm{PPG}} = A\,exp[(B\,[\mathrm{GB\text{-}IL}]_{\mathrm{PPG}}^{0.5}) - (C\,[\mathrm{GB\text{-}IL}]_{\mathrm{PPG}}^{3})] \quad (2)$$

$$[\mathrm{PPG}]_{\mathrm{GB\text{-}IL}} = A\,exp[(B\,[\mathrm{GB\text{-}IL}]_{\mathrm{GB\text{-}IL}}^{0.5}) - (C\,[\mathrm{GB\text{-}IL}]_{\mathrm{GB\text{-}IL}}^{3})] \quad (3)$$

$$[\mathrm{PPG}]_{\mathrm{PPG}} = \frac{[\mathrm{PPG}]_{\mathrm{M}}}{\alpha} - \left(\frac{1-\alpha}{\alpha}\right)[\mathrm{PPG}]_{\mathrm{GB\text{-}IL}} \quad (4)$$

$$[\mathrm{GB\text{-}IL}]_{\mathrm{PPG}} = \frac{[\mathrm{GB\text{-}IL}]_{\mathrm{M}}}{\alpha} - \left(\frac{1-\alpha}{\alpha}\right)[\mathrm{GB\text{-}IL}]_{\mathrm{GB\text{-}IL}} \quad (5)$$

where the subscripts "PPG", "GB-IL" and "M" represent the top and the bottom phases, and the mixture composition, respectively. The parameter α is the ratio between the weight of the top phase and the weight of the total mixture. The solution of the referred system gives the concentration of PPG and GB-IL in the top and bottom phases. In the PPG 400 + GB-IL systems, the bottom phase corresponds to the phase enriched in the IL, whereas the top phase is majorly composed of the polymer and water.

For the calculation of the tie-line lengths (TLLs) equation 6 was used,

$$\mathrm{TLL} = \sqrt{([\mathrm{GB\text{-}IL}]_{\mathrm{PPG}} - [\mathrm{GB\text{-}IL}]_{\mathrm{GB\text{-}IL}})^2 + ([\mathrm{PPG}]_{\mathrm{PPG}} - [\mathrm{PPG}]_{\mathrm{GB\text{-}IL}})^2} \quad (6)$$

For the GB-IL + $K_3C_6H_5O_7$ ABS, the experimental binodal curves were fitted using equation 7 [43]:

$$[\mathrm{IL}] = A\,exp[(B[\mathrm{salt}]^{0.5}) - (C[\mathrm{salt}]^3)] \quad (7)$$

where [IL] and [salt] are the IL and salt weight fractions percentages, respectively, and *A*, *B,* and *C* are fitted constants obtained by least-squares regression.

For the determination of the TLs, the following system of four equations (equations 8 to 11) were used to estimate the concentration of IL and salt at each phase ($[\mathrm{IL}]_{\mathrm{IL}}$, $[\mathrm{IL}]_{\mathrm{salt}}$, $[\mathrm{salt}]_{\mathrm{salt}}$ and $[\mathrm{salt}]_{\mathrm{IL}}$),

$$[\mathrm{IL}]_{\mathrm{IL}} = A\,exp[(B[\mathrm{salt}]_{\mathrm{IL}}^{0.5}) - (C\,[\mathrm{salt}]_{\mathrm{IL}}^{3})] \quad (8)$$

$$[\mathrm{IL}]_{\mathrm{salt}} = A\,exp[(B\,[\mathrm{salt}]_{\mathrm{salt}}^{0.5}) - (C\,[\mathrm{salt}]_{\mathrm{salt}}^{3})] \quad (9)$$

$$[\mathrm{IL}]_{\mathrm{IL}} = \frac{[\mathrm{IL}]_{\mathrm{M}}}{\alpha} - \left(\frac{1-\alpha}{\alpha}\right)[\mathrm{IL}]_{\mathrm{salt}} \quad (10)$$

$$[\mathrm{salt}]_{\mathrm{IL}} = \frac{[\mathrm{salt}]_{\mathrm{M}}}{\alpha} - \left(\frac{1-\alpha}{\alpha}\right)[\mathrm{salt}]_{\mathrm{salt}} \quad (11)$$

where the subscripts salt and IL designate the salt- and IL-rich phases, respectively, and M is the initial mixture composition. The parameter α is the ratio between the weight of the top phase and the total weight of the mixture. The solution of the described system provides the concentration of the IL and salt in the top and bottom phases. In the GB-IL + $K_3C_6H_5O_7$ systems, the top phase corresponds to the phase enriched in the IL, whereas the bottom phase is majorly composed of the salt and water.

In order to calculate each the TLL equation 12 was applied,

$$\mathrm{TLL} = \sqrt{([\mathrm{salt}]_{\mathrm{IL}} - [\mathrm{salt}]_{\mathrm{salt}})^2 + ([\mathrm{IL}]_{\mathrm{IL}} - [\mathrm{IL}]_{\mathrm{salt}})^2} \quad (12)$$

The correlation parameters of equations 1 and 7 were determined using the software Sigmaplot.v11.0, while the compositions of the top and bottom phases were determined using the software Matlab R2013a.

All the calculations considering the mass fraction or molality of the citrate-based salt were carried out discounting the complexed water.

Extraction and purification of IgY from the egg yolk WSPF using ABS

PPG 400 + GB-IL ABS

To obtain the WSPF from egg yolk, the protocol described previously by Polson et al. [8] was adopted. A common tie-line length (TLL = 38 - 41 wt%) for given mixture compositions of the PPG 400 + GB-IL ABS (≈ 50 wt% PPG 400 + ≈ 7 - 10 wt% IL + ≈ 40 - 43 wt% WSPF) was chosen based on the phase diagrams determined in advance. As the phase diagrams are very similar to each other, the similar tie-line lengths (TLL = 38 - 42 wt%) also correspond to similar mixture compositions. Table 3.1.1. describes the mixture compositions and TLL of each ternary mixture/ABS investigated.

Table 3.1.1. Ternary mixture compositions of ABS composed of PPG 400 + GB-IL for the purification of IgY from the WSPF, and respective TLL.

ABS	[PPG 400]/ wt%	[GB-IL]/ wt%	[WSPF]/ wt%	TLL ≈
[Ch][Tricine]	50.0	7.0	43.0	41.3
[Ch][HEPES]	50.0	8.0	42.0	42.2
[Ch][TES]	50.0	8.5	41.5	38.5
[Ch][MES]	50.0	10.0	40.0	42.4

Each mixture was prepared gravimetrically within ± 10^{-4} g, vigorously stirred and left to equilibrate for at least 12 h (a time period established in previous optimizing experiments) at (25 ± 1) °C to achieve the complete partitioning of IgY and other contaminant proteins between the two phases. After the careful separation of the phases, using small glass ampoules designed for the purpose, the total amount of proteins was quantified in each phase. At least three individual experiments were carried out for each ABS, allowing the determination of the average partition coefficients and extraction efficiencies and respective standard deviations. The protein content was quantified through UV-spectroscopy, using a SHIMADZU UV-1800 UV Spectrometer, at a wavelength of 280 nm.

The partition coefficients of the proteins, *K*, were determined according to equation 13:

$$K = \frac{[\text{Protein}]_{\text{bottom phase}}}{[\text{Protein}]_{\text{top phase}}} \quad (13)$$

where $[\mathrm{Protein}]_{\mathrm{bottom\ phase}}$ and $[\mathrm{Protein}]_{\mathrm{top\ phase}}$ are the concentration of proteins in the IL-rich (bottom phase) and in the PPG-rich (top phase) aqueous phases, respectively. The percentage extraction efficiencies of the proteins, *EE%*, are defined as the percentage ratio between the amount of protein in the IL-rich aqueous phase and that in the total mixture, according to equation 14:

$$EE\% = \frac{\mathrm{W}_{\mathrm{Protein}}^{\mathrm{bottom\ phase}}}{\mathrm{W}_{\mathrm{Protein}}^{\mathrm{top\ phase}} + \mathrm{W}_{\mathrm{Protein}}^{\mathrm{bottom\ phase}}} \times 100 \quad (14)$$

where $\mathrm{W}_{\mathrm{Protein}}^{\mathrm{top\ phase}}$ and $\mathrm{W}_{\mathrm{Protein}}^{\mathrm{bottom\ phase}}$ are the weight of protein in the PPG-rich (top phase) and in the IL-rich (bottom phase) aqueous phases, respectively.
Control or "blank" solutions at the same mixture point used for the extraction studies (with no WSPF/proteins added) were used in all systems.

GB-IL + $K_3C_6H_5O_7$ ABS

The WSPF of egg yolk was prepared from fresh eggs, following the protocol described by Liu et al. [44] in the literature, and then applied in the ABS composition. The ternary mixtures compositions used were chosen based on the phase diagrams determined for each GB-IL + $K_3C_6H_5O_7$ + water system. A ternary mixture within the biphasic region was prepared, using a common composition of IL (20 wt%) and variable concentrations of salt (from 13.5 to 30 wt%) to achieve a similar TLL (39.6 ≥ TLL ≤ 44.3 for systems composed of $[P_{4444}][GB]$ and 39.1 ≥ TLL ≤ 58.1 for those composed of $[N_{4444}][GB]$). These differences in the TLL are due to the differences in the phase diagrams between the two classes of GB-ILs and mixture compositions range able to form two liquid phases. Table 3.1.2. describes the mixture compositions and TLL of each ternary mixture investigated.

Table 3.1.2. Ternary mixture composition of ABS composed of GB-IL + $K_3C_6H_5O_7$ for the purification of IgY from the WSPF, and respective TLL.

ABS	[GB-IL]/ wt%	[$K_3C_6H_5O_7$]/ wt%	[WSPF]/ wt%	TLL ≈
[P_{4444}][CHES]	20.0	13.5	66.5	44.3
[P_{4444}][HEPES]	20.0	22.0	48.0	41.6
[P_{4444}][MES]	20.0	21.0	49.0	39.6
[P_{4444}][TES]	20.0	28.0	52.0	42.9
[P_{4444}][Tricine]	20.0	29.0	51.0	41.6
[N_{4444}][CHES]	20.0	17.0	63.0	39.1
[N_{4444}][HEPES]	20.0	23.0	47.0	42.3
[N_{4444}][MES]	20.0	22.0	48.0	40.5
[N_{4444}][TES]	20.0	28.5	51.5	43.9
[N_{4444}][Tricine]	25.0	30.0	45.0	58.0

Each mixture was vigorously stirred and left to equilibrate for at least 3 h, at (25 ± 1) °C, to achieve a complete IgY partitioning and other contaminant proteins between the two phases. At least three individual experiments were carried out for each ABS. After a careful separation of the phases, the SE-HPLC technique was used with the aim of quantifying IgY in each phase and to address its purity by the analysis of the peaks corresponding to the remaining proteins. A phosphate buffer solution (1000 mL) was prepared using 47 mL of a Solution A (27.8 g NaH_2PO_4), 203 mL of a Solution B (53.65 g $Na_2HPO_4{\cdot}7H_2O$) and 35 g of NaCl. Each phase was diluted at a 1:9 (v:v) ratio in the phosphate buffer solution before injection. A Chromaster HPLC (VWR Hitachi) was used for IgY quantification. The SE-HPLC was performed on an analytical column Shodex Protein KW- 802.5 (8 mm x 300 mm). A 100 mM phosphate buffer + NaCl 0.3 M was run isocratically with a flow rate of 0.5 $mL{\cdot}min^{-1}$. The column oven and autosampler temperatures were kept at 25 °C and at 10 °C, respectively. The injection volume was 25 µL. The wavelength was set at 280 nm using a DAD detector. The obtained chromatograms were treated and analyzed using the PeakFit (version 4) software. The calibration curve for IgY is provided in Annex A – Figure A1. Control or "blank" solutions at the same mixture point used for the extraction studies (with no WSPF/proteins added) were used in all systems.

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE)

The protein profile of the coexisting phases was investigated by SDS-PAGE using an Amersham ECLTM Gel from GE Healthcare Life Sciences. The protein concentration in both phases was determined by the Bio-Rad protein assay (Bio-Rad), diluted in order to achieve a total protein content of 0.005 mg, and further mixed with the Laemmli buffer. Both phases were then subjected to SDS-PAGE in 20% polyacrylamide gels. The proteins were stained with Coomassie Brilliant Blue G-250 for 2 - 3 h and then distained at room temperature. All gels were analyzed using the Image Lab 3.0 (BIO-RAD) analysis tool.

pH measurements

The pH values of both the IL-rich and PPG 400 or $K_3C_6H_5O_7$-rich aqueous phases were measured at (25 ± 1) °C using a METTLER TOLEDO SevenMulti pH meter within an uncertainty of ± 0.02. The calibration of the pH meter was carried out with two buffers (pH values of 4.00 and 7.00).

Enzyme-linked immunosorbent assays (ELISA)

Aqueous solutions of polymers (PEG 200, 400, 600 and PPG 400), salts (NaCl, Na_2SO_4, Na_2CO_3, $Na_3C_6H_5O_7$ and $NaHPO_4$) and ILs ([Ch][MES], [C_4mim]Cl, [Ch][Ac] and [N_{4444}]Cl) with a final concentration of 10, 30 and 50 wt% were prepared and used to infer their impact on the IgY activity. IgY purified by the Pierce® Chicken IgY Purification Kit was dissolved in PBS and then diluted with ELISA diluent to a final concentration of 100 ng·mL^{-1}. The IgY solution was added to the aqueous solution of polymers, salts, and ILs in a proportion of 1:1 (v:v), resulting in aqueous solutions of 5, 15 and 25 wt% of the polymer, salt or IL. Due to the IgY precipitation at higher concentrations of salt, it was only possible to prepare solutions with 5 wt% of NaCl, Na_2CO_3 and $NaHPO_4$, 5 and 10 wt% of Na_2SO_4, and 5 and 15 wt% of $Na_3C_6H_5O_7$. According to the supplier instructions (Abnova Corporation), the aqueous solutions of polymers, salts, and ILs with IgY were submitted to the IgY (chicken) ELISA kit.

Results and discussion

PPG 400 + GB-IL ABS

The ternary phase diagrams of the ABS composed of PPG 400 + [Ch][HEPES], [Ch][Tricine], [Ch][TES] or [Ch][MES] + water at (25 ± 1) °C and atmospheric pressure are depicted in Figure 3.1.4. Formation of ABS was not found for the PPG 400/ [Ch][CHES] mixture. The experimental weight fraction data are reported in Annex A – Tables A1 and A2. In all studied ABS, the top phase corresponds to the PPG-rich phase, while the bottom phase is mainly composed of GB-IL and water. Figure 3.1.4 depicts the solubility curves, displayed in molality of polymer (mole of polymer per kg of solvent) versus molality of GB-IL (mole of GB-IL per kg of solvent). Molality was chosen in order to avoid distortions in the comparisons that could be a consequence of the different molecular weights of the GB-ILs involved. For mixtures with compositions above the solubility curve, formation of two aqueous phases occurs; below the solubility curve, the concentration of each component is not sufficient to induce liquid-liquid demixing, thus falling within the monophasic regime. Figure 3.1.4 depicts the ability of each GB-IL to induce the phase separation. As all the GB-ILs share a common cation, the differences in the solubility curves are a result of the anion nature. The GB-IL anions aptitude to form ABS follows the order: $[HEPES]^- \approx [Tricine]^- > [TES]^- > [MES]^-$. As PPG 400 is a moderately hydrophobic polymer, the higher the affinity that each GB has for water, the greater the ability of the IL to promote two-phase formation. This trend closely follows the GBs octanol-water partition coefficients ($\log(K_{ow})$) [45], meaning that the IL exerts the salting-out over the polymer in aqueous media, and being in agreement with data previously published on phase diagrams for ABS composed of other cholinium-based ILs and PPG 400 [35].

The experimental binodal data for the studied systems were fitted by the empirical relationship described by equation 1. The regression parameters *A*, *B,* and *C*, which were estimated by the least-squares regression method, are provided in Annex A – Table A3, along with their corresponding standard deviations (σ). Overall, good correlation coefficients were obtained, indicating that these fittings can be used to predict data in a given region of the phase diagram where no experimental results are available. The

experimental data for the TLs and their respective length (TLL) are reported in Annex A – Tale A4.

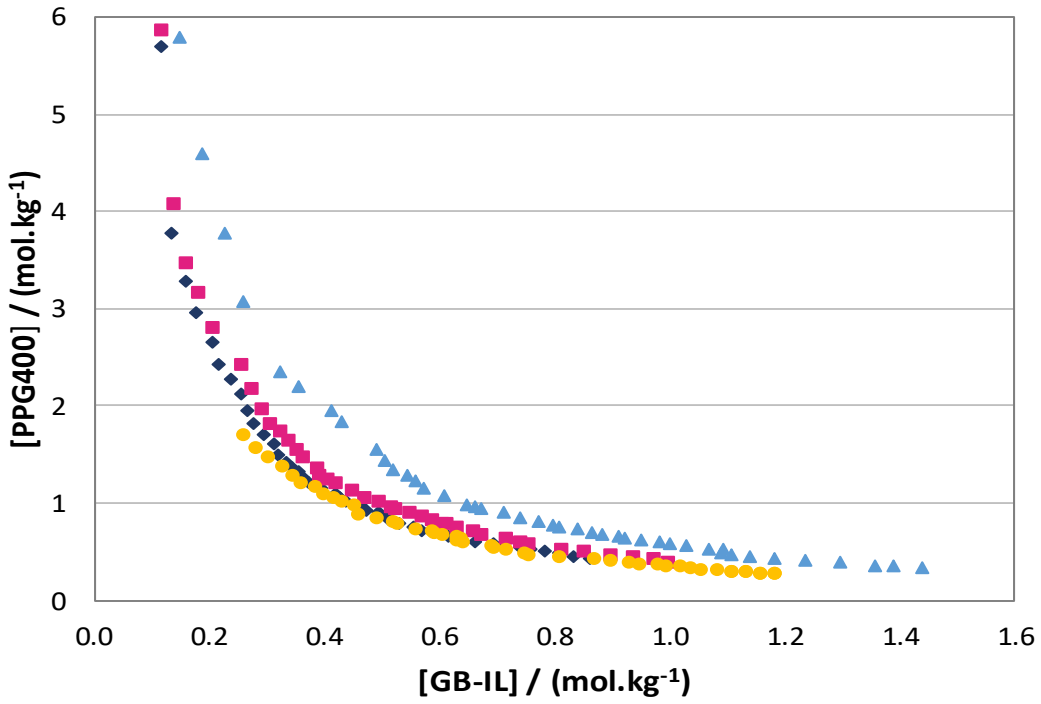

Figure 3.1.4. Ternary phase diagrams for the systems composed of PPG 400 + GB-IL + water at (25 ± 1) °C and atmospheric pressure: (▲) [Ch][MES], (◆) [Ch][Tricine], (■) [Ch[TES] and (●) [Ch][HEPES].

The novel ABS proposed herein were investigated for the purification of IgY from the WSPF obtained from egg yolk, characterized in the previous section of this work. The mixture compositions (50 wt% of PPG 400 + 7 - 10 wt% of each GB-IL + 40 - 43 wt% of an aqueous solution containing the water-soluble proteins), which fall within the biphasic region, were chosen according to a fixed TLL to avoid differences in the compositions of the coexisting phases amongst the four IL-based ABS. Table 3.1.1. provides the mixture compositions and TLL of each ternary mixture investigated.

The *K* and *EE%* of total proteins, namely IgY and remaining water-soluble proteins, at (25 ± 1) °C, are depicted in Figure 3.1.5. (see Annex A – Table A5 for detailed data). For all the investigated systems, the partition coefficient is higher than 1.0, confirming the preferential partitioning of the proteins into the IL-rich phase (bottom phase). The extraction efficiencies for the IL-rich phase range from 79 to 94%. The ABS composed of PPG 400 and [Ch][Tricine] or [Ch][HEPES] led to the highest extraction efficiencies, above 90%, in a single step. These GB-ILs are indeed those that present a higher ability to form

ABS with PPG 400, and as provided in Annex A – Table A4, corresponding to the systems with a higher water content at the IL-rich phase, meaning that the WSPF proteins, including IgY, tend to enrich in phases with a higher water content.

The coexisting phases of the investigated ABS were also analyzed by SDS-PAGE, under reducing conditions, to identify the several proteins present, and thus to have indications on these systems to purify IgY (Figure 3.1.6). It is important to mention that, under reducing conditions, IgY presents two major bands, namely at 65 kDa and 25 kDa, corresponding to the two heavy chains and the two light chains. In all the studied systems, no proteins were detected at the polymer-rich phase. Nevertheless, three major proteins were identified in the IL-rich phase, namely IgY (heavy chain), vitellogenin 1 and 2 (contaminant proteins of the water-soluble fraction). In the previous section, six main water-soluble proteins were identified in the WSPF: IgY, serum albumin, ovalbumin, ovotransferrin, vitellogenin 1, and vitellogenin 2. IgY and serum albumin are in the WSPF in a higher concentration, followed by vitellogenin 2 and vitellogenin 1. Ovalbumin and ovotransferrin are the proteins detected in a lower concentration in the WSPF. It should be however remarked that in this section a different protocol to obtain the WSPF was applied [8]. Comparing the WSPF obtained in the previous chapter, Figure 2.3., with the WSPF obtained in this section, Figure 3.1.6., it is notorious that a lower number of proteins and their lower concentration is observed. The modification of the protocol to obtain the WSPF and its low recovery yield may explain the absence of ovalbumin, ovotransferrin and serum albumin.

For the system composed of PPG 400 and [Ch][MES], a reduction in the intensity of the bands corresponding to vitellogenin 1 and 2 was observed, indicating that this system may induce the contaminant proteins precipitation, and thus an increase of the IgY purity. In summary, all the studied systems display a high extraction efficiency for proteins, but they, however, fail in selectivity, particularly to be achieved in an ABS equilibrium step. Further studies on the manipulation of the mixtures compositions, nature of the phase-forming components and pH and temperature should be addressed to identify favorable conditions to selectively recover IgY at the IL-rich phase.

Given that the best results in terms of IgY selectivity were obtained with the most hydrophobic IL ([Ch][MES]) investigated and aiming at increasing the differences in polarities between the phases, novel ABS composed of $[P_{4444}][GB]$ or $[N_{4444}][GB]$ ILs and

$K_3C_6H_5O_7$ were studied, and their extractive performance for IgY investigated. According to the literature [P_{4444}]- and [N_{4444}]-based ILs are more hydrophobic than the respective cholinium-based counterparts [46].

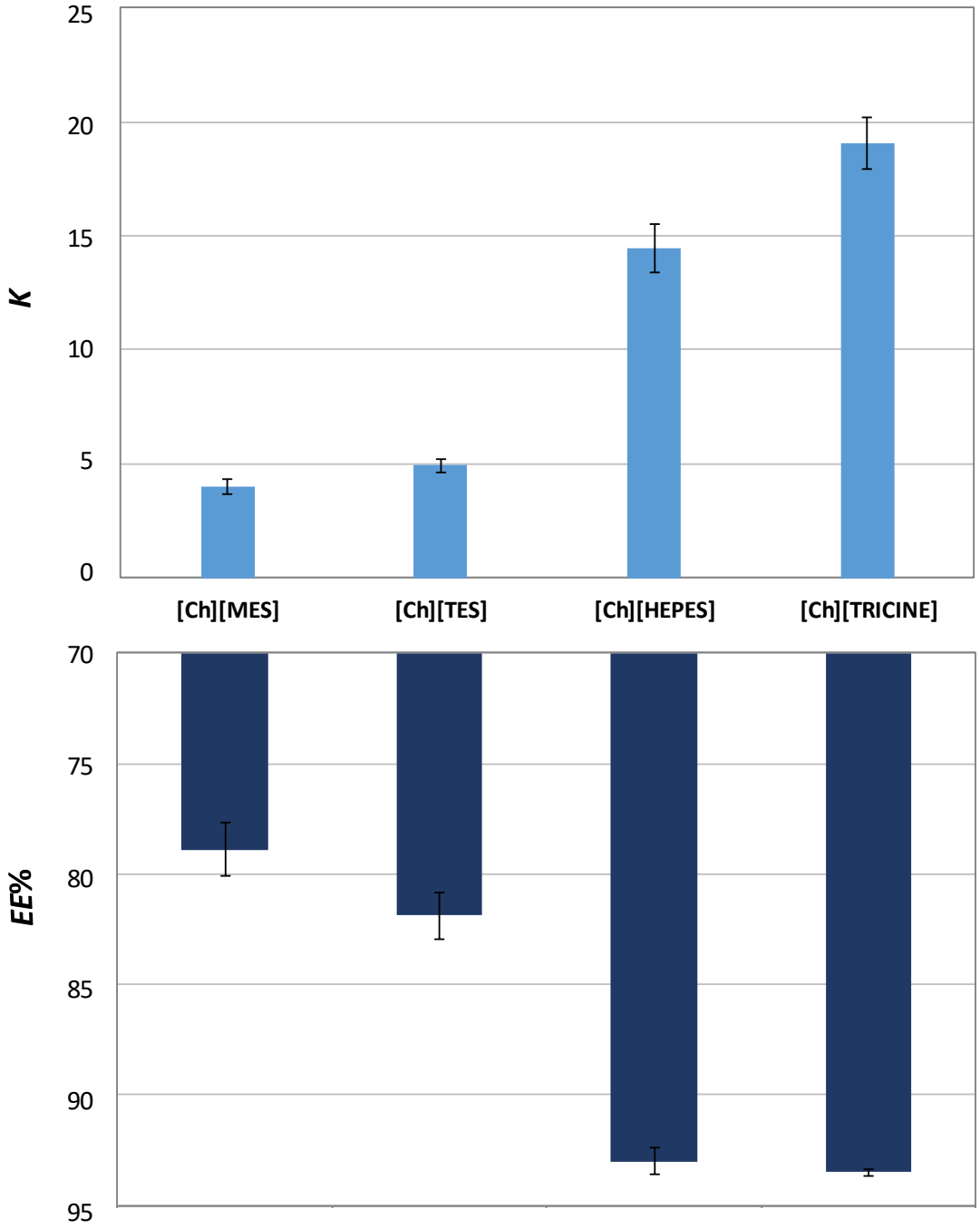

Figure 3.1.5. Partition coefficients (*K*) and extraction efficiencies (*EE*%) of the total proteins of the WSPF from egg yolk using ABS composed of PPG 400 + GB-IL + water at (25 ± 1) °C.

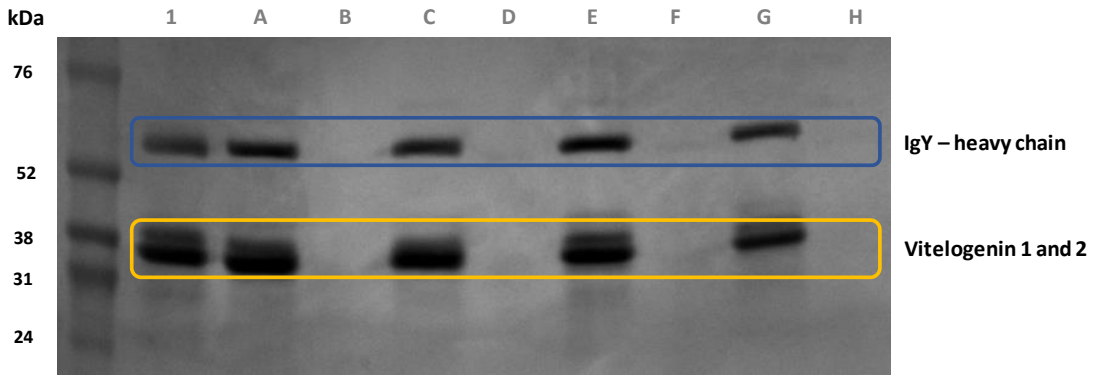

Figure 3.1.6. SDS-PAGE stained with Coomassie blue: 1 - water-soluble proteins from egg yolk; A and B - bottom and top phases, respectively, of the PPG 400 + [Ch][HEPES] ABS; C and D - bottom and top phases, respectively, of the PPG 400 + [Ch][TES] ABS; E and F - bottom and top phases, respectively, of the PPG 400 + [Ch][Tricine] ABS; G and H - bottom and top phases, respectively, of the PPG 400 + [Ch][MES] ABS.

GB-IL + $K_3C_6H_5O_7$ ABS

The phase diagrams for the systems composed of $[P_{4444}][GB]$ or $[N_{4444}][GB]$ + $K_3C_6H_5O_7$ + H_2O are illustrated in Figures 3.1.7. and 3.1.8., respectively. As before, the experimental data are shown in molality units in order to avoid differences that could result from different molecular weights, allowing a better understanding of the impact of the ILs molecular structure on the phase diagrams behavior. The detailed experimental data corresponding to these ternary phase diagrams are given in Annex A – Tables A6 to A10. For all studied ABS, the bottom phase is mostly composed of salt and water while the top phase corresponds to the IL-rich phase (IL phase inversion when compared with the last set of ABS investigated).

For both classes of ILs sharing the same cation (Figures 3.1.7. and 3.1.8.), the ILs anion ability to form an ABS, for instance at 1.0 mol·kg^{-1} of $K_3C_6H_5O_7$, follows the order: $[CHES]^-$ > $[MES]^-$ ≈ $[HEPES]^-$ > $[TES]^-$ ≈ $[Tricine]^-$. For ammonium-based ILs (Figure 3.1.8.), the anions order closely follows the same trend: $[CHES]^-$ > $[MES]^-$ ≈ $[HEPES]^-$ > $[TES]^-$ ≈ $[Tricine]^-$. This trend is in an opposite order to that shown in Figure 3.1.4., meaning that the IL does not act as the salting-out species in IL + salt mixtures in aqueous media. Instead, the IL is salted-out by the strong salting-out salt used ($K_3C_6H_5O_7$), as appraised by the Hofmeister series [47]. As expected, ILs with anions with higher log(K_{ow}) values, discussed above, are more easily separated into two phases, and their phase diagrams

are more near located to the binodal origin. This trend is in close agreement with previous results on ABS formed by ILs and salts [38, 45].

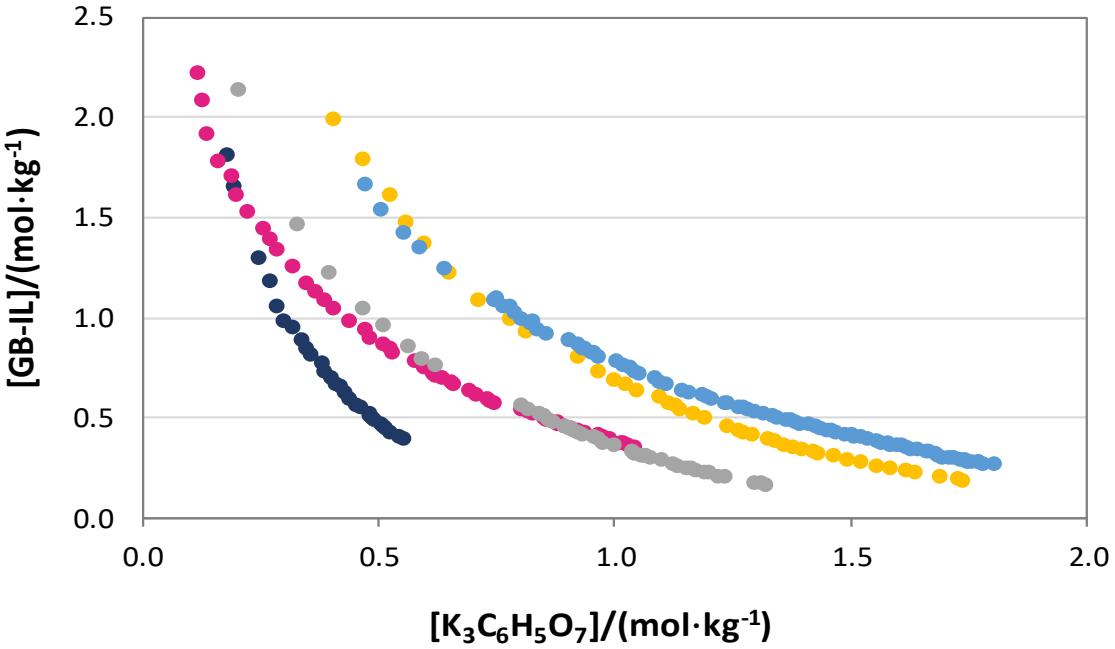

Figure 3.1.7. Ternary phase diagrams for systems composed of GB-IL + $K_3C_6H_5O_7$ + H_2O at (25 ± 1) °C and atmospheric pressure: (●) [P_{4444}][CHES], (●) [P_{4444}][HEPES], (●) [P_{4444}][MES], (●) [P_{4444}][TES] and (●) [P_{4444}][Tricine].

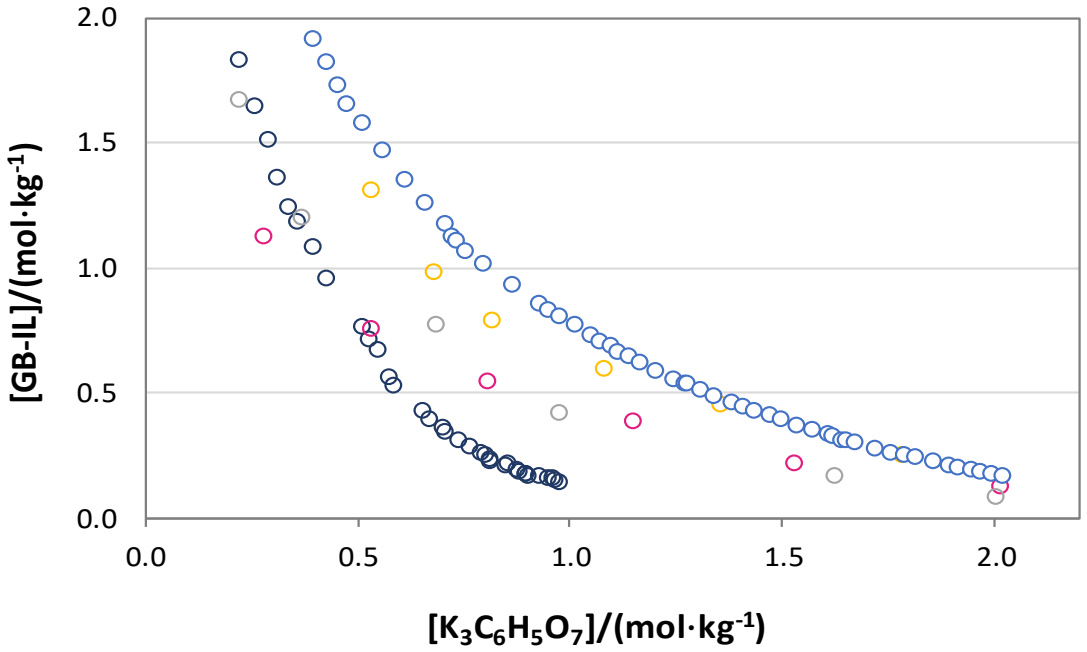

Figure 3.1.8. Ternary phase diagrams for systems composed of GB-IL + $K_3C_6H_5O_7$ + H_2O at (25 ± 1) °C and atmospheric pressure: (o) [N_{4444}][CHES], (o) [N_{4444}][HEPES], (o) [N_{4444}][MES], (o) [N_{4444}][TES] and (o) [N_{4444}][Tricine].

The effect of the IL cation on the phase diagrams behavior is presented in Figure 3.1.9. Both cations, $[P_{4444}]^+$ and $[N_{4444}]^+$, reveal a high ability to form ABS in the presence of $K_3C_6H_5O_7$ aqueous solutions. For instance, there is no two-phase formation by combining [Ch][GB] ILs and the same salt. In general, although both types of compounds are composed of four butyl chains, the $[P_{4444}]^+$ ability to form ABS is higher than that displayed by $[N_{4444}]$-based ILs. Analogous results were reported for ABS composed of more conventional ILs, such as $[P_{4444}]Cl$ and $[N_{4444}]Cl$, combined with Na_2CO_3 and $K_3C_6H_5O_7$ [48, 49]. Due to their highly-shielded charges, located mostly on the heteroatom that is surrounded by four alkyl chains and no aromatic character which may is responsible for their low affinity for water molecules, both cations are known for their strong ability in ABS formation with salts. The lower the affinity for water and/or hydrophobic nature of the IL, the more effective is the IL in promoting the phase separation [48, 50]. However, similar phase formation ability is shown by the ILs composed of the $[Tricine]^-$ and $[HEPES]^-$ anions. In these systems, no meaningful differences were observed concerning their capability to form ABS in presence of $K_3C_6H_5O_7$, indicating that in these ILs the contribution of the central atom at the cation core plays a less relevant role.

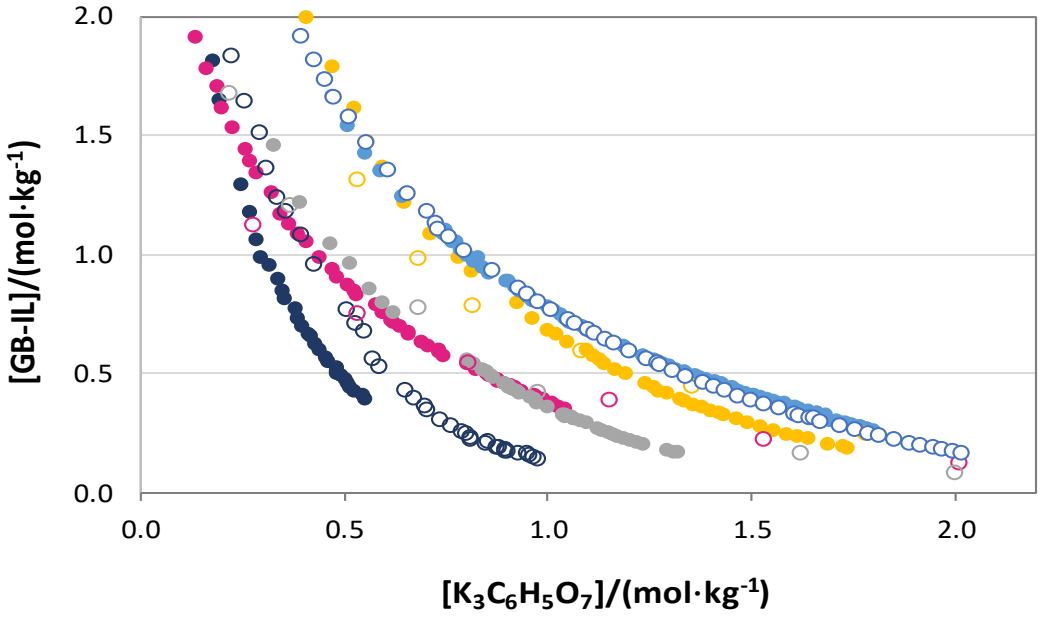

Figure 3.1.9. Ternary phase diagrams for systems composed of GB-IL + $K_3C_6H_5O_7$ + H_2O at (25 ± 1) °C and atmospheric pressure: (●) $[P_{4444}]$[CHES], (●) $[P_{4444}]$[HEPES], (●) $[P_{4444}]$[MES], (●) $[P_{4444}]$[TES] and (●) $[P_{4444}]$[Tricine], (○) $[N_{4444}]$[CHES], (○) $[N_{4444}]$[HEPES], (○) $[N_{4444}]$[MES], (○) $[N_{4444}]$[TES] and (○) $[N_{4444}]$[Tricine].

The experimental binodal data for the studied systems were fitted by the empirical relationship described by equation 7. The regression parameters *A*, *B* and *C*, which were estimated by least-squares regression, are provided in Annex A – Table A11, along with the corresponding standard deviations (σ). The experimental data for the TLs and TLL are reported in Annex A -Table A12.

The attempts of purification of IgY from the WSPF using this second set of ABS was performed at a fixed TLL (≈ 40 ± 5) and GB-IL concentration (20 wt%), with the exception of the system composed of GB-IL [N_{4444}][Tricine] where the GB-IL concentration was fixed at 25 wt% (due to the lower ability of this IL to create ABS with salts). The implementation of a fixed TLL allows keeping the difference in compositions between the coexisting phases constant, leading to a more accurate comparison in the extractive performance of these systems for IgY. Table 3.1.2. describes the mixtures compositions and TLL of each ternary mixture investigated.

All the extractions were carried out in a range of pH 7.7 - 10.8 and the pH values of both phases in each ABS are presented in Table 3.1.3. Although the GB-IL-based systems studied allowed to maintain the pH values, some precipitation and/or denaturation was observed in some systems, namely in those composed of [P_{4444}][HEPES], [N_{4444}][HEPES] and [N_{4444}][TES] (Figures 3.1.10. and 3.1.11.).

Table 3.1.3. pH values of the coexisting phases of the ABS composed of GB-ILs + $K_3C_6H_5O_7$.

IL	pH (IL-rich phase)	pH (salt-rich phase)
[P_{4444}][CHES]	10.8	10.4
[P_{4444}][HEPES]	9.2	9.1
[P_{4444}][MES]	8.0	7.8
[P_{4444}][TES]	8.8	8.4
[P_{4444}][Tricine]	10.2	10.2
[N_{4444}][CHES]	10.9	10.5
[N_{4444}][HEPES]	9.9	9.5
[N_{4444}][MES]	7.9	7.7
[N_{4444}][TES]	9.4	9.2
[N_{4444}][Tricine]	9.9	9.5

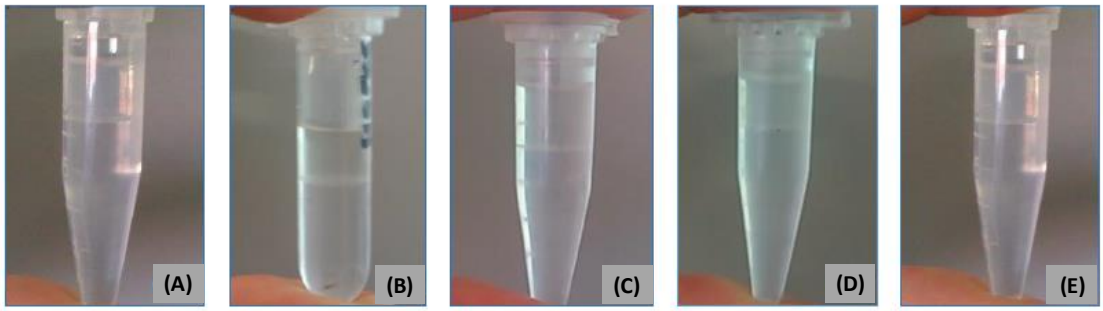

Figure 3.1.10. ABS formed by $[P_{4444}][GB]$ + $K_3C_6H_5O_7$ + WSPF: (A) $[P_{4444}][CHES]$, (B) $[P_{4444}][HEPES]$, (C) $[P_{4444}][MES]$, (D) $[P_{4444}][TES]$, (E) $[P_{4444}][Tricine]$.

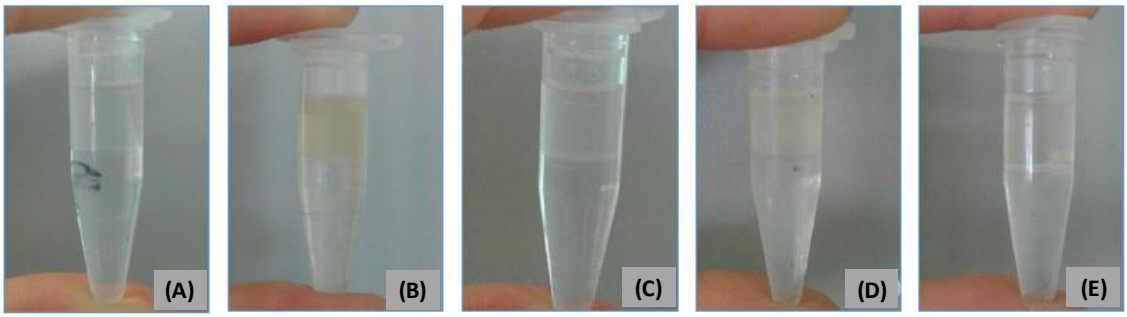

Figure 3.1.11. ABS formed by $[N_{4444}][GB]$ + $K_3C_6H_5O_7$ + WSPF: (A) $[N_{4444}][CHES]$, (B) $[N_{4444}][HEPES]$, (C) $[N_{4444}][MES]$, (D) $[N_{4444}][TES]$, (E) $[N_{4444}][Tricine]$.

The SDS-PAGE of the coexisting phases of the ten GB-IL-based ABS investigated was carried out to address the proteins profile in each phase and to infer these systems selectivity (Figure 3.1.12. and Figure 3.1.13.). As highlighted before, pure IgY under reducing conditions presents two major bands, namely at 65 kDa and 25 kDa, corresponding to the two heavy chains and the two light chains. According to the results shown in Figures 3.1.12. and 3.1.13., in all the studied systems no proteins were detected at the salt-rich phase, and two or three bands of proteins were detected in the IL-rich phase. These results confirm the high extraction ability of IL-based ABS for proteins. Independently of the second phase-forming component (PPG or salt), all proteins tend to migrate preferentially to the IL-rich phase. The bands at 65 and 25 kDa correspond to IgY, and the third band at 38 kDa confirm the presence of vitellogenin 1 and 2. In the previous section, six main water-soluble proteins from WSPF (IgY, serum albumin, ovalbumin, ovotransferrin, vitellogenin 1, and vitellogenin 2) were identified. In Figures 3.1.12. and 3.1.13., (lane 2)-WSPF, the presence of the six proteins is clear,

although IgY (heavy chain) and serum albumin are overlapping. However, in the SDS-PAGE of the coexisting phases, it is notorious the absence of serum albumin and ovotransferrin. For the systems composed of $[P_{4444}][HEPES]$, $[P_{4444}][Tricine]$, $[N_{4444}][HEPES]$, $[N_{4444}][TES]$, $[N_{4444}][MES]$ or $[N_{4444}][Tricine]$, also a lower intensity or even absence of the bands corresponding to the IgY heavy chain and vitellogenin 1 and 2, is observed. This fact may be explained by the proteins denaturation, which was particularly evident for the ABS composed of $[P_{4444}][HEPES]$, $[N_{4444}][HEPES]$ and $[N_{4444}][TES]$, where a significant amount of precipitated proteins was formed at the interface (Figures 3.1.10. and 3.1.11). Although no high selectivity for IgY was identified with these systems, their coexisting phases were also analyzed by SE-HPLC in order to address the IgY purification factor and recovery yield.

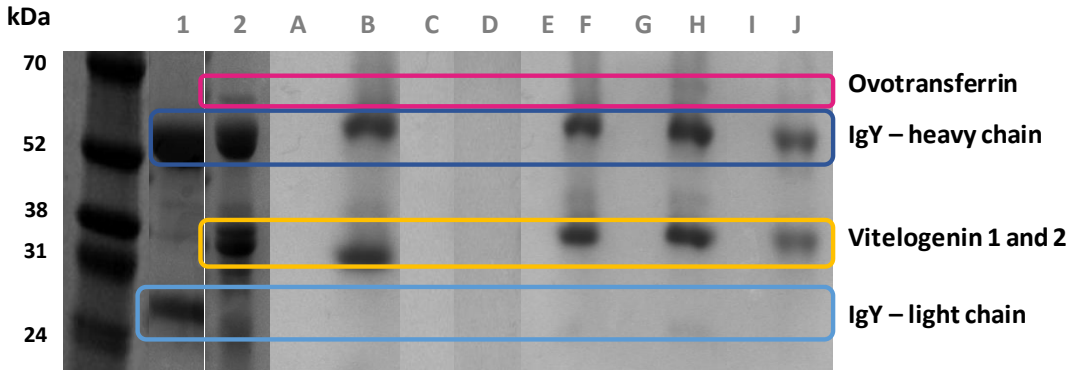

Figure 3.1.12. SDS-PAGE of a gel loaded with samples of bottom and top phases of systems composed of $[P_{4444}]$ GB-ILs + $K_3C_6H_5O_7$ stained with Coomassie blue: 1 - pure IgY; 2 – water-soluble proteins from egg yolk; A and B - bottom and top phases, respectively, of the $[P_{4444}][CHES]$ + $K_3C_6H_5O_7$ ABS; C and D - bottom and top phases, respectively, of the $[P_{4444}][HEPES]$ + $K_3C_6H_5O_7$ ABS; E and F - bottom and top phases, respectively, of the $[P_{4444}][MES]$ + $K_3C_6H_5O_7$ ABS; G and H - bottom and top phases, respectively, of the $[P_{4444}][TES]$ + $K_3C_6H_5O_7$ ABS; I and J - bottom and top phases, respectively, of the $[P_{4444}][Tricine]$ + $K_3C_6H_5O_7$ ABS.

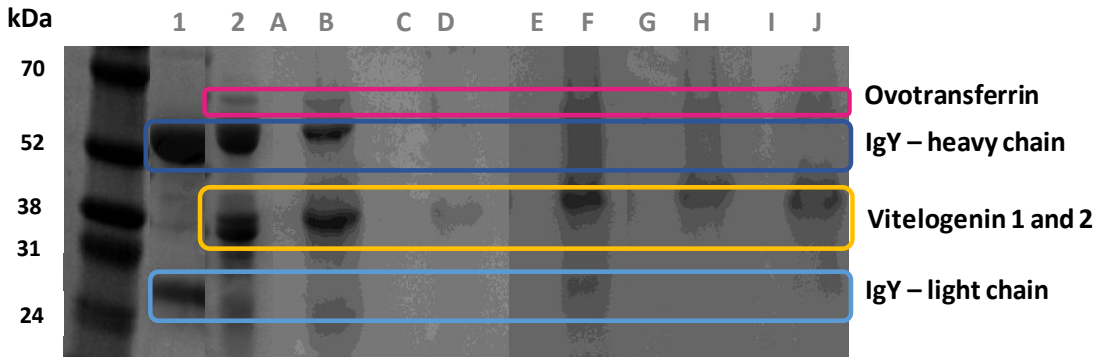

Figure 3.1.13. SDS-PAGE of a gel loaded with samples of bottom and top phases of systems composed of [N_{4444}] GB-ILs + $K_3C_6H_5O_7$ stained with Coomassie blue: 1 – pure IgY; 2 – water-soluble proteins from egg yolk; A and B - bottom and top phases, respectively, of the [N_{4444}][CHES] + $K_3C_6H_5O_7$ ABS; C and D - bottom and top phases, respectively, of the [N_{4444}][HEPES] + $K_3C_6H_5O_7$ ABS; E and F - bottom and top phases, respectively, of the [N_{4444}][MES] + $K_3C_6H_5O_7$ ABS; G and H - bottom and top phases, respectively, of the [N_{4444}][TES] + $K_3C_6H_5O_7$ ABS; I and J - bottom and top phases, respectively, of the [N_{4444}][Tricine] + $K_3C_6H_5O_7$ ABS.

The results obtained by SE-HPLC were similar in all the studied systems. Figure 3.1.14. depicts an example of the SE-HPLC chromatograms of the top and bottom phases of the ABS composed of [N_{4444}][Tricine] + $K_3C_6H_5O_7$ + WSPF. The remaining SE-HPLC chromatograms are shown in Annex - Figures A2 to A7. The SE-HPLC chromatogram of the WSPF shows the three main peaks corresponding to proteins discussed in the previous section, namely IgY, with 15 min of retention time, and the remaining proteins at ≈16-18 min. In general, no proteins were found in the chromatograms corresponding to the bottom phases, agreeing with the results discussed before and attained by SDS-PAGE. Regarding the top phase samples, it was expected the presence of a peak at 15 min of retention time, corresponding to IgY. Instead, it was detected a new peak at a lower retention time (≈12 min), which we admit being an IL-protein complex resulting from the high interaction of each GB-IL with IgY confirmed by an additional SE-HPLC study performed using mixtures composed of pure IgY + GB-ILs or $K_3C_6H_5O_7$ (Annex A - Figures A8 and A9). This IL-IgY strong interaction is also confirmed by molecular docking studies previously carried out [51]. Further attempts to characterize this complex were carried out. For instance, two different protein precipitation techniques, acetone, and trichloroacetic acid precipitation, plus ultrafiltration and dialysis, were applied to recover the IgY from the IL-rich and analyze the proteins profile by SE-HPLC. However,

no successful results were obtained. The failure of these procedures was mainly due to difficulties regarding the pellet solubilization in an adequate phosphate buffer aqueous solution preventing further studies on the identification of proteins by SE-HPLC. The peaks after 20 min belong mostly to the IL and salt present in the system, as confirmed by the SE-HPLC chromatograms of the IL-based ABS for which no IgY or WSPF was added (Annex A - Figures A10). In summary, it was not possible the quantification of IgY in the IL-rich phase and to address its purification factor by SE-HPLC.

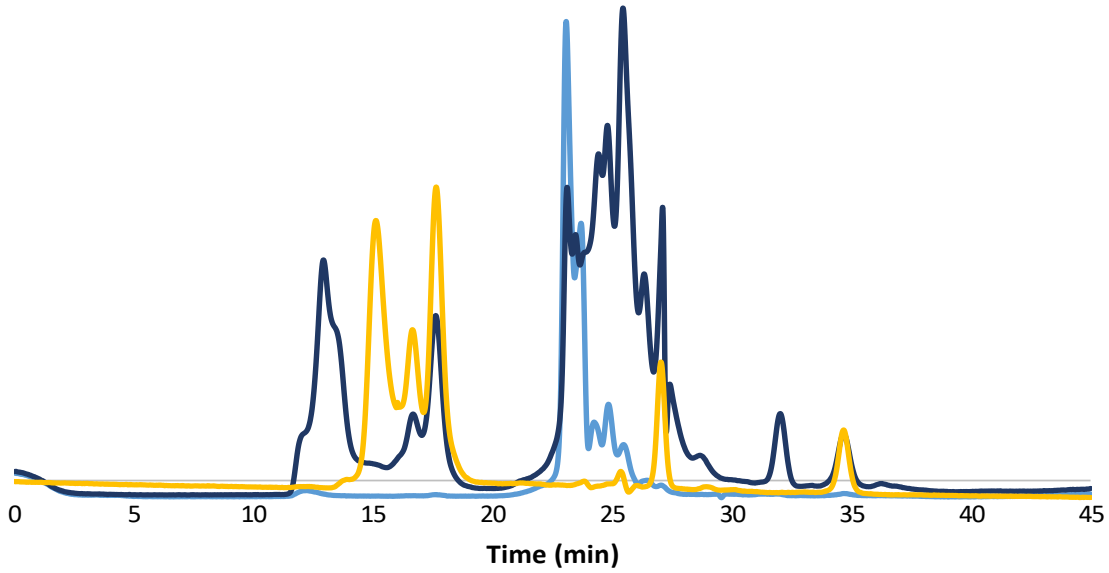

Figure 3.1.14. SE-HPLC chromatogram of the system composed of [N_{4444}][Tricine] + $K_3C_6H_5O_7$ + WSPF: bottom phase (light blue line), top phase (blue line) and original WSPF (yellow line).

Based on the results comprising the purification of IgY from the WSPF using PPG 400 + GB-IL and [P_{4444}][GB]/[N_{4444}][GB] + $K_3C_6H_5O_7$ it is possible to conclude that these systems are enhanced strategies to extract proteins to the IL-rich phase, independently of the second phase-forming components, but yet lack in selectivity for IgY. Moreover, some of the ABS composed of $K_3C_6H_5O_7$ and GB-ILs lead to the WSPF proteins precipitation (Figures 3.1.10. and 3.1.11.), which in some cases corresponds to IgY lost.

Aiming at better understand the precipitation phenomenon and loss of IgY in some ABS and to better identify the most appropriate phase-forming components to be used in further IgY purification studies, the IgY stability/activity in various aqueous solutions of ILs, salts, and polymers were addressed by ELISA.

Enzyme-linked immunosorbent assays (ELISA)

When foreseeing the use of antibodies as therapeutic agents, it is required a high purity level of these target compounds. However, to recover a high IgY pure sample, usually different and multi-stage techniques are required, which may affect the antibodies structure and activity [52]. By studying the antibody functionality in the presence of several compounds, namely ILs, salts, and polymers, it may be possible to select the better ABS phase-forming components in further studies. The use of immunodetection methods (based on the antigen-antibody interaction), specifically ELISA, allows the evaluation of the antibody's activity and function, i.e., if the antigen recognizes the antibody.

Sandwich ELISA was used to evaluate the activity of IgY in aqueous solutions of 5, 15 and 25 wt% of ILs ([Ch][MES], [C_4mim]Cl, [Ch][Ac] and [N_{4444}]Cl) and polymers (PEG 200, PEG 400, PEG 600 and PPG 400). Nevertheless, for aqueous solutions of salts, different concentrations were tested: 5 wt% for NaCl, Na_2CO_3 and $NaHPO_4$, 5 and 10 wt% for Na_2SO_4, and 5 and 15 wt% for $Na_3C_6H_5O_7$. This fact was due to the IgY precipitation in solutions with a higher salts concentration. It should be remarked that additional ILs and polymers to those tested in ABS discussed before were investigated, mainly because our aim was to identify the most appropriate ILs, polymers, and salts to be used in following purification experiments.

Active IgY in all aqueous solutions was quantified using the calibration curve given in Annex A - Figure A11. The concentration of IgY in aqueous solutions of polymers, salts, and ILs are given in Figures 3.1.15., 3.1.16. and 3.1.17., respectively. The IgY concentration is higher in aqueous polymer solutions, followed by salts and ILs. These results are in agreement with those provided by Rosa et al. [2], where it is mentioned that some polymers have a stabilizing effect on the protein tertiary structure and biological activity. By increasing the polymer or IL content, a lower concentration of IgY is detected. However, with higher concentrations of salt, particularly in Na_2SO_4 and $Na_3C_6H_5O_7$ aqueous solutions, a higher concentration of salt leads to a higher amount of active IgY; however, higher concentrations of these salts also lead to IgY precipitation.

In summary, amongst all the studied possible phase-forming components of ABS, ILs are those that lead to a poorer IgY activity. Better results are indeed obtained with polymers

and salts. Amongst all the studied polymers, PEG 600 allows higher preservation of the IgY activity, whereas, amongst the studied salts, Na_2SO_4 was the salt that allows the highest recognition of IgY by ELISA. Based on these results, our further studies on the purification of IgY from the egg yolk WSPF were focused on ABS formed by polymers and salts. This type of systems, particularly if PEG is used, also lead to a decrease on the process cost, which is advantageous when large-scale and CPC applications are envisaged.

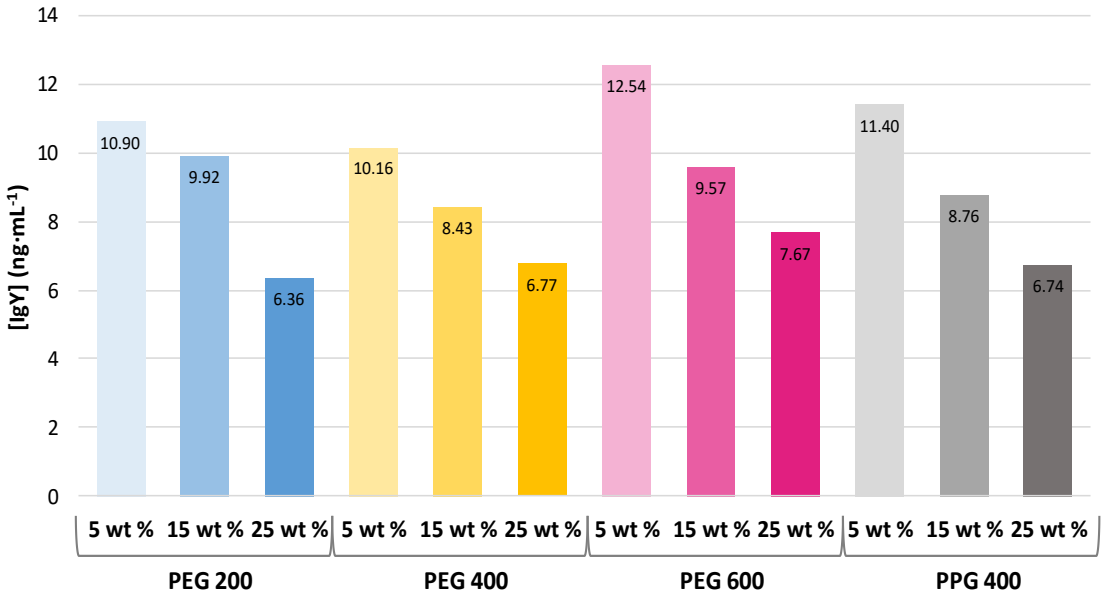

Figure 3.1.15. Concentration of IgY determined by ELISA in aqueous solutions of polymers.

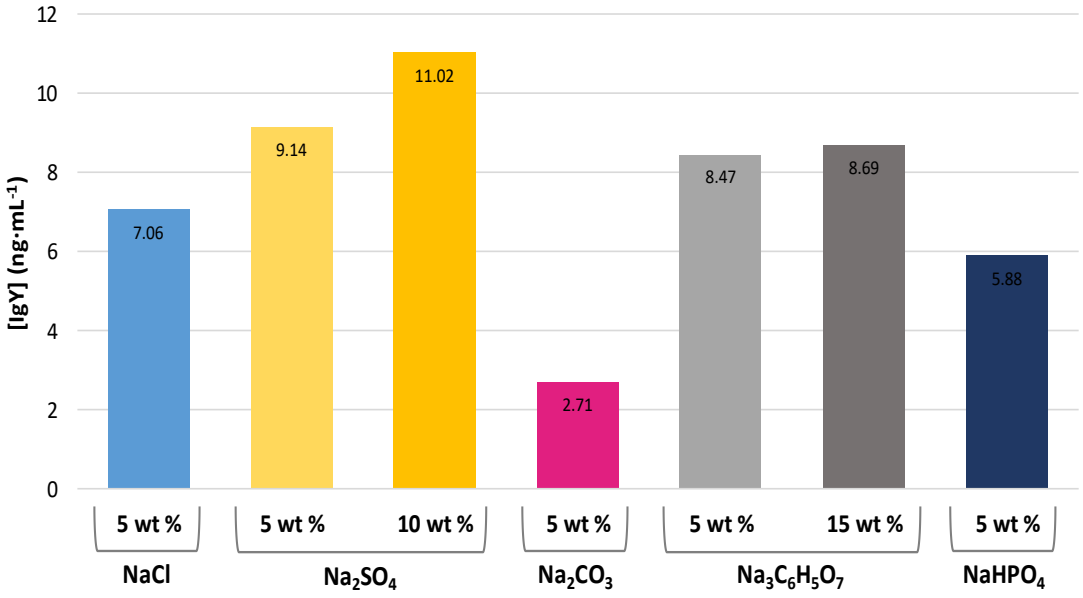

Figure 3.1.16. Concentration of IgY determined by ELISA in aqueous solutions of salts.

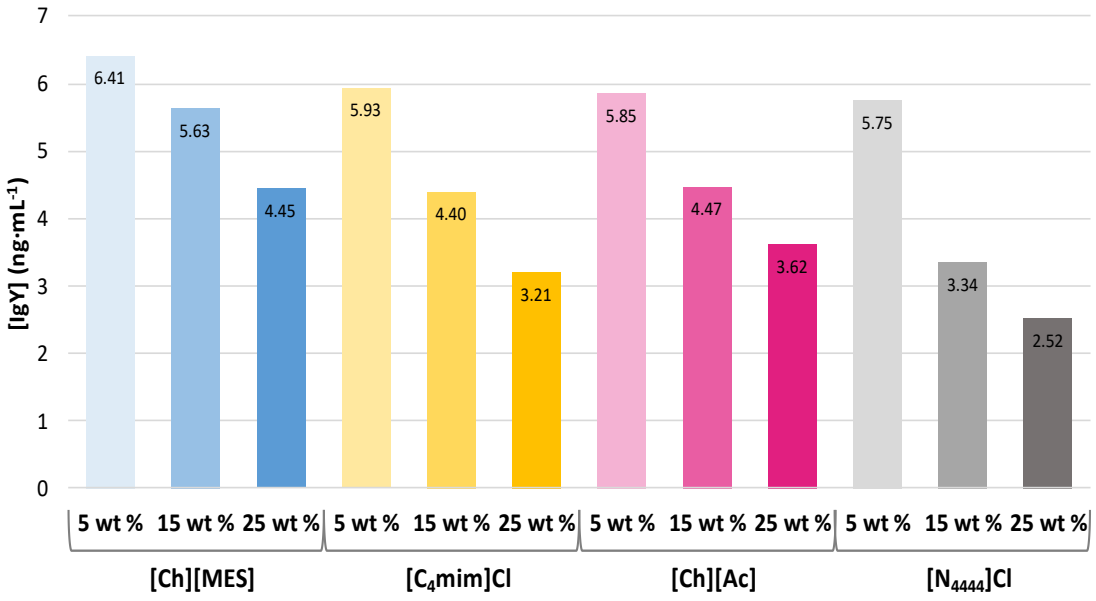

Figure 3.1.17. Concentration of IgY determined by ELISA in aqueous solutions of ILs.

Conclusions

In this section, it was evaluated the capacity of GB-IL-based ABS combined with PPG 400 or $K_3C_6H_5O_7$ for the extraction and purification of IgY from the WSPF of egg yolk. In this chapter, the synthesis of novel GB-ILs was described, together with the determination of the respective ABS phase diagrams, including TLs and TLLs. The diversity of GB-ILs investigated allowed to study the influence of the IL cation and anion on the ABS formation ability. Generally, an increase in the hydrophobicity of the GB-IL promotes the ABS formation, following the same behavior as other more conventional ILs and salts ABS. Overall, and although some selectivity of IgY was identified, the purification of IgY with the investigated systems was far from being achieved in a single-step using ABS. All of the systems investigated led to the migration of most proteins to the IL-rich phase, independently of the second ABS phase-forming component. In some cases, the presence of an IL-IgY complex and/or precipitation of the proteins of the WSPF was also identified. By ELISA it was possible to prove that the antibody activity is higher in aqueous polymer solutions, followed by salts and ILs, leading us to a new research direction focused on the use of ABS formed by polymers and salts - presented in the subsequent chapter.

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3.2. Purification of IgY using ABS formed by polymers and salts combined with centrifugal partition chromatography

Some of the experimental work presented in this section was developed with the collaboration and inputs of Filipe Ferreira.

Abstract

Typical polymer + salt aqueous biphasic systems (ABS) are recurrently used in the extraction/purification of biomolecules, such as antibodies, mainly due to their high-water content and biocompatibility and because most of the polymers used have a stabilizing effect on the proteins tertiary structure. However, these systems were not studied hitherto to purify immunoglobulin Y (IgY). On the other hand, only less than 2 % of the total polyclonal antibodies produced worldwide are IgY due to the difficulties in isolating antibodies from egg yolk. Based on the exposed, ABS composed of polyethylene glycol (PEG) + Na_2SO_4 and polyethylene glycol 1000 $g \cdot mol^{-1}$ (PEG 1000) + K_2HPO_4/ KH_2PO_4 buffer were here studied for the extraction and purification of IgY from the water-soluble proteins fraction (WSPF) recovered from egg yolk. New phase diagrams for these ternary systems were determined. The effect of the molecular weight of PEG (200, 300, 400 and 600 $g \cdot mol^{-1}$) and mixture compositions in PEG + Na_2SO_4 ABS were investigated, whereas in PEG 1000 + K_2HPO_4/ KH_2PO_4 buffer ABS the effect of the pH value (5.5, 6.0, 6.5, 7.5 and 8.0) was studied. All systems were investigated as one-step ABS platforms to purify IgY. The results obtained for both types of systems revealed a high affinity of IgY and remaining proteins to the polymer-rich phase. By changing the PEG and salt concentration, a higher selectivity was observed for IgY, with the contaminant proteins displaying a similar partition between the top and bottom phases. Based on the selectivity of these systems for IgY, PEG + Na_2SO_4 and PEG 1000 + K_2HPO_4/ KH_2PO_4 buffer ABS were applied in centrifugal partition chromatography (CPC) to allow multi-stage partition and to address the technology scale-up. ABS formed by PEG and Na_2SO_4 display a small difference in the coexisting phases densities, not allowing their proper application in CPC. On the other hand, the ABS formed by PEG 1000 + K_2HPO_4/ KH_2PO_4 buffer display proper retention of the stationary phase, allowing to increase the IgY purity by CPC. Several operating conditions were optimized, allowing to obtain IgY with 51% of purity by CPC.

Introduction

Conventional polymer-based aqueous biphasic systems (ABS) have been successfully used in the downstream processing of immunoglobulin (IgG) [1-5]. In 2007, Rosa et al. [3] described the partitioning of immunoglobulins in conventional ABS (polymer + polymer and polymer + salt). IgG was first extracted into a PEG-rich phase, and then to a phosphate-rich phase [3]. The purification factor achieved was 2.7 and 5.9, respectively [3]. The same authors [4] reported the recovery of human IgG from Chinese hamster ovary (CHO) and hybridoma cell culture supernatants using PEG 6000 + K_2HPO_4/ NaH_2PO4 buffer ABS. IgG with an 88% recovery yield in the polymer-rich phase was obtained [4]. In 2009, Azevedo et al. [5] studied the partitioning and purification of human IgG from a clarified hybridoma supernatant with PEG + $Na_3C_6H_5O_7$/ citric acid buffer ABS. The effect of PEG molecular weight and the addition of a neutral salt, like NaCl, on the partition of IgG, were addressed. Using an ABS composed of 8 wt% PEG 3350 + 8 wt% $Na_3C_6H_5O_7$/ citric acid buffer + 15 wt% NaCl at pH 6, IgG was recovered with a 99% yield, 44% of purity and with an IgG/protein ratio of 0.9. Later, Mao et al. [6] investigated the extraction of antibodies from host cell proteins (HCP) from clarified cell culture media using PEG + $Na_3C_6H_5O_7$/ citric acid buffer ABS. An optimal ABS composed of 14 wt% PEG + 8.4 wt% $Na_3C_6H_5O_7$/ citric acid buffer + 7.2 wt% NaCl at pH 7.2 resulted in a product yield of 89%, a 7.6-fold reduction in HCP levels relative to the clarified cell culture fluid before extraction, and overall purity of 70%. However, among all the works described, the scale-up of the ABS applied, for instance by applying centrifugal partition chromatography (CPC) or countercurrent chromatography (CCC), is scarce.

CPC is a type of liquid-liquid chromatography requiring two immiscible liquid phases, one of which acts as the stationary phase and the other acts as the mobile phase (CPC uses centrifugal force in order to hold the stationary liquid phase) [7]. The high volume of the stationary phase that can be loaded into the CPC is a significant advantage, making this technique suitable for industrial applications [8]. CPC works by the exploration of the partition trend of different compounds between the two immiscible solvents/phases, as long as their densities are sufficiently different [7]. The CPC apparatus can be used in two operation modes, ascending and descending, with the key difference being the density of the phase being used as the mobile one: in the ascending

mode the lightest phase is the mobile phase, flowing upwards through the heavier stationary phase, while the opposite occurs in the descending mode [8]. The main advantage of using CPC comes from the liquid-liquid nature of this process, making it unnecessary to use solid chromatographic supports, therefore guaranteeing almost 100% of the target compound recovery and easy recyclability of the solvents [7]. This technique also avoids the need to acquire, maintain and clean high-cost solid columns [9]. While this adaptability is seen as an advantage in terms of potential applications, it should be stressed the importance of a careful choice of the solvents/phases, which should be defined according to the following criteria: easiness of two-phase formation; capacity to be retained by the CPC; and separation/purification effectiveness [8]. Despite all the CPC advantages, the application of polymer + salt ABS in CPC for the separation/purification of antibodies has not been deeply explored. The only work found corresponds to Oelmeier et al. [10], who described CPC as an alternative separation step that combined with other precipitation and resolubilization techniques allow to purify monoclonal antibodies. The researchers found that after removing the cells from culture supernatant, either via regular means like centrifugation or by using liquid-liquid separation approaches by ABS and performing a CPC run in dual-mode, an upper phase rich in antibodies could be obtained while removing host cell proteins. The ABS investigated in this study was formed by PEG 400 + $Na_3C_6H_5O_7$/ citric acid buffer.

Based on three evidences, namely (i) the main drawbacks associated with the purification of immunoglobulin (IgY), (ii) the several reports using polymer + salt ABS for the extraction and purification of antibodies, and (iii) the advantages of CPC as a viable scaled-up technique, ABS formed by polymers and salts combined with CPC were here investigated aiming at purifying IgY from the water-soluble proteins fraction (WSPF) of egg yolk. ABS formed by PEG + Na_2SO_4 and PEG 1000 + K_2HPO_4/ KH_2PO_4 buffer were studied. The ternary phase diagrams of ABS were firstly determined, and their selective extractive performance for IgY further evaluated. The most promising ABS were applied in CPC aiming the complete purification of IgY from the WSPF. Although further work is still required, CPC is an important technique to obtain low-cost biopharmaceuticals.

Experimental Section

Materials

Fresh eggs were periodically provided by Dr. Ricardo Pires from Biocant, located in Cantanhede, Portugal. IgY to establish the SE-HPLC calibration curve was purified using the Pierce® Chicken IgY Purification Kit (Thermo Scientific, EUA).

The ABS studied in this work were formed by using Na_2SO_4 (anhydrous, 99 wt% pure from Sigma Aldrich) or phosphate buffer salts composed of potassium phosphate dibasic trihydrate ($K_2HPO_4{\cdot}3H_2O$, purity > 99%) and monopotassium phosphate (KH_2PO_4, purity > 99.5%), acquired from PanReac and Sigma-Aldrich, respectively. Poly(ethylene)glycol (PEG) with a molecular weight of 200 $g{\cdot}mol^{-1}$, 300 $g{\cdot}mol^{-1}$, 400 $g{\cdot}mol^{-1}$, 600 $g{\cdot}mol^{-1}$ and 1000 $g{\cdot}mol^{-1}$ and here abbreviated as PEG 200, PEG 300, PEG 400, PEG 600 and PEG 1000, respectively, were studied. All polymers were acquired from Fluka, with the exception of PEG 300 and PEG 600 that was from Sigma-Aldrich.

The solvents/salts required to perform the SE-HPLC analysis comprise sodium phosphate monobasic (NaH_2PO_4, purity: 99 – 100.5%), and sodium phosphate dibasic heptahydrate ($Na_2HPO_4{\cdot}7H_2O$, purity: 98.2 – 102.0%) and sodium chloride (NaCl), obtained from Sigma–Aldrich. The water employed was double distilled, passed through a reverse osmosis system and treated with a Milli-Q plus 185 water purification apparatus.

ABS phase diagrams and tie-lines

The binodal curves for the ABS formed by PEG + Na_2SO_4 + water were not determined here since they are already reported in the literature [11]. The binodal curves of the ABS composed of PEG 1000 + K_2HPO_4/ KH_2PO_4 buffer at different pH values were determined through the cloud point titration method at (25 ± 1) °C and atmospheric pressure [12]. Aqueous solutions of PEG 1000 at 60 wt% and K_2HPO_4/ KH_2PO_4 buffer at 25 - 40% were used in the determination of the phase diagrams. The phosphate buffer was prepared at different pH values - 5.5 (±0.22), 6.0 (±0.16), 6.5 (±0.16), 7.5 (±0.02) and 8.0 (±0.05) - using different molar ratios of the two phosphate-based salts. The composition of each buffer solution is given in Annex B - Table B1.

The tie-lines (TLs) were determined by a gravimetric method originally described by Merchuk et al. [13]. A mixture at the biphasic region was gravimetrically prepared with

PEG + salt + water, vigorously stirred, and allowed to reach equilibrium by separation into the two phases over at least 12 h at 25 °C. After the separation of the coexisting phases, the phases were further weighted. Finally, each individual TL was determined by the application of the lever-arm rule to the relationship between the weight of the top and bottom phases and the overall system composition. TLs were determined for both types of ABS evaluated, namely formed by PEG + Na_2SO_4 and PEG 1000 + K_2HPO_4/ KH_2PO_4 buffer.

The experimental binodal curves were fitted using equation 15 [13],

$$[\mathrm{PEG}] = A\,exp[(B[\mathrm{salt}]^{0.5}) - (C[\mathrm{salt}]^3)] \quad (15)$$

where [PEG] and [salt] are the PEG and salt weight fractions percentages, respectively, and *A*, *B* and *C* are fitted constants obtained by least-squares regression.

For the determination of the TLs, the following system of four equations (equations 16 to 19) was used to estimate the concentration of IL and salt at each phase ($[\mathrm{PEG}]_{\mathrm{PEG}}$, $[\mathrm{PEG}]_{\mathrm{salt}}$, $[\mathrm{salt}]_{\mathrm{salt}}$ and $[\mathrm{salt}]_{\mathrm{PEG}}$),

$$[\mathrm{PEG}]_{\mathrm{PEG}} = A\,exp[(B\,[\mathrm{salt}]_{\mathrm{PEG}}^{0.5}) - (C\,[\mathrm{salt}]_{\mathrm{PEG}}^{3})] \quad (16)$$

$$[\mathrm{PEG}]_{\mathrm{salt}} = A\,exp[(B\,[\mathrm{salt}]_{\mathrm{salt}}^{0.5}) - (C\,[\mathrm{salt}]_{\mathrm{salt}}^{3})] \quad (17)$$

$$[\mathrm{PEG}]_{\mathrm{PEG}} = \frac{[\mathrm{PEG}]_{\mathrm{M}}}{\alpha} - \left(\frac{1-\alpha}{\alpha}\right)[\mathrm{PEG}]_{\mathrm{salt}} \quad (18)$$

$$[\mathrm{salt}]_{\mathrm{PEG}} = \frac{[\mathrm{salt}]_{\mathrm{M}}}{\alpha} - \left(\frac{1-\alpha}{\alpha}\right)[\mathrm{salt}]_{\mathrm{salt}} \quad (19)$$

where the subscripts salt and PEG designate the salt- and PEG-rich phases, respectively, and M is the initial mixture composition. The parameter α is the ratio between the weight of the top phase and the total weight of the mixture. The solution of the described system provides the concentration of the PEG and salt in the top and bottom phases.

In order to calculate each the tie-line length (TLL), equation 20 was applied,

$$\mathrm{TLL} = \sqrt{([\mathrm{salt}]_{\mathrm{PEG}} - [\mathrm{salt}]_{\mathrm{salt}})^2 + ([\mathrm{PEG}]_{\mathrm{PEG}} - [\mathrm{PEG}]_{\mathrm{salt}})^2} \quad (20)$$

The correlation parameters of equation 15 and the compositions of the top and bottom phases, were determined using the software Matlab R2015a.

Extraction and purification of IgY from the egg yolk WSPF using ABS

PEG + Na_2SO_4 ABS

The WSPF of egg yolk was prepared from fresh eggs, following the protocol described in the literature [14], and then applied in the ABS composition. The ternary mixture compositions for the PEG + Na_2SO_4 + water systems were chosen according to the phase diagrams reported in the literature [11]. To evaluate the effect of the PEG molecular weight on the water-soluble proteins partitioning, ternary mixtures within the biphasic region and using a common composition of salt (10 wt%) and variable concentrations of the different polymers (from 24 to 30 wt%) were prepared. Table 3.2.1 describes the mixture compositions and TLL of each ternary mixture determined by Almeida et al. [11] to address the PEG molecular weight effect. After identifying the most promising PEG as a phase-forming component to selectively extract IgY from the WSPF to the polymer-rich phase, several mixture compositions of the ABS formed by PEG 600 and Na_2SO_4 were investigated, given in Table 3.2.2. Each mixture was gravimetrically prepared (± 10^{-4} g), gently stirred and left to equilibrate for at least 4 h, at (25 ± 1) °C, to achieve the complete IgY partitioning and remaining proteins between the two phases. In all the ternary mixtures evaluated, the polymer-rich aqueous phase corresponds to the top phase, while the bottom phase is mainly composed of salt and water.

Table 3.2.1. Ternary mixture composition of ABS composed of PEG + Na_2SO_4 for the purification of IgY from the WSPF, and respective TLL.

PEG	[PEG]/ wt%	[Na_2SO_4]/ wt%	[WSPF]/ wt%	TLL ≈
200	25.0	10.0	65.0	32.5
300	24.0	10.0	63.0	37.5
400	27.0	10.0	63.0	35.9
600	24.0	10.0	66.0	43.3

Table 3.2.2. Ternary mixture composition of ABS composed of PEG 600 + Na_2SO_4 for the purification of IgY from the WSPF, and respective TLL.

	[PEG]/ wt%	[Na_2SO_4]/ wt%	[WSPF]/ wt%	TLL≈
PEG range	24.0	10.0	66.0	42.2
	22.0	10.0	68.0	37.9
	20.0	10.0	70.0	33.4
	18.0	10.0	72.0	31.7
Na_2SO_4 range	24.0	9.0	67.0	39.4
		8.0	67.8	35.2
		7.0	68.5	20.9
Minimum PEG and salt concentrations	21.0	8.0	71.0	22.0

PEG 1000 + K_2HPO_4/ KH_2PO_4 buffer ABS

The WSPF of egg yolk was prepared from fresh eggs, following the protocol described in the literature [14], and then applied in the ABS composition. The ternary mixtures compositions to be applied as ABS to purify IgY from the WSPF were chosen based on the phase diagrams determined for each PEG 1000 + K_2HPO_4/ KH_2PO_4 buffer systems at different pH values. A common ternary mixture composition was prepared using 18 wt% PEG 1000 + 15 wt% K_2HPO_4/ KH_2PO_4 buffer + WSPF. Table 3.2.3 describes the mixture compositions and TLL of each ternary mixture determined in this work to address the pH value effect. With the optimum pH value identified (6.0), different concentrations of PEG and K_2HPO_4/ KH_2PO_4 buffer were then studied (4 mixture points). These experimental mixture compositions are presented in Table 3.2.4.

Table 3.2.3. Ternary mixture composition of ABS composed of PEG 1000 + K_2HPO_4/ KH_2PO_4 buffer for the purification of IgY from the WSPF, and respective TLL.

pH	[PEG]/ wt%	[K_2HPO_4/ KH_2PO_4 buffer]/ wt%	[WSPF]/ wt%	TLL≈
5.5	18.0	15.0	67.0	28.9
6.0	18.0	15.0	67.0	38.3
6.5	18.0	15.0	67.0	38.6
7.5	18.0	15.0	67.0	41.4
8.0	18.0	15.0	67.0	42.3

Table 3.2.4. Ternary mixture composition of ABS composed of PEG 1000 + K_2HPO_4/ KH_2PO_4 buffer pH 6.0 for the purification of IgY from the WSPF, and respective TLL.

	[PEG 1000]/ wt%	[K_2HPO_4/ KH_2PO_4 buffer]/ wt%	[WSPF]/ wt%	TLL≈
pH 6.0	18.0	15.0	67.0	42.3
	18.0	13.0	69.0	29.9
	21.0	16.0	63.0	47.6
	21.0	14.0	65.0	42.5

Each mixture was vigorously stirred and left to equilibrate for at least 4 h, at (25 ± 1) °C, to achieve the complete IgY partitioning and other contaminant proteins between the two phases. In all the ternary mixtures evaluated, the polymer-rich aqueous phase is the top phase, while the bottom phase is mainly composed of salt and water.

pH measurements

The pH values of both the PEG-rich and salt-rich aqueous phases were measured at (25 ± 1) °C using a METTLER TOLEDO SevenMulti pH meter within an uncertainty of ± 0.02. The calibration of the pH meter was carried out with two buffers (pH values of 4.00 and 7.00).

Size exclusion-high performance liquid chromatography (SE-HPLC)

After a careful separation of the phases, SE-HPLC was used with the aim of quantifying IgY in each phase and inferring its purity. A calibration curve for IgY was determined for

this purpose and is reported in Annex B – Figure B1. The remaining steps of the SE-HPLC protocol are described in "Chapter 3. – subchapter 3.1. – Experimental section.

The extraction efficiency, *EE*%, of the studied systems for IgY from the WSPF was determined according to equation 21,

$$EE\% = \frac{w_{IgY}^{\text{top phase}}}{w_{IgY}^{\text{top phase}} + w_{IgY}^{\text{bottom phase}}} \times 100 \quad (21)$$

where $w_{IgY}^{\text{top phase}}$ and $w_{IgY}^{\text{bottom phase}}$ are the weight of IgY in the PEG-rich (top phase) and in the salt-rich (bottom phase) aqueous phases, respectively.

The partition coefficients of IgY and contaminant proteins in the WSPF, $K_{IgY,}$ and $K_{\text{contaminant protein}}$, were determined according to equations 22 and 23, respectively,

$$K_{IgY} = \frac{\text{peak area of IgY}_{\text{top phase}}}{\text{peak area of IgY}_{\text{bottom phase}}} \quad (22)$$

$$K_{\text{contaminant protein}} = \frac{\text{peak area of contaminant protein}_{\text{top phase}}}{\text{peak area of contaminant protein}_{\text{bottom phase}}} \quad (23)$$

where peak area of IgY/contaminant protein$_{\text{top phase}}$ and peak area of IgY/contaminant *protein$_{bottom\ phase}$* are the peak area of the IgY or contaminant protein in PEG-rich (top phase) and in the salt-rich (bottom phase) aqueous phases, respectively.

The purity of IgY, Purity%, was determined according to equation 24,

$$\text{Purity\%} = \frac{\text{peak area of IgY}_{\text{top phase}}}{\text{peaks area of contaminant proteins} + \text{peak area of IgY}_{\text{top phase}}} \quad (24)$$

where, peak area of IgY$_{\text{top phase}}$ and peak area of contaminant proteins + peak area of IgY$_{\text{top phase}}$ are the peak area of IgY and peak areas of contaminant proteins and IgY in PEG-rich (top phase) aqueous phase, respectively.

The recovery yield of IgY was determined by the ratio between the amount of IgY in the PEG-rich phase (top phase) and the total amount of IgY present in the WSPF added to each system, by the application of equation 25,

$$\text{Yield}\ (\%) = \frac{\text{WIgY}_{\text{top phase}}}{\text{WIgY}_{\text{WSPF}}} \times 100 \quad (25)$$

At least three individual experiments were carried out for each ABS, allowing the determination of the average extraction efficiencies, partition coefficients, purity and recovery yield, and respective standard deviations. Control or "blank" solutions at the same mixture point used for the extraction studies (with no WSPF/proteins added) were used in all systems.

Centrifugal partition chromatography (CPC)

A Fast Centrifugal Partition Chromatography (FCPC)® system, model FCPC-C, from Kromaton Rousselet-Robatel (Annonay, France), was used for the IgY purification from the WSPF by applying ABS. The equipment design comprises a pattern of cells interconnected by ducts and dug in a stainless-steel disk. The cell design, also called twin cells, contains a restriction in the middle ducts of the canal creating two superimposed chambers. The total cell volume is about 50 mL, with 10 mL or 20 % of the column volume corresponding to the connecting ducts. The maximum theoretical liquid stationary phase retention factor (S_f = VS/VC) is 80% since 20% of connecting ducts volume can only contain the mobile phase. The maximum rotor rotation is 3000 rpm, generating a maximum centrifugal field of ~ 1500 G. Two rotating seals are displayed at the rotor entrance and exit, and they can withstand a maximum pressure of 70 bar (7 MPa or 1000 psi). The FCPC system was connected to an ECOM ECB2004 Gradient box with degasser, an ECOM ECP2010 Analytical HPLC pump, an ECOM Flash 14 DAD detector (four wavelengths are simultaneously being analyzed), and to a continuous scan (ECOM spol. S.r.o., Czech Republic). Several fractions were collected with an ADVANTEC® Super Fraction Collector CHF122SC (Advantec Toyo Kaisha, Ltd., Tokyo, Japan). Each sample was injected manually using a Rheodyne valve model 3055-023 through a 10 mL sample loop. Analogical detector signals were processed using the ECOMAC software (ECOM spol. S.r.o., Czech Republic).

The CPC assays were carried out using the PEG 600 + Na_2SO_4 and PEG 1000 + K_2HPO_4/ KH_2PO_4 buffer at pH 6.0 ABS. The systems were set to work in the ascending mode. The

rotor was entirely filled with the bottom-rich phase (salt-rich phase) at 600 rpm to achieve the homogeneous solvent re-equilibration on the rotor. Then, the rotation was set to the intended speed, needed for the appropriate stationary phase retention. After the working rotational speed was set up, the PEG-rich phase (top phase) was pumped through the stationary phase to reach the equilibrium, *i.e.,* when only the mobile phase came out of the column, and the signal baseline is stabilized. The stationary phase retention, S_f%, was calculated by the ratio of the stationary phase volume (VS) and the column volume (VC), as described in equation 26,

$$S_f\% = \frac{VS}{VC} \times 100 \quad (26)$$

Results and Discussion

PEG + Na_2SO_4 ABS

The effect of the molecular weight of the polymer in the partitioning of water-soluble proteins from egg yolk, including IgY, in PEG + Na_2SO_4 systems, was evaluated using four PEGs with distinct molecular weights (PEG 600, 400, 300 and 200). The mixture compositions used in partitioning experiments and the respective phases' compositions and TLLs are presented in Table 3.2.1. As an example, in Figure 3.2.1., are represented the SE-HPLC chromatograms corresponding to original WSPF and both phases of the PEG 600 + Na_2SO_4 ABS, with the peaks of the contaminant proteins present in the WSPF from egg yolk identified. As described in Chapter 2, six main water-soluble proteins were identified in the WSPF: IgY, serum albumin, ovalbumin, ovotransferrin, vitellogenin 1, and vitellogenin 2. IgY it was identified in the first peak, as represented in Figure 3.2.1., while serum albumin and ovotransferrin were the main proteins identified in the second peak (Figure 3.2.1. – contaminant 1). In the last peak, in Figure 3.2.1. – contaminant 2, were identified vitellogenin 1 and 2.

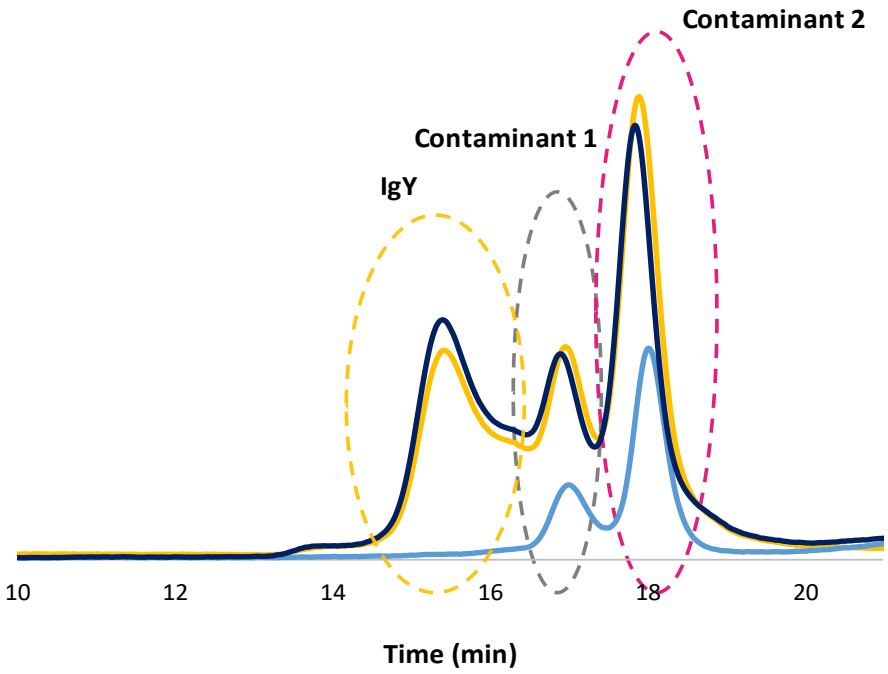

Figure 3.2.1. SE-HPLC chromatogram of the system composed of PEG 600 + Na_2SO_4 + WSPF: bottom phase (light blue line), top phase (blue line) and original WSPF (yellow line).

The extraction efficiency (*EE*%), yield and purity of IgY, and partition coefficients of contaminant proteins, at (25 ± 1) °C in the PEG + Na_2SO_4 ABS are given in Table 3.2.5. In all systems, with PEG 600 + Na_2SO_4 as an exception, it is observed the total partitioning of all proteins present in the WSPF (IgY and contaminant proteins) to the PEG-rich phase, with an *EE*% of IgY of 100.0%. However, with the system composed of the polymer with higher molecular weight (PEG 600), the extraction efficiency decreases to 68.7%. The lower affinity of IgY to the PEG-rich phase in ABS constituted by PEGs of higher molecular weight may be related with its lower water content - as addressed by the TL data given in Annex B – Table B2. These results are in agreement with the ones reported by Ferreira et al. [15], where it was found that IgG has a higher tendency to migrate to the polymer-rich phase in ABS composed of lower molecular weight polymers.

The partitioning coefficients of contaminant proteins range between 1.6 and 2.3, being highly dependent on the molecular weight of the PEG used. Furthermore, the partition of contaminant proteins between the two-phases of the ABS occur only with the PEG of higher molecular weight, meaning a higher selectivity of this system. The purity and yield

of IgY range between 18.8 and 27.1%, and 27.5 and 98.8%, respectively. The lower values of purity and yield are obtained with the system composed of PEG 600 + Na_2SO_4, i.e. with the system composed with the PEG with the higher molecular weight. The lower yield of IgY obtained with this system could be related with the lower water concentration in the PEG-rich phase (Annex B – Table B2), leading to the WSPF proteins denaturation. Figure 3.2.2. shows the macroscopic appearance of the systems prepared constituted by PEG + Na_2SO_4 + WSPF, proving the WSPF proteins denaturation only in the PEG 600 + Na_2SO_4 ABS. Antibodies precipitation and lower yields in ABS composed of polymers of higher molecular weight were previously described by Rosa et al. [16]. However, only with this ternary mixture and system (PEG 600 + Na_2SO_4) it is visible the partition of the contaminant proteins in the WSPF to the bottom (salt-rich) phase. Thus, the PEG 600 + Na_2SO_4 ABS was selected for the following investigations, in which different mixture compositions of the ABS formed by PEG 600 + Na_2SO_4 were prepared. Seven different mixtures compositions by varying the concentration of PEG and salt were prepared. These experimental mixture points are presented in Table 3.2.2.

Table 3.2.5. Extraction efficiency, purity, yield and partition coefficient of IgY; partition coefficients of each contaminant protein, $K_{contaminnat1}$ and $K_{contaminant2}$, in the PEG 600 + Na_2SO_4 + WSPF systems at (25 ± 1) °C.

PEG	IgY			Contaminant Proteins		
	EE%	Purity%	Yield%	K_{IgY}	$K_{contaminant1}$	$K_{contaminant2}$
200	100.0 ± 0.1	26.6 ± 1.1	98.8 ± 1.1	-	-	-
300	100.0 ± 0.1	26.8 ± 0.9	85.4 ± 2.0	-	-	-
400	100.0 ± 0.1	27.1 ± 1.5	81.1± 2.4	-	-	-
600	63.6 ± 0.9	18.8 ± 2.8	27.5 ± 3.6	1.1 ± 0.2	2.3 ± 0.5	1.6 ± 0.1

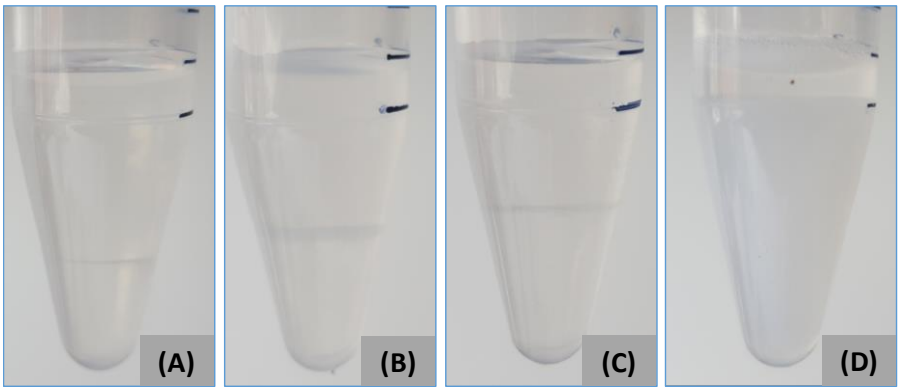

Figure 3.2.2. ABS composed of PEG + Na_2SO_4 + WSPF at (25 ± 1) °C. A – PEG 200; B – PEG 300; C – PEG 400; D – PEG 600.

The *EE*%, purity, yield and partition coefficient of IgY, partition coefficients of each contaminant protein ($K_{contaminant1}$ and $K_{contaminant2}$) and pH of the PEG-rich phase (top phase) in the different PEG 600 + Na_2SO_4 systems prepared are given in Table 3.2.6. The partition coefficients of IgY and contaminant proteins, and IgY yield dependence on the mixture point at (25 ± 1) °C, is displayed in Figure 3.2.3. In all the studied mixtures, the partition coefficients are higher than 1.0, confirming the IgY and contaminant proteins preferential partitioning to the polymer-rich phase (more hydrophobic phase when compared with the salt-enriched phase).

According to Table 3.2.6., the *EE*% of IgY ranges between 63.6 and 100.0%. For the systems with lower concentrations of polymer (20 wt% PEG or 18 wt% PEG), salt (8 wt% Na_2SO_4 or 7 wt% Na_2SO_4) or both polymer and salt (21 wt% PEG + 8 wt% Na_2SO_4), an *EE*% of 100% is achieved, meaning that there is no IgY at the salt-rich phase. However, when higher concentrations of PEG are used (24 wt% PEG or 22 wt% PEG), a lower *EE*% of IgY to the PEG-rich phase is observed. The lower affinity of IgY to the PEG-rich phase in ABS constituted by higher concentrations of PEG may be related with its lower water content - as addressed by the TL data given in Annex B – Table B3.

Regarding the recovery yield, values ranging from 27.5 and 100.0% were obtained. The systems with higher concentrations of polymer and salt led to lower yield values. This is due to the lower water concentration in the PEG-rich phase leading to the WSPF proteins denaturation, as highlighted before. Figure 3.2.4. shows the macroscopic appearance of the systems PEG 600 + Na_2SO_4 + WSPF prepared, showing the WSPF proteins denaturation in the PEG 600 + Na_2SO_4 ABS with higher concentrations of polymer (24

wt% PEG 600/ 22 wt% PEG + 10 wt% Na_2SO_4) and salt (24 wt% PEG 600 + 9 wt% Na_2SO_4/ 8 wt% Na_2SO_4), with low recovery yield values.

The IgY purity obtained in all the mixtures studied ranges between 18.8 and 42.5% (Table 3.2.6.). Higher concentrations of PEG and salt in the ABS lead to a lower IgY purity in the polymer-rich phase, as occurs with the IgY yield. This fact is also justified by the protein precipitation and turbidity macroscopically seen in the systems constituted by higher concentrations of polymers (Figure 3.2.4.).

The $K_{contaminant1}$ and $K_{contaminant2}$ range between 1.2 and 4.3, and 1.1 and 2.3, for the contaminant proteins 1 and 2, respectively (Table 3.2.6 and Figure 3.2.3.). All the contaminant proteins display a partition coefficient higher than 1 and a preferential migration to the PEG-rich phase. The $K_{contaminant1}$ and $K_{contaminant2}$ increase with a decrease of the PEG concentration in the overall mixture composition, with the mixture point 18 wt% PEG + 10 wt% salt, as an exception. On the other hand, the $K_{contaminant1}$ and $K_{contaminant2}$ decrease by decreasing the concentration of salt in the mixture composition. In summary, higher amounts of polymer in the mixture composition lead to a lower partition of IgY to the polymer-rich phase, while higher concentrations of salt lead to a higher partition of the contaminant proteins to the polymer-rich phase. These evidences suggest that the partitioning of IgY seems to be mainly driven by the water content at the polymer-rich phase, and salting-out effects (exerted by the salt-rich phase). These results corroborate with previous partitioning results of IgG in PEG-salt ABS [15]. However, electrostatic interactions cannot be discarded if extractions are being carried out at pH values different from the protein pI (the pI of the chicken IgY in this work is 5.5 – determined experimentally by dynamic light scattering) as well as hydrogen-bonding interactions, which seem particularly relevant in water-rich media. According to the results obtained, and taking into account that the pH of all system is between 5.4 and 5.6 - Table 3.2.6, it seems that the partitioning of IgY and contaminant proteins is mainly related with the water content in the PEG-rich phase and salting-out effects.

Among all the mixtures studied, the best result was achieved with the system composed of 24 wt% PEG 600 + 7 wt% Na_2SO_4, with an *EE*% and Yield for IgY of 100.0% and 100.0%, respectively, and a $K_{contaminant1}$ and $K_{contaminant2}$ of 1.8 and 1.1, respectively. With the IgY completely retained in the PEG-rich phase and the contaminant proteins partitioning

between the top and bottom phases, the application of this system in CPC is expected to allow the complete purification of IgY and the two other contaminant proteins.

Table 3.2.6. Extraction efficiency, purity, yield and partition coefficient of IgY; partition coefficients of each contaminant protein, $K_{contaminnat1}$ and $K_{contaminant2}$, and pH of the PEG-rich phase (top phase), in the PEG 600 + Na_2SO_4 + WSPF systems at (25 ± 1) °C.

Mixture composition	$pH_{PEG\text{-}rich\ phase}$	IgY				Contaminant Proteins	
		EE%	Purity%	Yield%	K_{IgY}	$K_{contaminant1}$	$K_{contaminant2}$
24 wt% PEG + 10 wt% salt	5.6	63.6 ± 0.9	18.8 ± 2.8	27.5 ± 3.6	1.1 ± 0.2	2.3 ± 0.5	1.6 ± 0.1
22 wt% PEG + 10 wt% salt	5.5	66.2 ± 2.6	25.2 ± 0.8	42.4 ± 1.4	1.2 ± 0.1	2.9 ± 0.2	1.8 ± 0.1
20 wt% PEG + 10 wt% salt	5.5	100.0 ± 0.1	37.7 ± 1.8	74.1 ± 2.4	-	4.3 ± 0.6	2.1 ± 0.7
18 wt% PEG + 10 wt% salt	5.4	100.0 ± 0.1	42.5 ± 0.1	85.7 ± 0.9	-	2.7 ± 0.2	2.3 ± 0.1
24 wt% PEG + 9 wt % salt	5.5	87.2 ± 0.6	22.8 ± 1.5	40.1 ± 1.2	3.5 ± 0.5	4.3 ± 0.4	1.7 ± 0.3
24 wt% PEG + 8 wt% salt	5.5	100.0 ± 0.1	37.6 ± 0.8	83.4 ± 3.3	-	3.2 ± 0.2	1.4 ± 0.1
24 wt% PEG + 7 wt% salt	5.4	100.0 ± 0.1	40.3 ± 0.1	100.0 ± 0.1	-	1.8 ± 0.2	1.1 ± 0.2
21 wt% PEG + 8 wt% salt	5.4	100.0 ± 0.1	40.7 ± 0.3	96.37 ± 0.1	-	1.2 ± 0.5	1.1 ± 0.6

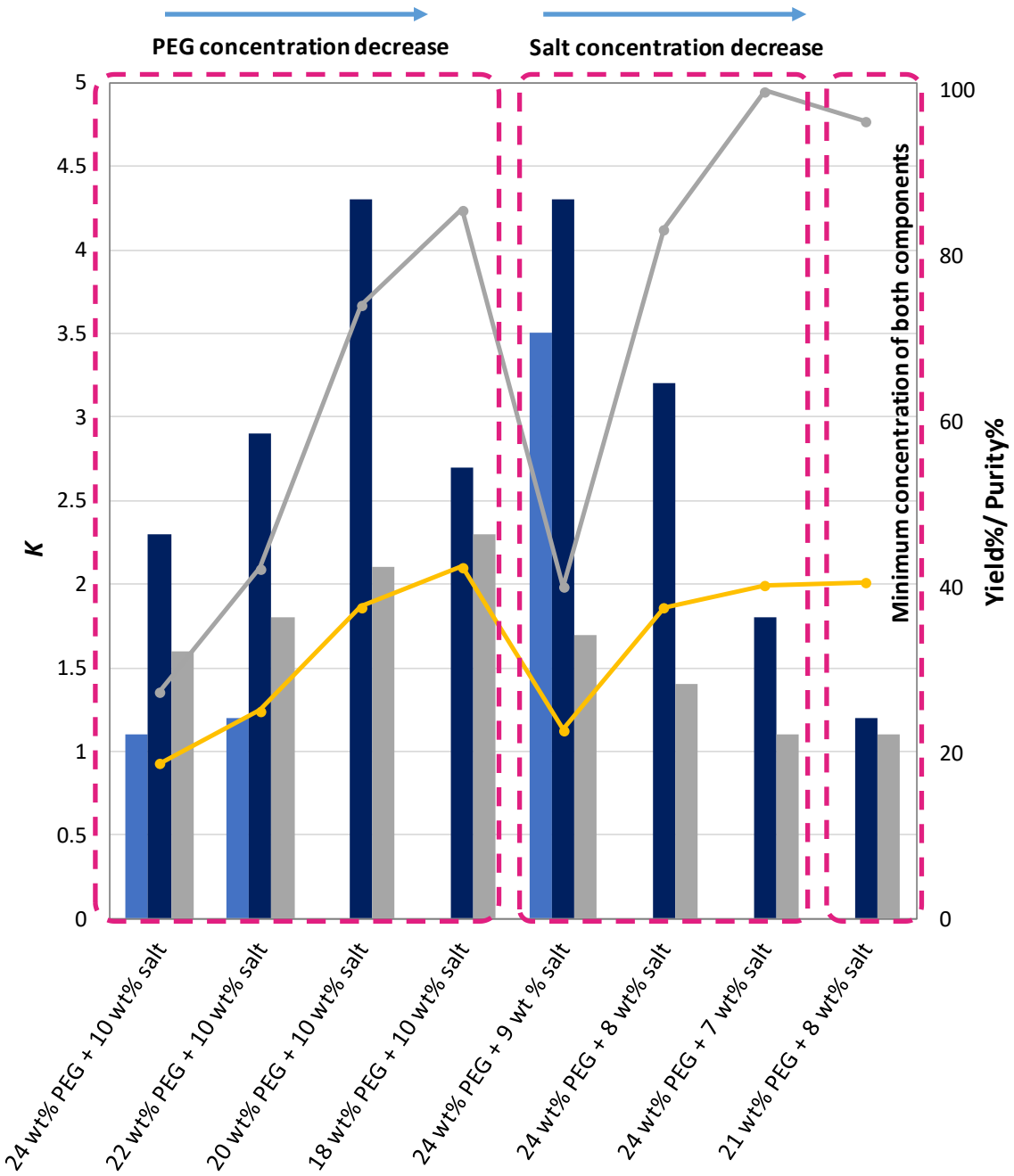

Figure 3.2.3. Yield and purity of IgY (grey and yellow lines, respectively) and partition coefficients of IgY (light blue bar), proteins contaminant 1 (blue bar) and contaminant 2 (grey bar), in the PEG 600 + Na_2SO_4 ABS at (25 ± 1) °C.

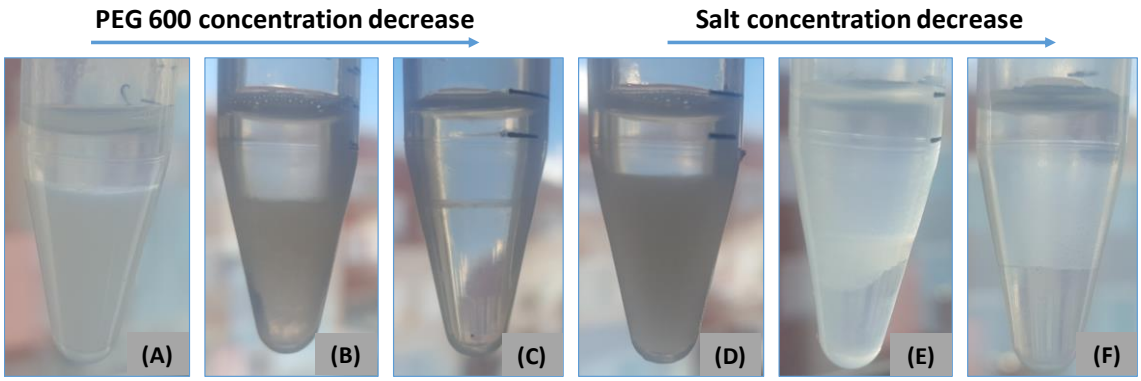

Figure 3.2.4. ABS composed of PEG 600 + Na_2SO_4 + WSPF at (25 ± 1) °C. A – 24 wt% PEG 600 + 10 wt% Na_2SO_4; B – 22 wt% PEG 600 + 10 wt% Na_2SO_4; C – 20 wt% PEG 600 + 10 wt% Na_2SO_4; D – 24 wt% PEG 600 + 9 wt% Na_2SO_4; E – 24 wt% PEG 600 + 8 wt% Na_2SO_4; F – 24 wt% PEG 600 + 7 wt% Na_2SO_4.

In an attempt to purify IgY from the WSPF using ABS, the CPC technique was applied. In a first stage several operating conditions, such as mode (ascending or descending), flow rate and rotation speed were applied to verify the PEG 600 + Na_2SO_4 + water ABS behavior in the equipment (Table 3.2.7.). In these experiments covering the equipment optimization, the WSPF was not used. Taking into account the complete partitioning of IgY into the PEG-rich phase, the ascending mode was first investigated. In this mode, the stationary phase is the salt-rich phase, and the mobile phase the PEG-rich phase; thus, IgY is expected to be the first protein being purified (IgY has the higher affinity to the PEG-rich phase). However, despite all the operating conditions studied (different flow rates and rotation speed), in this mode, it was not possible to retain the stationary phase in the CPC rotor (Table 3.2.7.). Thus, a new strategy was implemented, and the descending mode was explored. The rotor was first filled with the PEG-rich phase (stationary phase), and then the salt-rich phase (mobile phase) was pumped to equilibrate the system. Table 3.2.7. shows the stationary phase retention obtained at different operating conditions. With the increase of flow-rate, a loss of stationary phase inside the column is observed; however, increasing the rotation speed, this fact is surpassed. Considering the information summarized in Table 3.2.7., the descending mode was selected for the following experiments.

Table 3.2.7. Stationary phase retention (S_f%) achieved for the CPC assays with the 24 wt% PEG 600 + 7 wt% Na_2SO_4 + 69 wt% H_2O systems, and respective operating conditions.

FCPC operation mode	Rotation speed/ (rpm)	Flow rate/ (mL/min)	S_f (%)
Ascending	2500	2.0	No retention of the stationary phase
Ascending	2500	1.0	
Ascending	3000	2.0	
Ascending	3000	1.0	
Descending	2000	1.0	33.82
Descending	2000	2.0	14.22
Descending	2500	1.0	28.82
Descending	2500	2.0	30.15
Descending	2500	3.0	10.54
Descending	3000	1.0	33.82

The CPC separations were carried out using the system composed of 24 wt% PEG 600 + 7 wt% Na_2SO_4 + 69 wt% H_2O in descending mode. For the separation of IgY from the contaminant proteins, the sample loop was filled with 5 mL of the ABS composed of 24 wt% PEG 600 + 7 wt% Na_2SO_4 + 69 wt% WSPF. Four operations conditions were investigated (2500 rpm at 2.0 mL/min, 2500 at 1.0 mL/min, 3000 rpm at 2.0 mL/min, and 3000 rpm at 1.0 mL/min). In all studies, after 20 min of elution with the bottom salt-rich phase to extract the contaminants proteins, the mobile phase was changed to the PEG-rich (top) phase to elute IgY, by applying an elution-extrusion process [18]. Although several operating conditions were studied, the results were similar, and it was impossible to purify IgY by CPC using the selected ABS. During the elution, and only when the contaminating proteins were expected to be eluted, IgY was also eluted. This fact may be due to the loss of the stationary phase (PEG-rich phase) during the elution, the phase for which IgY has more affinity. The high amount of water in the system used to maintain the proteins integrity makes the phases density too similar, thus decreasing the stationary-phase retention and compromising the success of the assays on the purification of IgY. Therefore, the search for an adequate system composition to avoid the loss of stationary phase during this type of assays is desired. Further attempts on this direction are described below.

PEG 1000 + K_2HPO_4/ KH_2PO_4 buffer ABS

To a proper ABS to be used in the CPC equipment, three main parameters should be analyzed, namely the easiness of two-phase formation, the retention capacity in the CPC rotor, and the separation/purification effectiveness [8]. In order to move on with an adequate system composition, a literature review was carried out not to identify systems which could be successful in the purification of antibodies, but instead relevant and feasible to be applied in CPC. Foucault et al. [19] compared several polymers + salt and polymer + polymer ABS in CPC assays, concluding that while all of the systems analyzed have an effective retention capacity, the major problem is the mass transfer effect, which requires significant reductions in the apparatus' flow-rate to overcome this fact. Advances in CPC technology [20] have allowed the use of higher flow rates with polymer + salt ABS, as demonstrated by Sutherland et al. [21], using ABS composed of 12.5 wt% of PEG 1000 + 12.5 wt% of K_2HPO_4, at a 10.0 mL/min flow-rate, with an effective retention capacity.

Based on the effectiveness of the PEG 1000 + K_2HPO_4 ABS, when considering the retention capacity of the phases in CPC [21], the remarkable results provided by this system for the purification of enzymes [22] and IgG antibodies [23], this system was selected and investigated here in an attempt to purify IgY from the WSPF by CPC. To initially evaluate the effectiveness of the 12.5 wt% PEG 1000 + 12.5 wt% K_2HPO_4 + 75 wt% H_2O ABS in the CPC apparatus, several operating conditions were tested (rotation speed, flow-rate, and mode - Table 3.2.8.). In general, higher retention values were obtained with stationary phase retention (S_f%) above 40%, which are higher than the values obtained with PEG 600 + Na_2SO_4 ABS discussed above. However, as described before, the increase of the flow-rate decreases the stationary phase retention, which is not significant with this system (PEG 1000 + K_2HPO_4) since high stationary phase retention is obtained. This system has an effective retention in CPC enabling its further use in this work for the purification of IgY from the egg yolk WSPF.

Table 3.2.8. Stationary phase retention (S_f %) achieved for the CPC assays with the 12.5 wt% PEG 1000 + 12.5 wt% K_2HPO_4 + 75 wt% H_2O ABS, and respective operating conditions.

CPC operation mode	Rotation speed/ (rpm)	Flow rate/ (mL/min)	S_f (%)
Descending	2000	1.5	48.52
Descending	2500	2.0	44.90
Ascending	2000	1.5	47.30
Ascending	2500	2.0	46.00

After demonstrating that the PEG 1000 + K_2HPO_4 + water ABS has sufficient retention to be used in CPC assays, further optimizations were carried out in order to purify IgY. Since the pH is a crucial parameter when dealing with the extraction of proteins, K_2HPO_4/ KH_2PO_4 buffer solutions at different pH values were prepared, namely 5.5, 6.0, 6.5, 7.5 and 8.0, to appraise the effect of the pH on these ABS extraction capacity and selectivity for IgY. The respective ternary ABS phase diagrams were determined at (25 ± 1) °C and atmospheric pressure, and are illustrated in Figure 3.2.5. The experimental weight fraction data are given in Annex B (Tables B4 to B6). In the studied ABS, the top phase is the PEG-rich phase, while the bottom phase is primarily composed of salt and water.

Regarding the phase diagrams presented in Figure 3.2.5., there is an increase in the biphasic region area with the increase of pH, meaning that the capacity to form ABS follows the trend: pH 5.5 < 6.0 < 6.5 < 7.5 ≈ 8.0. This behavior is due to the salting-out of the salt over the polymer, in which ions that are more easily hydrated are more able to induce phase splitting. With the increase of pH in the system there is a higher concentration of K_2HPO_4, and as such, higher concentrations of HPO_4^{2-}, a strongly hydrated anion according to the Hofmeister series [24], causing an increase of the salting-out effect. Similar trends have been observed in other ABS composed of polymers and salts [25, 26].

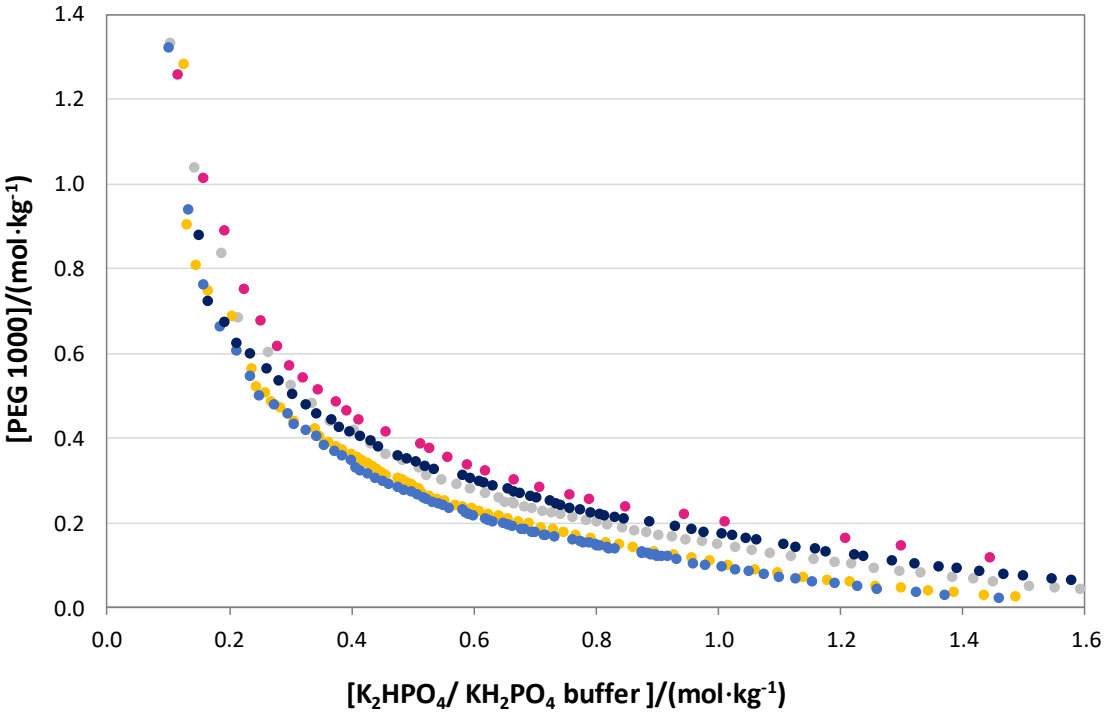

Figure 3.2.5. Phase diagrams of the ABS composed of PEG 1000 + K_2HPO_4/ KH_2PO_4 buffer + H_2O at different pH values: 5.5 (●); 6.0 (●); 6.5 (●); 7.5 (●); 8.0 (●), at (25 ± 1) °C and atmospheric pressure.

The experimental binodal data were fitted according to the empirical relationship described by equation 15. The regression parameters *A*, *B,* and *C*, which were estimated by the least-squares regression, are provided in Annex B – Table B7, along with their corresponding standard deviations (σ). Overall, good correlation coefficients were obtained, indicating that these fittings can be used to predict data in any given region of the phase diagram, without the need to resort to experimental data. The experimental data for the TLs and their respective length (TLL) are reported in Annex B -Table B8.

After characterizing the PEG 1000 + phosphate buffer ABS by their phase diagrams, a fixed mixture point for the extraction of IgY was selected: 18 wt% PEG 1000 + 15 wt% of K_2HPO_4/ KH_2PO_4 buffer + 67 wt% of WSPF. This mixture point was selected to fit within the biphasic area of all phase diagrams, but as close as possible to the binodal curve to maximize the water content on the systems and avoid the proteins denaturation. Figure 2.3.6. shows the macroscopic appearance of the systems prepared at different pH values. The appearance of these systems suggests that proteins in the WSPF suffer denaturation and precipitate at the interface. In general, there is an increase in the amount of proteins precipitated by the increase of the pH values. The occurrence of

proteins denaturation may be related with the strong salting-out capacity of the K_2HPO_4 salt used, as previously described in the literature [27], and with the low water content at the polymer-rich phase - as addressed by the TL data given in Annex B – Table B8. Another important macroscopic characteristic is the precipitation of salt present in the ABS at pH 5.5, as can be seen in Figure 2.3.6.

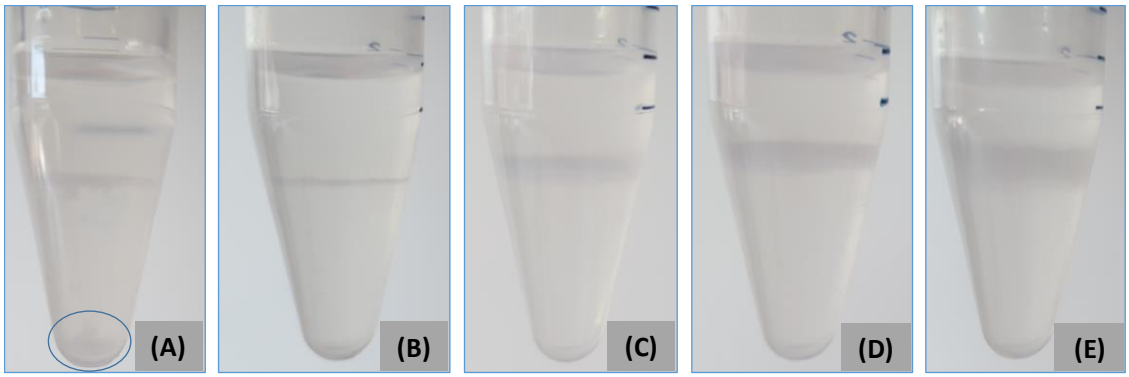

Figure 3.2.6. ABS composed of 18 wt% PEG 1000 + 15 wt% of K_2HPO_4/ KH_2PO_4 buffer + 67 wt% of WSPF at (25 ± 1) °C, at several pH values. A – pH 5.5; B – pH 6.0; C – pH 6.5; D – pH 7.5; E – pH 8.0. The blue circle highlights the presence of precipitated salt.

Despite the proteins denaturation, the purification performance of these systems cannot be ruled out, as the denatured proteins may correspond to contaminant proteins present in the WSPF. Both phases of the systems were collected and analyzed by SE-HPLC, without removing the precipitate in the interphase which was then discarded, in order to calculate the partition coefficients of the contaminant proteins, IgY recovery yield, and purity. The results obtained are shown in Table 3.2.9. and Figure 3.2.7. The partition coefficient of IgY was not determined because a remarkable complete partition (selectivity) of IgY to the PEG-rich phase was observed, as in the PEG 600 + Na_2SO_4 ABS discussed above.

The recovery yield of IgY ranges between 74.9 and 89.1%, with the lower values obtained with the systems composed of K_2HPO_4/ KH_2PO_4 buffer with higher pH values. The lower yield of IgY is due to the lower water concentration in the PEG-rich phase (Annex B – Table B8), leading to a WSPF proteins denaturation.

The values of the partition coefficients of the contaminant proteins to the polymer-rich phase tend to increase with the pH increase (Table 3.2.9.), and thus the selectivity of the

systems decreases with an increase in pH. The partition coefficients of the contaminant proteins are all higher than 1, meaning that the contaminant proteins are preferentially migrating to the top phase, which explains the low purity of IgY obtained in a single-step with all systems. Overall, the pH value and/or the K_2HPO_4/ KH_2PO_4 buffer used affects the purity of IgY, whereas extractions performed at lower pH values lead to the best results. The extractions with ABS formed with the K_2HPO_4/ KH_2PO_4 buffer at pH 5.5 and 6.0 result in similar purity levels. However, the ABS composed of phosphate buffer at 5.5 lead to some salt precipitation, making this system less feasible to work with when it is foreseen to be applied in CPC. Therefore, the ABS buffered to pH 6.0 was selected to perform the following investigations.

Table 3.2.9. Purity and yield of IgY and partition coefficients of each contaminant protein, $K_{contaminant1}$ and $K_{contaminant2}$, in the 18 wt% PEG 1000 + 15 wt% K_2HPO_4/ KH_2PO_4 buffer + 69 wt% WSPF systems at (25 ± 1) °C.

pH	IgY		Contaminant Proteins	
	Purity%	Yield%	$K_{contaminant1}$	$K_{contaminant2}$
5.5	41.8 ± 0.1	89.1 ± 3.4	1.6 ± 0.1	1.4 ± 0.1
6.0	41.1 ± 0.5	79.8 ± 3.1	2.0 ± 0.1	1.7 ± 0.1
6.5	33.9 ± 3.6	79.9 ± 0.7	12.7 ± 3.8	2.5 ± 0.9
7.5	27.2 ± 4.0	74.9 ± 3.4	10.8 ± 3.1	3.0 ± 0.7
8.0	28.1 ± 1.6	79.9 ± 5.6	25.5 ± 2.4	3.5 ± 0.6

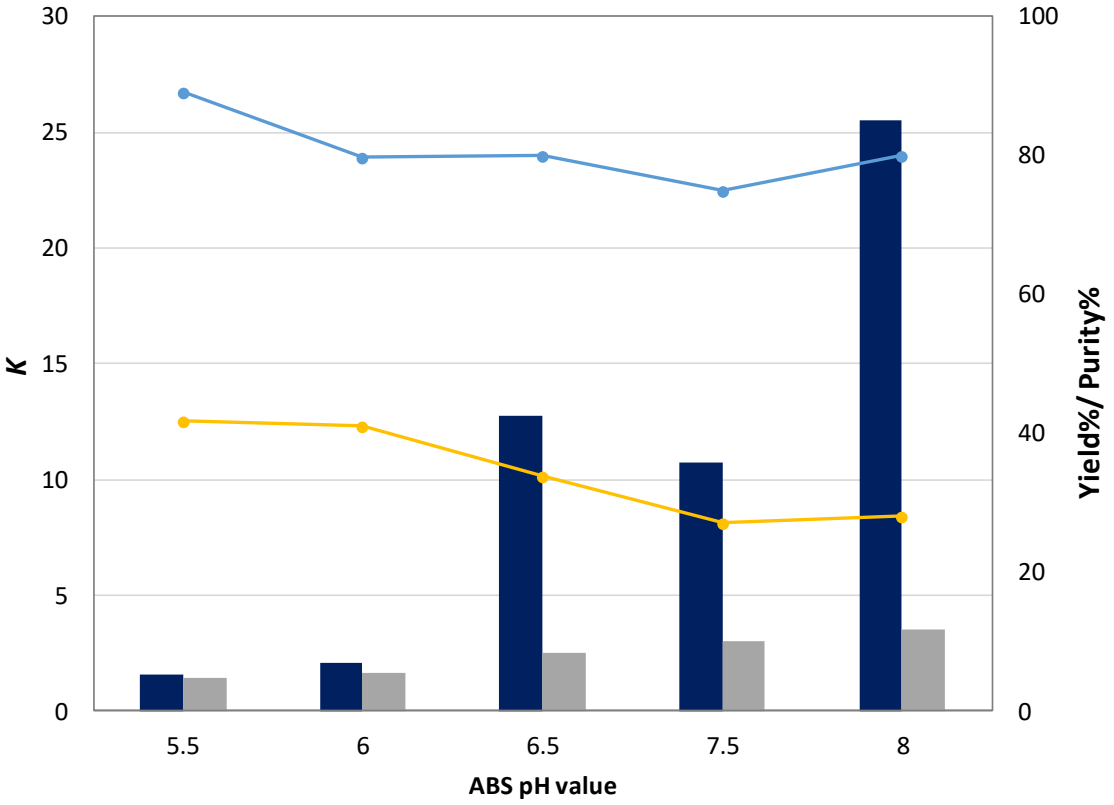

Figure 3.2.7. Yield and purity of IgY (light blue and yellow lines, respectively) and partition coefficients of proteins contaminant 1 (blue bar) and contaminant 2 (grey bar), in 18 wt% PEG 1000 + 13 wt% phosphate buffer + 69 wt% WSPF systems at (25 ± 1) °C.

Taking into account that the best results are achieved with ABS with pH 6.0, and in order to decrease the protein denaturation, several mixture compositions were evaluated by changing the concentration of PEG 1000 and K_2HPO_4/ KH_2PO_4 buffer at pH 6.0. Three new mixture compositions were tested: 18 wt% PEG 1000 + 13 wt% K_2HPO_4/ KH_2PO_4 buffer; 21 wt% PEG 1000 + 16 wt% K_2HPO_4/ KH_2PO_4 buffer and 21 wt% PEG 1000 + 14 wt% K_2HPO_4/ KH_2PO_4 buffer (Table 3.2.4.). The extraction results, in terms of IgY purity and yield, and partition coefficient of the contaminant proteins are described in Table 3.2.10.

Comparing the results obtained with the three new mixture compositions with the initially studied ABS, better results have been identified feasible to be applied in further CPC trials. In terms of purity, no mixture surpassed the first studied ABS (18 wt% PEG 1000 + K_2HPO_4/ KH_2PO_4 buffer), with only the 18 wt% PEG 1000 + 13 wt% K_2HPO_4/ KH_2PO_4 buffer system achieving comparable results (41.1 ± 0.5% *versus* 39.3 ± 1.0 %). However, since the CPC will be applied to purify IgY, the purity of IgY is not a key-element

to choose the ABS for further experiments. Regarding the IgY yield, the 18 wt% PEG 1000 + 13 wt% K_2HPO_4/ KH_2PO_4 buffer system clearly performs better. The yield value of this system is 100.0 ± 2.4 %, meaning not only that this system is partitioning IgY exclusively to its top phase, but that there is also no loss of IgY. In comparison, the first studied system (18 wt% PEG 1000 + 15 wt% K_2HPO_4/ KH_2PO_4 buffer) leads to a yield of IgY 79.8 ± 3.1 %. The last parameter that demonstrates the 18 wt% PEG 1000 + 13 wt% K_2HPO_4/ KH_2PO_4 buffer system as a more suitable liquid-liquid system for CPC assays is the partition coefficient of the contaminant proteins. The partition coefficients of the contaminant proteins are related to the elution time in a chromatographic assay due to their relative affinity between the mobile and stationary phases. Therefore, a higher difference between $K_{contaminant1}$ and $K_{contaminant2}$ will allow easier separation of the different contaminant proteins by CPC. In fact, the partition coefficients of both contaminant proteins, 3.9 ± 0.1 and 1.8 ± 0.4, are more distinct than the ones reported for first system studied and discussed above (2.0 ± 0.1 and 1.7 ± 0.1). It should be remarked that it was not possible to determine the partition coefficients for contaminant protein 1 in the 21 wt% PEG 1000 + 16 wt% K_2HPO_4/ KH_2PO_4 buffer system, and for both contaminant proteins in the 21 wt% PEG 1000 + 14 wt% K_2HPO_4/ KH_2PO_4 buffer. ABS since the contaminant proteins totally partitioned to the PEG-rich phase. In these systems, there is no selectivity IgY, with all proteins present in the WSPF partitioning to the same (PEG-rich) phase. In summary, the 18 wt% PEG 1000 + 13 wt% K_2HPO_4/ KH_2PO_4 buffer ABS is the best system to apply in the CPC trials due to the high extraction yield of IgY and the higher difference between the partition coefficients for the contaminant proteins.

Table 3.2.10. Purity and yield of IgY and partition coefficients of each contaminant protein, $K_{contaminant1}$ and $K_{contaminant2}$, in the PEG 1000 + K_2HPO_4/ KH_2PO_4 buffer at pH 6.0 + WSPF systems at (25 ± 1) °C.

Mixture composition	IgY		Contaminant Proteins	
	Purity%	Yield%	$K_{contaminant1}$	$K_{contaminant2}$
18 wt% PEG 1000 + 15 wt% phosphate buffer	41.1 ± 0.5	79.8 ± 3.1	2.0 ± 0.1	1.7 ± 0.1
18 wt % PEG 1000 + 13 wt% phosphate buffer	39.3 ± 1.0	100.0 ± 2.4	3.9 ± 0.1	1.8 ± 0.4
21 wt% PEG 1000 + 16 wt% phosphate buffer	27.2 ± 0.1	53.8 ± 0.6	n.d.*	8.8 ± 2.7
21 wt% PEG 1000 + 14 wt% phosphate buffer	22.8 ± 2.0	35.9 ± 3.3	n.d.*	n.d.*

With the previous screening on the ABS ability to purify IgY, and after confirming their feasibility of these ABS for use in CPC, the systems formed by PEG 1000 and K_2HPO_4/ KH_2PO_4 buffer were finally tested for their capacity to purify IgY from the egg yolk WSPF by CPC. Since the feasibility of these ABS involving PEG 1000 in CPC was carried out using an ABS composed of 12.5 wt% of PEG 1000 + 12.5 wt% of K_2HPO_4, as described above, it is imperative to confirm that the behavior is the same for the ABS constituted by 18 wt% of PEG 1000 + 13 wt% of phosphate buffer when it comes to the stationary phase retention. A process similar to the one previously described was carried out, mainly regarding the determination of the S_f value for the CPC assay with this system composition. The first purification trial was performed with the injection of a sample with 8 g of WSPF plus 2 g of mobile phase with an ABS composed of 18 wt% of PEG 1000 + 13 wt% of K_2HPO_4/ KH_2PO_4 buffer at pH 6.0 + 69 wt% of WSPF, using the following operating conditions: ascending mode, 2500 rpm at a 2.05 mL/min flow rate. The ascending mode of operation was chosen since IgY partition exclusively to the top phase. In this first assay, it was obtained a remarkably low S_f value, 16.7%, lower than the 20% usually required. However, some IgY purification was indeed achieved. As the purity of the end, IgY sample was of 29.5%, possibly due to the low retention of the stationary phase, further optimizations were performed. Since a rotation speed of 2500 rpm leads to an insufficient S_f value, an additional assay was performed in the ascending mode using 2000 rpm and a 3.0 mL/min flow rate. This assay allowed to obtain more promising

results, not only presenting a higher S_f than the previous one, but also a higher IgY purity (50.6%). Although further work is still required in this direction, this IgY purity surpassed all extraction assays using only one-step ABS investigated in this work, disclosing the high potential of CPC for the purification of biopharmaceuticals and its scale-up feasibility.

Conclusions

In this work PEG + Na_2SO_4 and PEG 1000 + K_2HPO_4/ KH_2PO_4 buffer, ABS were investigated as alternative platforms to purify IgY from the WSPF obtained from egg yolk. Initially, PEG + Na_2SO_4 ABS were investigated, while addressing the effect of the PEG molecular weight on the IgY selective partition to the PEG-rich phase. For the systems composed of PEG 200, 300 and 400, the total partitioning of all proteins present in the water-soluble protein fraction to the PEG-rich phase was observed. However, with PEG 600, it was observed the migration of the remaining water-soluble proteins between the top and bottom phases. By changing the PEG and salt concentration on the mixture composition, a higher selectivity of these systems for IgY was achieved. Based on these results, the improved PEG + Na_2SO_4 ABS (24 wt% PEG 600 + 7 wt% Na_2SO_4) was applied in CPC aiming at completely purifying IgY. However, with this system, it was not possible to purify IgY by CPC due to a significant loss of the stationary phase (PEG-rich phase) during the elution step, which even after testing different operating conditions could not be avoided.

A different strategy was then adopted by identifying promising ABS feasible to be applied in CPC. PEG 1000 + K_2HPO_4/ KH_2PO_4 buffer ABS were finally evaluated for the purification of IgY from the WSPF from egg yolk. The respective phase diagrams for PEG 1000 + K_2HPO_4/ KH_2PO_4 buffer + water were determined, and the effect of the pH value (5.5, 6.0, 6.5, .5, 8.0) s studied. These systems revealed a high affinity of IgY and reaming proteins in the WSPF to the polymer-rich phase, with the best results obtained with the phosphate buffer at a pH of 6.0. Additional studies on the purification of IgY were carried out by the change of PEG and salt concentration or mixture composition at this fixed pH. The best results in one-step ABS were obtained with the system formed by 18 wt% PEG 1000 + 13 wt% K_2HPO_4/ KH_2PO_4 buffer at pH 6.0. This ABS was finally applied in CPC, and

after the study of several operating conditions, it was possible to purify IgY from the WSPF obtained from egg yolk with a purity level of 50.6%.

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4. Final remarks and future work

The present work reports the progress carried out towards the development of alternative purification platforms for the selective extraction, and thus purification, of IgY from egg yolk using ABS. Taking in account the complexity of the IgY source (egg yolk), a first step for the removal of lipoproteins and lipids was used, and the WSPF that contains the target antibody was characterized by mass spectrometry. Six proteins were identified in the WSP, namely IgY, ovotransferrin, serum albumin (α-livetin), ovalbumin, vitellogenin 1 and vitellogenin 2.

After identification by mass spectrometry of the main the egg yolk water-soluble proteins and appraisal of this fraction complexity, the capability of ABS composed of GB-ILs combined with PPG 400 or $K_3C_6H_5O_7$ to selective extract IgY was evaluated. The GB-ILs synthesis and characterization was carried out, and the phase diagrams for each ABS and respective TLs and TLLs were determined. Despite the different PPG 400 + [Ch][GB] and $[P_{4444}]$/$[N_{4444}]$[GB] + $K_3C_6H_5O_7$ ABS investigated, the selective extraction of IgY to the IL-rich phase was not successfully achieved. Moreover, the precipitation of IgY and other water-soluble proteins was observed with some systems, as well as the formation of a GB-IL-IgY complex. In order to investigate the best phase-forming components to be used in ABS for the extraction and purification of IgY, the antibody stability/activity was evaluated by ELISA in several aqueous solutions of ILs, salts, and polymers. The IgY activity is higher in polymer aqueous solutions, followed by salts and ILs aqueous solutions. Since polymers and salts are the compounds that allowed higher preservation of the IgY activity, and since polymer-salt systems were reported in the literature for the separation of mammal antibodies, the use of ABS composed of polymers and salts for the selective extraction and purification of IgY from the WSPF was then investigated.

PEG + Na_2SO_4 ABS were initially tested and the effect of the PEG molecular weight (200, 300, 400 and 600 $g \cdot mol^{-1}$) was studied. The results obtained reveal the total partitioning of all the proteins in the WSPF fraction to the PEG-rich phase when low molecular weight PEGs were used. However, with PEG 600, it was achieved a higher selectivity towards IgY. By changing the PEG and salt concentration or mixture composition, a higher selectivity was attained, with the contaminant proteins displaying an almost equal migration between the top and bottom phases. Based on these results, the application of the improved PEG + Na_2SO_4 ABS was applied in CPC with the goal of completely purifying IgY. Different operating conditions were applied and tested, but the loss of the

stationary phase (PEG-rich phase) during the elution step was always observed, turning the purification of IgY from the WSPF by CPC impossible. A literature review was then carried out in order to find system feasible to be used in CPC. ABS formed by PEG 1000 and K_2HPO_4/ KH_2PO_4 buffer were then investigated. Novel phase diagrams for PEG 1000 + K_2HPO_4/ KH_2PO_4 buffer + water were determined, and the effect of the pH value (5.5, 6.0, 6.5, 7.5, 8.0) studied. As with the PEG + Na_2SO_4 ABS, these systems led to the preferential partitioning of IgY and contaminant proteins to the polymer-rich phase, with the best results achieved at pH 6.0. Additional studies on the purification of IgY were carried out at this pH by changing the PEG and salt concentrations or mixture composition. The best system was applied in CPC, and after the optimization of several operating conditions, it was possible to obtain IgY with 51% of purity.

Based on the results obtained, ABS formed by polymers with a high molecular weight and low pH values are favorable for the selective partitioning of IgY into the PEG-rich phase. Therefore, as future work, ABS composed of polymers with a higher molecular weight and other salts capable of affording acidic media should be investigated. Furthermore, these ABS should allow the complete purification of IgY using higher flow-rates to turn the process faster and more profitable. In order to remove the IgY from the polymer-rich phase, ultrafiltration or dialysis should be investigated. After the purification step, the antibody structure and activity must be evaluated by circular dichroism and ELISA. Finally, the best systems identified should be tested to purify specific IgY obtained from immunized eggs.

List of publications/patents

List of publications in the current thesis

1. Mohamed Taha, Mafalda R. Almeida, Francisca A. e Silva, Pedro Domingues, Sónia P. M. Ventura, João A. P. Coutinho, Mara G. Freire; “Novel Biocompatible and self-buffering ionic liquids for biopharmaceutical applications”, Chemistry – A European Journal. 21 (2015) 4781–4788.

Other publications

1. Matheus M. Pereira, Mafalda R. Almeida, Joana Gomes, Ana F. C. S. Rufino, Marguerita E. Rosa, João A. P. Coutinho, Aminou Mohamadou and Mara G. Freire; “Glycine-betaine ionic liquid analogues as novel phase-forming components of aqueous biphasic systems”, Biotechnology Progress. 2018 DOI: 10.1002/btpr.2685;
2. João H. P. M. Santos, Mafalda R. Almeida, Cláudia I. R. Martins, Ana C. R. V. Dias, Mara G. Freire, João A. P. Coutinho, Sónia P.M. Ventura; “Separation of phenolic compunds by centrifugal partition chromatography”, Green Chemistry. 20 (2018) 1906-1916;
3. Matheus M. Pereira, Rafaela A. P. Cruz, Mafalda R. Almeida, Álvaro S. Lima, João A. P. Coutinho, Mara G. Freire; “Single-Step Purification of Ovalbumin from Egg White Using Aqueous Biphasic Systems”, Process Biochemistry. 51 (2016) 781-791;
4. Mafalda R. Almeida, Helena Passos, Matheus M. Pereira, Álvaro Lima, João A. P. Coutinho and Mara G. Freire; “Ionic liquids as additives to enhance the extraction of antioxidants in aqueous two-phase systems", Separation and Purification Technology. 128 (2014) 1-10.

Patents

1. Mafalda R. Almeida, Judite Resende, Ana P. M. Tavares, Pedro Domingues and Mara G. Freire. Provisional patent application (National, Portugal): “Purificação de imunoglobulina Y”. University of Aveiro, Portugal. Application date: 20-09-2018; request number: 115031.

Due to intellectual property rights, this work was not introduced in this PhD thesis.

Annex A

Characterization of the synthetized GB-ILs

The ^{1}H NMR characterization data of each GB-IL are presented below.

[Ch]-based ILs:

[Ch][HEPES]: ^{1}H RMN (300 MHz, TSP/D2O, ppm); 3,21 (s, 9H), 3,52 (m, 2H), 4,07 (m, 2H), 2,61 (t, 8H), 2,84 (t, 2H), 3,14 (t, 2H), 3,77 (t, 4H)

[Ch][MES]: ^{1}H RMN (300 MHz, TSP/D2O, ppm); 3,21 (s, 9H), 3,52 (m, 2H), 4,07 (m, 2H), 2,67 (t,4H), 2,84 (t, 2H), 3,13 (t, 2H), 3,76 (t,4H)

[Ch][TES]: ^{1}H RMN (300 MHz, TSP/D2O, ppm); 3,21 (s, 9H), 3,52 (m, 2H), 4,07 (m, 2H), 3,61 (s, 6H), 3,09 (t, 4H)

[Ch][Tricine]: ^{1}H RMN (300 MHz, TSP/D2O, ppm); 3,21 (s, 9H), 3,52 (m, 2H*)*, 4,07 (m, 2H), 3,57 (s, 6H), 3,34 (s, 2H)

[P_{4444}]-based ILs:

[P_{4444}][CHES]: ^{1}H NMR (300 MHz, D2O/TSP); 2.02 (m, 8H), 1.37-1.45 (m, 16H), 0.92 (t, 12H), 2.76 (m, 2H), 2.50-2.52 (*m*, 1*H*), 0.94 (m, 10H)

[P_{4444}][HEPES]: ^{1}H NMR (300 MHz, D2O/TSP); 2.18 (m, 8H), 1.35-1.52 (m, 16H), 0.92 (t, 12H), 3.59 (t,4H), 2.97 (t, 2H), 2.94 (t, 2H), 2.36 (t, 8H),

[P_{4444}][MES]: ^{1}H NMR (300 MHz, D2O/TSP); 2.02 (m, 8H), 1.27-1.42 (m, 16H), 0.92 (t,12H), 3.63 (t, 4H), 2.99 (t, 2H), , 2.72 (t, 2H), 2.50 (t, 4H)

[P_{4444}][TES]: ^{1}H NMR (300 MHz, D2O/TSP); 2.50 (m, 8H), 1.35-1.49 (m,16H), 0.92 (t, 12H), 3.31 (s, 6H), 2.81 (s, 2H)

[P_{4444}][Tricine]: ^{1}H NMR (300 MHz, D2O/TSP); 2.00 (m, 8H), 1.27-1.44 (m, 16H), 0.77 (t. 12H), 3.34 (s, 6H), 3.18 (s, 2H)

[N_{4444}]-based ILs:

[N_{4444}][CHES]: ^{1}H NMR (300 MHz, D2O/TSP); 2.3 (m, 8H), 1.27-1.37 (m, 16H,), 0.94 (t, 12H), 2.75 (m, 2H), 2.49-2.51 (*m*, 1*H*)

[N_{4444}][HEPES]: ^{1}H NMR (300 MHz, D2O/TSP); 3.34 (s,6H), 3.17 (t, 8H), 2.51 (t, 2H), 2.34 (2H, t), 1.31 (quin, 8H), 1.30 (sext,8 H), 0.94 (t,12 H).

[N_{4444}][MES]: ^{1}H NMR (300 MHz, D2O/TSP); 3.34 (t, 4H), 3.17 (t, 8H); 2.51 (t, 2H,), 2.49 (t,2H,), 2.32 (t, 4H), 1.55 (quin 8H), 1.31 (sext, 8H), 0.94 (t,12H).

[N_{4444}][TES]: ^{1}H NMR (300 MHz, D2O/TSP); 3.34 (s,6H), 3.27 (t, 8H), 2.86 (t, 2H), 2.51 (2H, t), 1.55 (quin, 8H), 1.30 (sext,8 H) 0.91 (t,12 H).

[N_{4444}][Tricine]: ^{1}H NMR (300 MHz, D2O/TSP); 3.53 (s, 6H) 3.27 (s, 2H), 3.21 (t, 8H), 1.65 (quin, 8*H*), 1.37 (sext, 8*H*), 0.93 (t,12H).

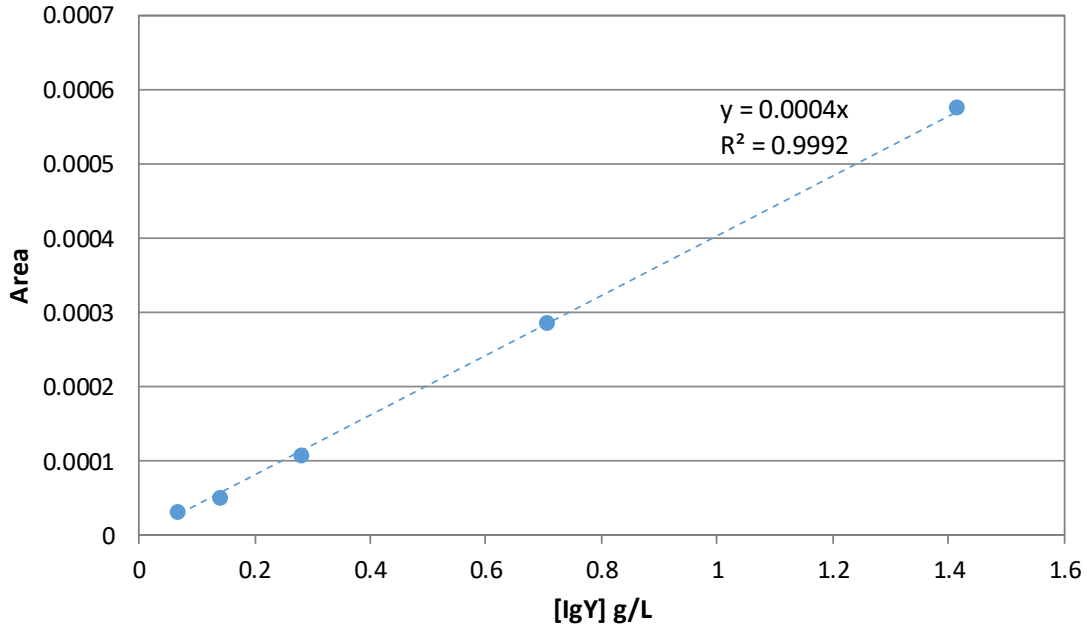

Figure A1. SE-HPLC calibration curve for IgY (purified using the Pierce® Chicken IgY Purification Kit).

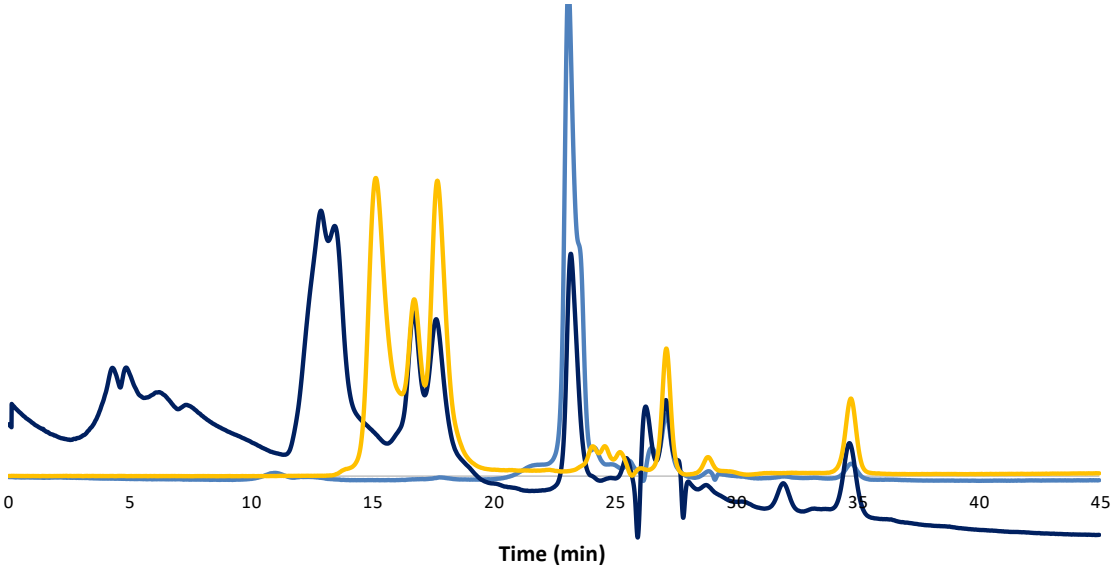

Figure A2. SE-HPLC chromatogram of the system composed of [N_{4444}][CHES] + $K_3C_6H_5O_7$ + WSPF: bottom phase (light blue line), top phase (blue line) and original WSPF (yellow line).

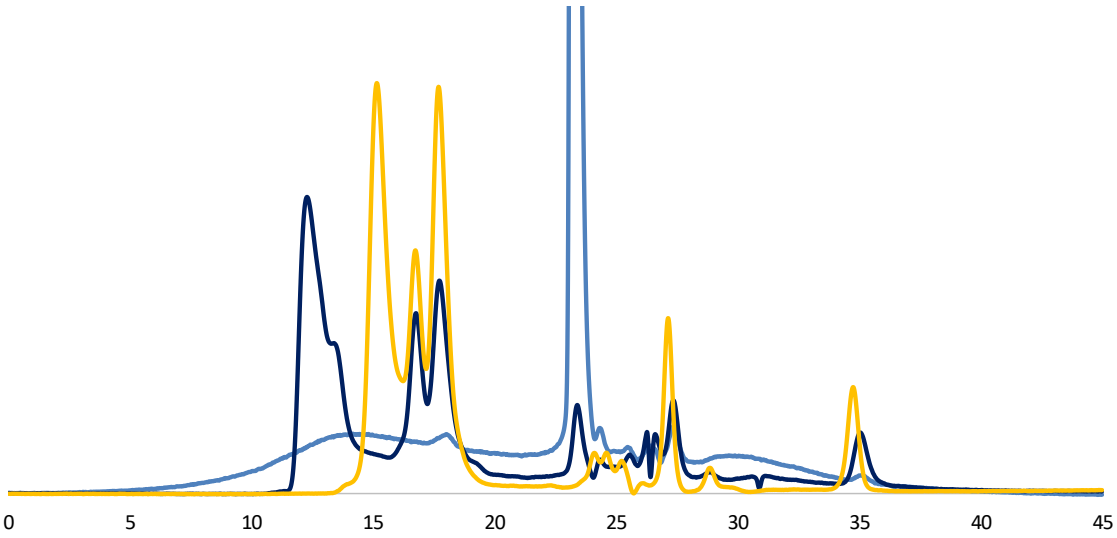

Figure A3. SE-HPLC chromatogram of the system composed of $[P_{4444}][CHES]$ + $K_3C_6H_5O_7$ + WSPF: bottom phase (light blue line), top phase (blue line) and original WSPF (yellow line).

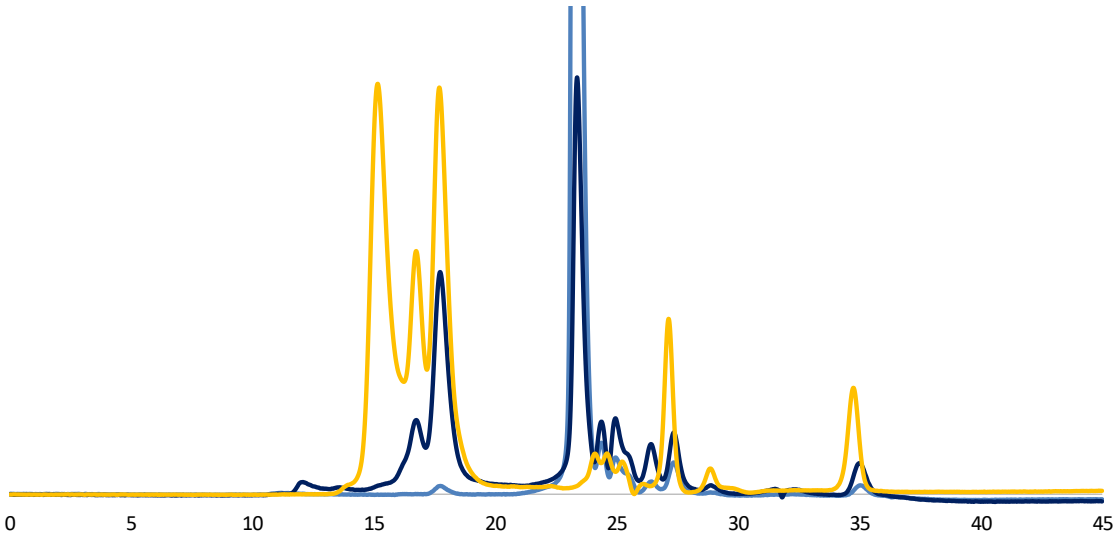

Figure A4. SE-HPLC chromatogram of the system composed of $[P_{4444}][HEPES]$ + $K_3C_6H_5O_7$ + WSPF: bottom phase (light blue line), top phase (blue line) and original WSPF (yellow line).

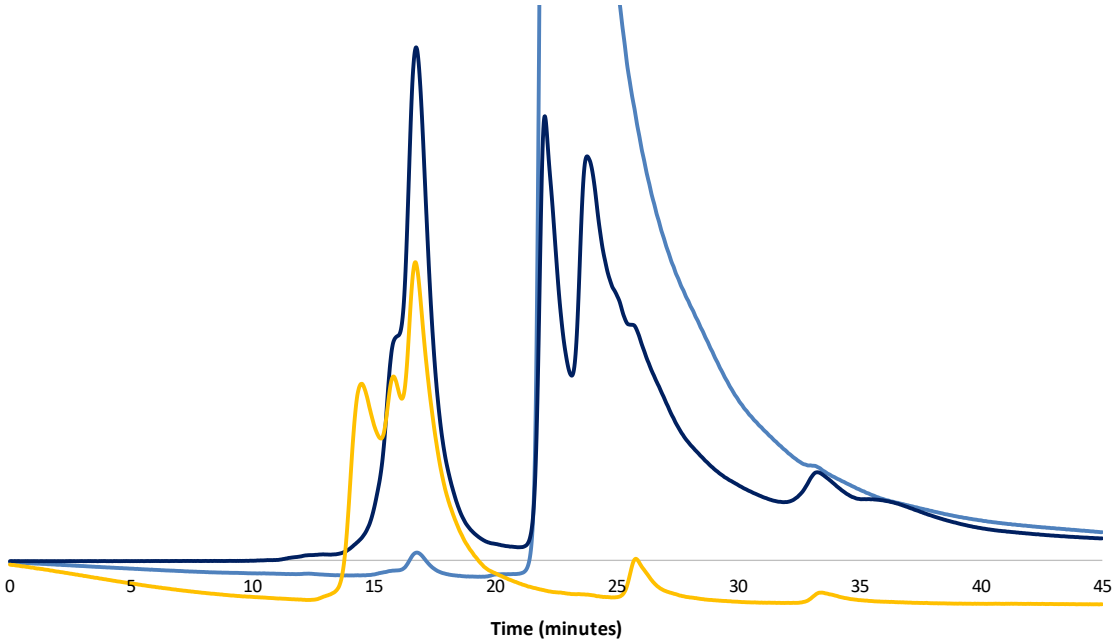

Figure A5. SE-HPLC chromatogram of the system composed of $[P_{4444}][MES]$ + $K_3C_6H_5O_7$ + WSPF: bottom phase (light blue line), top phase (blue line) and original WSPF (yellow line).

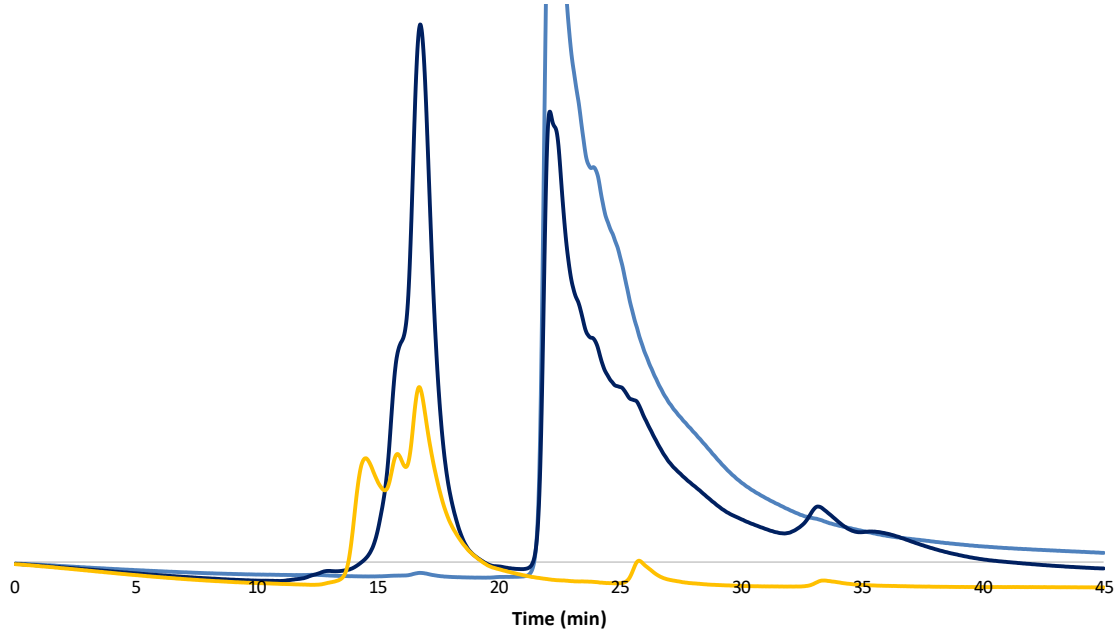

Figure A6. SE-HPLC chromatogram of the system composed of $[P_{4444}][TES]$ + $K_3C_6H_5O_7$ + WSPF: bottom phase (light blue line), top phase (blue line) and original WSPF (yellow line).

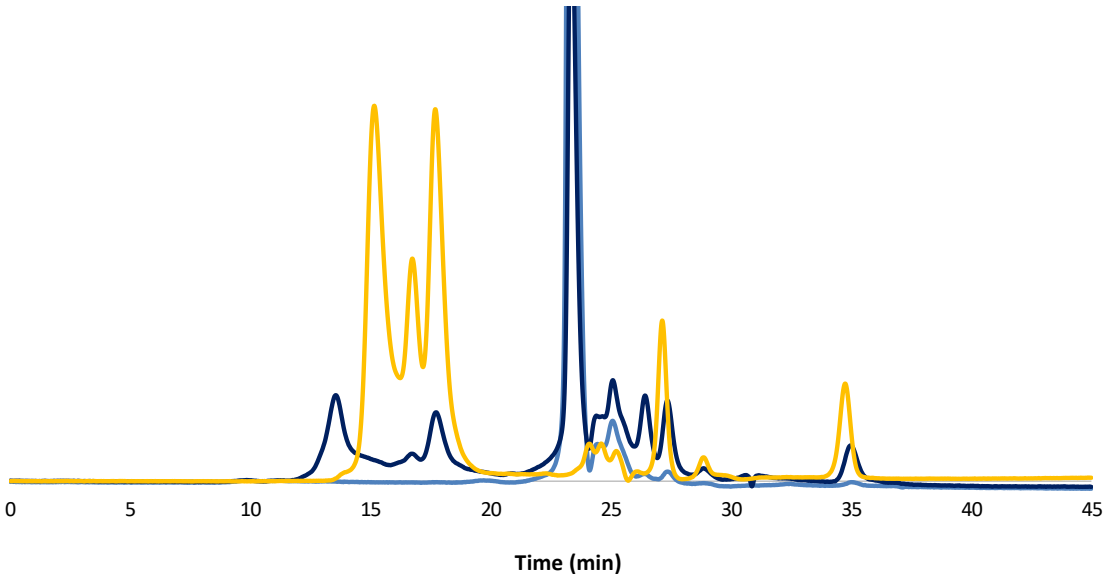

Figure A7. SE-HPLC chromatogram of the system composed of $[P_{4444}][Tricine]$ + $K_3C_6H_5O_7$ + WSPF: bottom phase (light blue line), top phase (blue line) and original WSPF (yellow line).

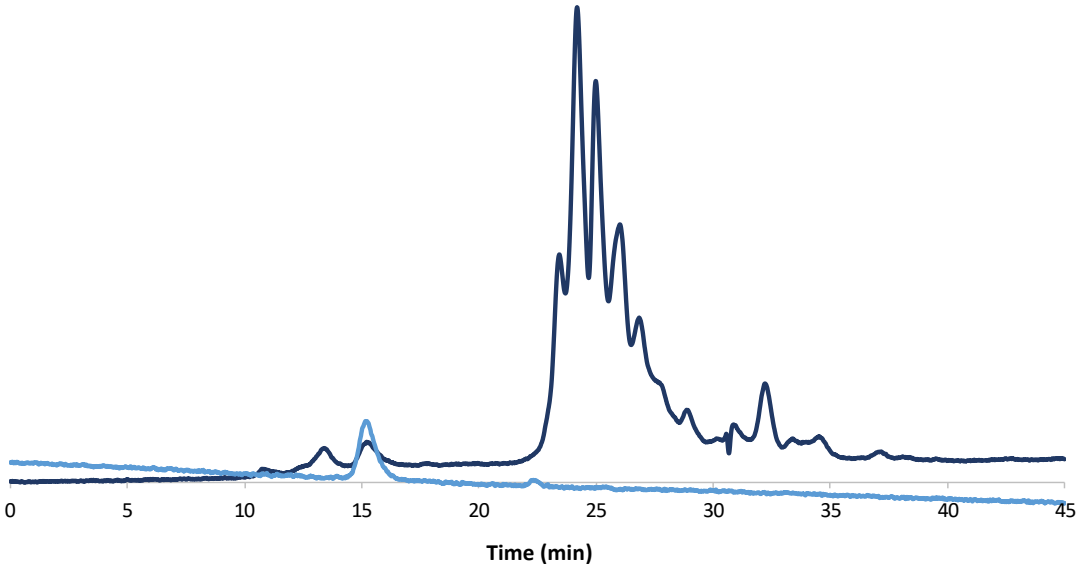

Figure A8. SE-HPLC chromatogram of the pure IgY solution (light blue line) and of a mixture composed of $[N_{4444}][CHES]$ + pure IgY (blue line).

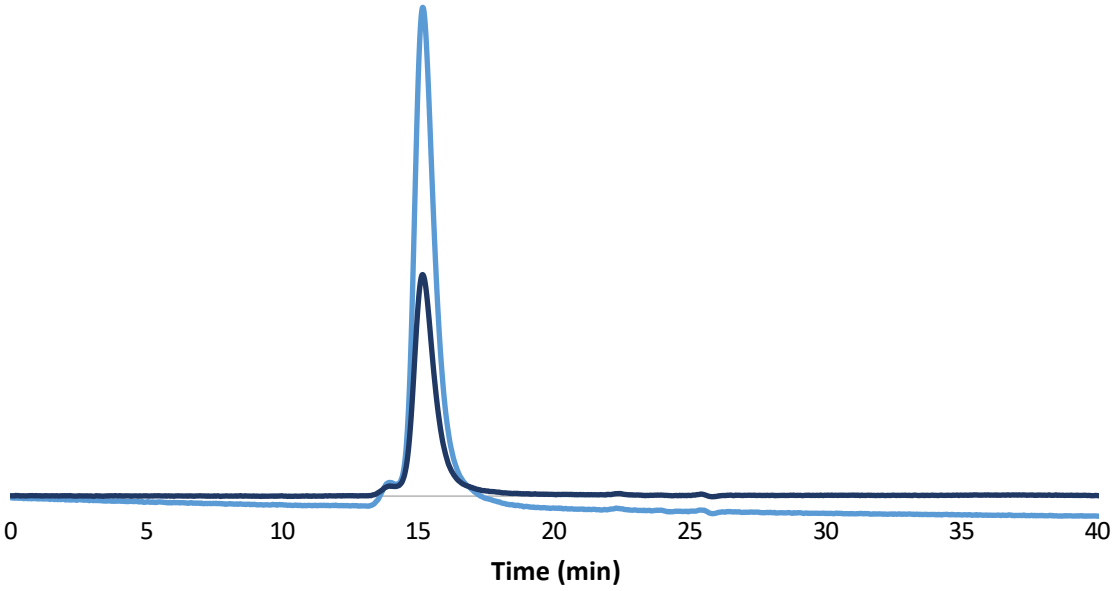

Figure A9. SE-HPLC chromatogram of the pure IgY solution (light blue line) and of a mixture composed of $K_3C_6H_5O_7$ + pure IgY (blue line).

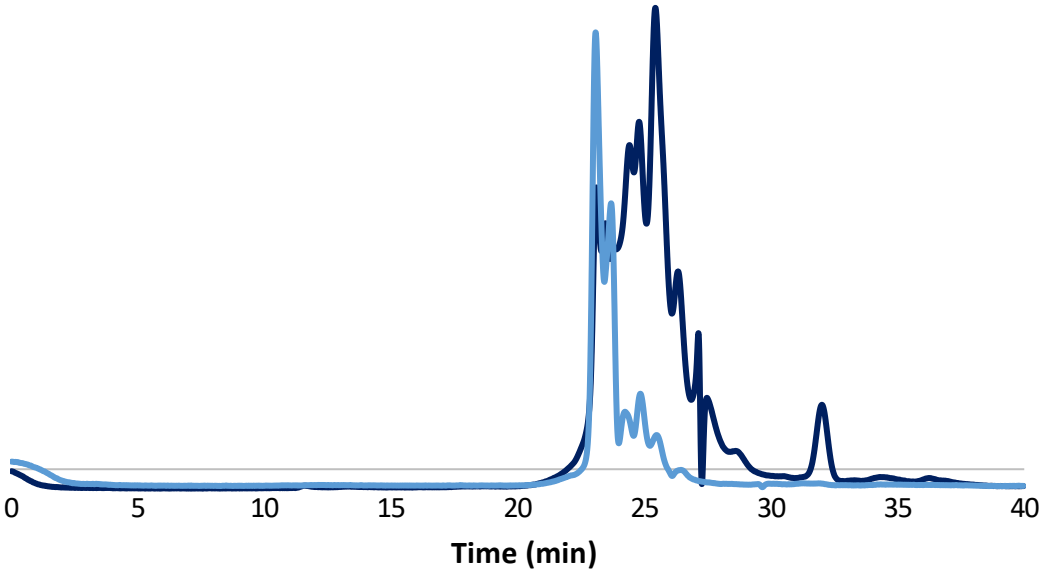

Figure A10. SE-HPLC chromatogram of the salt- rich phase (light blue line) and GB-IL-rich phase (blue line) with no protein added (blank phases).

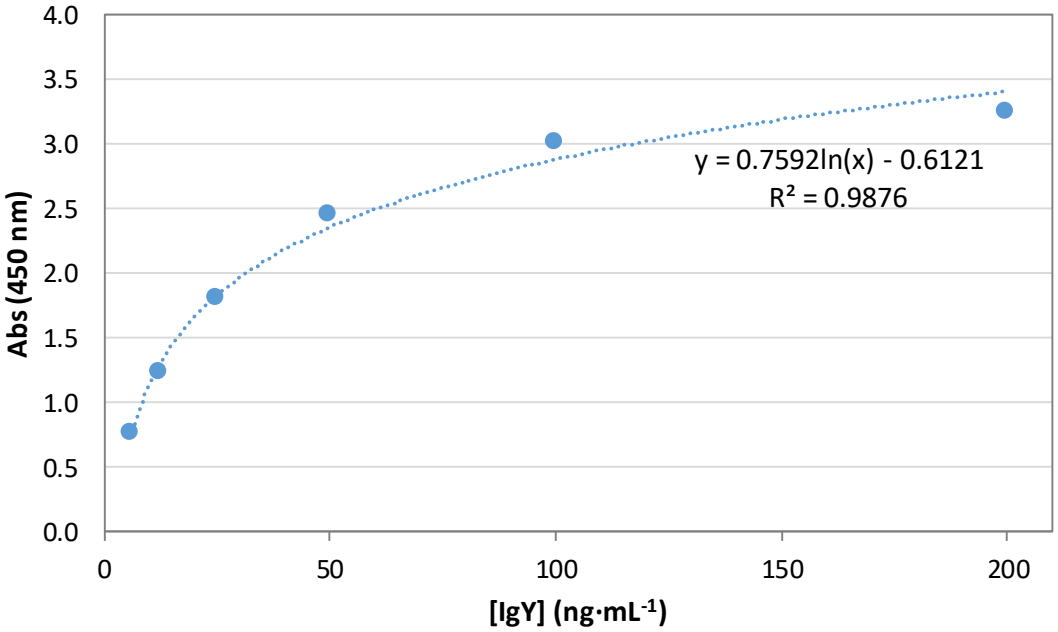

Figure A11. ELISA calibration curve for IgY.

Table A1. Experimental weight fraction data for the systems composed of PPG 400 (1) + GB-IL (2) + H_2O (3) at (25 ± 1) °C.

[Ch][Tricine]				[Ch][HEPES]			
100 w_1	*100* w_2	100 w_1	*100* w_2	100 w_1	*100* w_2	100 w_1	*100* w_2
97.87	1.30	22.41	13.85	86.73	2.30	15.91	20.48
69.49	3.13	21.74	14.31	65.64	3.68	15.26	21.64
60.19	3.63	20.96	14.78	40.58	8.07	14.63	22.83
56.78	4.28	20.32	15.21	38.56	8.67	14.16	23.41
54.16	4.70	19.60	15.76	37.15	9.29	13.78	23.87
51.55	5.45	19.00	16.35	35.58	9.97	13.45	24.09
49.26	5.76	18.46	16.66	34.04	10.47	13.15	24.44
47.60	6.23	17.79	17.26	32.69	10.90	12.76	25.03
45.85	6.66	17.26	17.62	31.83	11.53	12.45	25.35
43.78	6.95	16.70	18.07	30.65	11.99	12.15	25.81
42.23	7.26	16.07	18.58	29.80	12.37	11.68	26.14
40.60	7.66	15.46	19.03	28.92	12.74	11.42	26.48
39.15	8.11	14.77	19.53	28.11	13.36	11.08	26.97
37.48	8.28	14.23	20.03	26.36	13.52	10.81	27.45
36.35	8.55	13.68	20.60	25.45	14.38	10.47	27.87
35.38	8.81	12.93	21.38	24.60	15.01	10.15	28.30
34.55	9.06	12.40	21.90	24.00	15.26	9.89	28.78
33.51	9.29	11.77	22.68	23.34	15.57	10.81	27.45
32.67	9.49	10.96	24.44	22.90	15.99	10.47	27.87
31.59	10.00			22.34	16.66	10.15	28.30
30.46	10.58			21.76	16.77	9.89	28.78
29.60	10.74			21.40	17.07		
29.10	10.97			20.71	17.69		
28.25	11.51			20.17	17.81		
27.15	11.73			20.01	17.65		
26.59	12.23			19.18	17.91		
25.94	12.52			18.53	19.07		
25.14	12.63			17.69	19.14		
23.95	13.00			17.17	19.60		
23.04	13.54			16.56	20.32		

Table A2. Experimental weight fraction data for the systems composed of PPG 400 (1) + GB-IL (2) + H_2O (3) at (25 ± 1) °C.

[Ch][MES]				[Ch][TES]			
100 w_1	*100* w_2	100 w_1	*100* w_2	100 w_1	*100* w_2	100 w_1	*100* w_2
69.88	4.23	18.86	23.00	96.95	1.72	21.35	18.29
64.79	5.32	18.40	23.47	70.16	3.71	20.22	19.23
60.13	6.29	17.55	24.17	62.07	4.31	19.54	19.73
55.20	7.18	17.19	24.60	58.09	5.00	18.70	20.05
48.50	8.77	16.17	24.53	55.88	5.64	17.49	21.21
46.80	9.55	15.77	24.83	52.86	6.37	16.86	22.05
43.80	10.90	15.33	25.34	49.21	7.81	16.00	22.98
42.43	11.36	14.76	26.07	46.57	8.29	15.30	23.72
38.27	12.78	13.95	26.92	44.15	8.83	14.67	24.42
36.55	13.09	13.32	27.87	42.22	9.18	13.59	24.85
35.08	13.44	12.63	28.81	41.00	9.67		
34.13	13.98	12.22	29.31	39.81	10.09		
32.82	14.25	11.73	30.03	38.36	10.44		
31.70	14.55			37.08	10.75		
30.22	15.33			35.31	11.42		
28.40	16.19			34.03	11.49		
27.84	16.47			33.26	11.88		
27.27	16.73			32.54	12.20		
26.49	17.50			31.28	12.96		
25.49	18.05			29.92	13.47		
24.69	18.74			29.02	14.08		
23.73	19.23			27.98	14.58		
23.28	19.42			27.49	14.82		
22.63	20.02			26.66	15.40		
22.03	20.52			25.81	15.88		
21.35	20.87			24.93	16.31		
20.81	21.40			24.20	16.86		
20.46	21.59			23.97	16.69		
19.99	22.12			23.09	17.25		
19.50	22.67			22.10	17.92		

Table A3. Correlation parameters and respective standard deviations (σ) of equation 1 used to describe the experimental binodal data of the ABS composed of PPG 400 + GB-IL.

GB-ILs	$A \pm \sigma$	$B \pm \sigma$	$10^5 (C \pm \sigma)$	R^2
[Ch][Tricine]	180.5 ± 2.4	-0.52 ± 0.01	1.2 ± 0.3	0.998
[Ch][HEPES]	187.2 ± 4.7	-0.54 ± 0.01	1.0 ± 0.3	0.994
[Ch][MES]	169.2 ± 3.9	-0.42 ± 0.01	1.7 ± 0.2	0.997
[Ch][TES]	175.2 ± 3.4	-0.47 ± 0.01	1.2 ± 0.3	0.996

Table A4. Experimental TLs and TLLs of the ABS composed of PPG 400 + GB-ILs + H_2O at (25 ± 1) °C.

GB-ILs	Weight fraction composition/ wt%						
	$[PPG]_{PPG}$	$[IL]_{PPG}$	$[PPG]_M$	$[IL]_M$	$[PPG]_{IL}$	$[IL]_{IL}$	TLL
[Ch][Tricine]	80.08	2.22	49.90	10.17	15.05	19.35	67.24
	66.70	3.24	50.01	7.08	26.25	12.24	42.42
[Ch][HEPES]	77.52	2.93	49.73	9.82	21.64	16.79	57.57
	70.38	3.60	50.16	8.09	29.16	12.74	42.22
[Ch][MES]	63.57	5.48	50.62	9.88	25.45	18.41	40.25
	77.01	3.56	56.11	10.00	22.24	20.43	57.30
[Ch][TES]	72.16	3.54	49.73	10.06	21.94	18.13	52.29
	66.69	4.20	49.94	8.53	29.38	13.86	38.54

Table A5. Partition coefficients of total protein (*K*) and extraction efficiencies (*EE*%) for the IL-rich phase in the ABS composed of PPG 400 + GB-ILs + WPSF at (25 ± 1) °C.

GB-ILs	Weight fraction composition / wt%		*K*	*EE*%
	$[PPG\ 400]_M$	$[IL]_M$		
[Ch][Tricine]	50.0 ± 0.2	7.1 ± 0.1	19.1 ± 1.2	93.5 ± 0.1
[Ch][HEPES]	50.0 ± 0.2	8.1 ± 0.1	14.5 ± 1.1	93.0 ± 0.6
[Ch][MES]	49.7 ± 0.1	10.1 ± 0.2	4.0 ± 0.3	78.9 ± 1.2
[Ch][TES]	49.9 ± 0.1	8.5 ± 0.1	4.9 ± 0.3	81.9 ± 1.0

Table A6. Experimental weight fraction data for the binodal curve of the systems composed of [P_{4444}][GB] (1) + $K_3C_6H_5O_7$ (2) at (25 ± 1) °C.

[P_{4444}][CHES]		[P_{4444}][HEPES]			
100 w_1	*100* w_2	100 w_1	*100* w_2	100 w_1	*100* w_2
45.87	5.16	52.44	3.38	22.92	18.29
43.49	5.58	50.86	3.72	22.65	18.32
41.47	5.98	48.78	4.01	22.28	18.56
37.65	7.02	47.00	4.68	21.23	19.70
35.47	7.58	45.92	5.39	20.89	19.99
33.07	7.98	44.53	5.67	20.47	20.19
31.49	8.32	43.22	6.40	19.99	20.64
30.77	8.84	41.77	7.28	19.78	20.72
29.44	9.33	40.98	7.57	19.18	21.19
28.37	9.59	40.09	8.02	18.98	21.21
27.49	9.79	38.54	8.89	18.59	21.51
26.54	10.37	36.74	9.55	18.34	21.74
25.37	10.53	35.89	10.03	17.98	22.02
24.65	10.87	35.12	10.54	17.59	22.25
23.81	11.15	34.33	11.02	17.04	22.81
23.47	11.34	32.94	11.84	16.77	22.95
22.50	11.55	31.83	12.55	16.39	23.23
21.78	11.79	31.04	12.86	15.89	23.66
20.98	12.17	30.28	13.50	15.58	23.73
20.48	12.34	29.71	13.83	15.33	23.90
19.73	12.86	29.24	13.95	15.06	24.19
19.27	12.84	28.13	14.99		
19.00	12.90	27.30	15.43		
18.71	13.10	26.50	15.87		
18.11	13.39	26.20	15.94		
17.59	13.51	25.87	16.27		
17.28	13.62	25.23	16.72		
16.70	13.82	24.96	16.79		
16.00	14.27	24.00	17.49		
15.68	14.41	23.54	17.80		

Table A7. Experimental weight fraction data for the binodal curve of the systems composed of [P_{4444}][GB] (1) + $K_3C_6H_5O_7$ (2) at (25 ± 1) °C.

[P_{4444}][MES]				[P_{4444}][TES]			
100 w_1	100 w_2	100 w_1	100 w_2	100 w_1	100 w_2	100 w_1	100 w_2
56.09	3.72	12.35	24.58	49.33	11.04	13.82	30.48
49.20	5.88	12.08	24.80	46.68	12.54	13.13	30.97
39.91	9.05	11.72	25.16	44.08	13.78	12.57	31.38
35.68	10.71	10.94	25.62	41.93	14.61	12.00	31.80
32.23	12.45	10.69	25.77	40.11	15.40	11.49	32.25
30.45	13.54	10.30	26.06	37.34	16.53	10.83	32.69
28.03	14.65	10.08	26.23	34.74	17.86	10.44	33.09
26.54	15.34	9.82	26.41	32.60	19.25	10.01	33.41
25.64	15.97	9.53	26.69	31.24	19.90	9.11	34.10
20.35	19.68	9.29	26.88	28.11	22.04	8.70	34.58
19.84	20.02	8.79	27.16	26.38	22.80	8.33	34.70
19.12	20.44	8.51	27.40	25.11	23.46		
18.7	20.66	7.58	28.39	24.51	23.86		
18.25	20.86	7.26	28.63	23.69	24.30		
17.97	20.99	7.12	28.76	22.72	25.09		
17.40	21.47			22.07	25.41		
17.00	21.67			21.52	25.66		
16.81	21.70			20.98	25.85		
16.58	21.83			20.28	26.30		
16.33	22.04			19.60	26.74		
16.05	22.18			18.32	27.48		
15.52	22.65			17.76	27.85		
15.14	22.84			17.35	28.03		
14.75	22.98			16.87	28.34		
14.21	23.42			16.19	28.89		
13.06	24.10			15.76	29.05		
12.97	24.17			15.30	29.38		
12.80	24.22			14.87	29.68		
12.59	24.48			14.51	29.99		
12.46	24.53			14.10	30.35		

Table A8. Experimental weight fraction data for the binodal curve of the systems composed of [P_{4444}][GB] (1) + $K_3C_6H_5O_7$ (2) at (25 ± 1) °C.

[P_{4444}][Tricine]					
100 w_1	*100* w_2	100 w_1	*100* w_2	100 w_1	*100* w_2
42.13	12.60	22.78	25.31	14.54	32.24
40.36	13.42	21.98	25.95	14.27	32.41
38.46	14.47	21.69	26.17	14.09	32.60
37.25	15.23	21.24	26.62	13.97	32.64
35.34	16.39	20.99	26.78	13.78	32.85
32.61	18.66	20.71	26.94	13.64	32.97
31.57	19.22	20.26	27.41	13.47	33.15
30.19	20.22	20.04	27.47	13.27	33.23
28.07	21.65	19.646	27.85	13.10	33.42
27.18	22.17	19.44	28.02	12.88	33.71
26.48	22.66	19.21	28.17	12.77	33.76
32.27	18.52	18.90	28.45	12.49	33.98
31.59	18.97	18.60	28.74	12.03	34.02
30.98	19.39	18.30	29.02	11.87	34.18
30.45	19.68	18.11	29.14	11.71	34.36
29.86	20.06	17.78	29.48	11.58	34.49
29.39	20.41	17.58	29.57	11.43	34.66
28.80	20.78	17.41	29.75	11.31	34.79
27.97	21.61	17.19	29.88	11.16	34.83
27.50	21.99	16.93	30.15	11.03	35.003
27.05	22.29	16.67	30.38	10.89	35.17
26.61	22.58	16.51	30.52	10.81	35.17
26.18	22.80	16.25	30.77	10.70	35.31
25.52	23.47	16.09	30.87	10.46	35.57
25.11	23.75	15.86	31.06		
24.71	23.98	15.61	31.28		
24.29	24.15	15.36	31.53		
23.95	24.37	15.22	31.58		
23.41	24.94	14.99	31.80		
23.08	25.13	14.77	32.00		

Table A9. Experimental weight fraction data for the binodal curve of the systems composed of [N_{4444}][GB] (1) + $K_3C_6H_5O_7$ (2) at (25 ± 1) °C.

[N_{4444}][CHES]				[N_{4444}][HEPES]		[N_{4444}][MES]	
100 w_1	*100* w_2	100 w_1	*100* w_2	100 w_1	*100* w_2	100 w_1	*100* w_2
57.541	4.36	7.44	21.53	34.97	7.87	42.23	6.35
49.80	5.49	7.24	21.58	26.57	14.08	34.43	10.16
45.08	6.43	7.05	21.67	20.59	19.81	25.23	17.33
42.48	7.36	6.93	22.16	15.63	26.08	15.50	23.01
40.44	8.24	6.72	22.56	9.59	31.95	6.74	33.25
37.89	8.72	6.47	22.70	5.68	38.15	3.47	38.04
35.73	9.35	6.23	22.81				
34.68	9.91	6.03	23.06				
32.72	10.85						
29.99	11.62						
25.60	13.51						
24.14	13.97						
23.17	14.39						
20.05	14.96						
19.22	15.20						
16.05	16.66						
14.93	17.06						
13.90	17.68						
13.34	17.78						
12.14	18.41						
11.20	18.95						
10.36	19.54						
10.01	19.69						
9.45	19.90						
9.15	19.96						
8.75	20.74						
8.44	20.68						
7.87	21.14						
7.65	21.27						

Table A10. Experimental weight fraction data for the binodal curve of the systems composed of [N_{4444}][GB] (1) + $K_3C_6H_5O_7$ (2) at (25 ± 1) °C.

[P_{4444}][TES]		[P_{4444}][Tricine]			
100 w_1	*100* w_2	100 w_1	*100* w_2	100 w_1	*100* w_2
60.10	7.15	44.65	10.77	16.31	29.74
38.18	14.06	43.34	11.58	15.76	30.14
31.66	17.27	42.13	12.25	15.25	30.54
21.81	24.94	41.06	12.75	14.62	31.07
17.42	29.38	39.84	13.55	14.10	31.48
10.47	35.35	38.19	14.61	13.52	31.96
4.47	41.30	36.27	15.77	12.86	32.52
32.60	19.25	34.61	16.77	12.23	33.02
		33.14	17.79	12.01	33.18
		32.16	18.18	11.65	33.49
		31.75	18.34	11.50	33.63
		31.01	18.84	11.16	33.90
		29.98	19.63	10.50	34.50
		28.11	21.00	9.94	35.01
		26.48	22.20	9.50	35.38
		25.92	22.54	9.13	35.75
		25.21	23.03	8.61	36.24
		24.42	23.66	8.09	36.70
		23.48	24.34	7.76	36.99
		22.93	24.70	7.35	37.39
		22.34	25.18	7.14	37.63
		21.89	25.45	6.78	37.96
		21.30	25.90	6.50	38.21
		20.79	26.30		
		19.89	26.92		
		18.97	27.64		
		18.51	28.06		
		18.34	28.15		
		17.79	28.59		
		17.05	29.15		

Table A11. Correlation parameters and respective standard deviations (σ) of equation 7 used to describe the experimental binodal data of the ABS composed of GB-IL + $K_3C_6H_5O_7$.

GB-IL	$A \pm \sigma$	$B \pm \sigma$	$10^5(C \pm \sigma)$	R^2
$[P_{4444}][CHES]$	126.0 ± 2.0	- 0.44 ± 0.01	13.8 ± 0.1	0.999
$[P_{4444}][HEPES]$	82.3 ± 0.6	- 0.25 ± 0.01	3.3 ± 0.1	0.999
$[P_{4444}][MES]$	97.6 ± 1.5	- 0.29 ± 0.01	4.3 ± 0.1	0.998
$[P_{4444}][TES]$	109.8 ± 22.5	- 0.23 ± 0.06	2.9 ± 0.4	0.981
$[P_{4444}][Tricine]$	94.8 ±1.9	- 0.22 ± 0.01	1.9 ± 3.0	0.999
$[N_{4444}][CHES]$	120.1 ± 4.7	- 0.36 ± 0.02	1.1 ± 0.5	0.998
$[N_{4444}][HEPES]$	67.3 ± 4.7	- 0.23 ± 0.02	1.9 ± 0.2	0.999
$[N_{4444}][MES]$	80.1 ± 8.3	- 0.25 ± 0.04	3.0 ± 0.5	0.997
$[N_{4444}][TES]$	160.7 ± 16.0	- 0.37 ± 0.03	1.3 ± 0.3	0.996
$[N_{4444}][Tricine]$	90.4 ± 1.6	- 0.21 ± 0.01	2.3 ± 0.1	0.999

Table A12. Data for the tie-lines (TLs) and respective tie-line lengths (TLL) at (25 ± 1) °C. Initial mixture compositions are represented as $[salt]_M$ and $[IL]_M$, whereas $[salt]_{salt}$ and $[IL]_{salt}$ represent the composition of IL and salt at the IL-rich phase, respectively, and vice-versa.

Weight fraction composition/ wt%							
IL	**$[IL]_{IL}$**	**$[Salt]_{IL}$**	**$[IL]_M$**	**$[Salt]_M$**	**$[IL]_{Salt}$**	**$[Salt]_{Salt}$**	**TLL**
$[P_{4444}][CHES]$	58.42	2.99	21.57	15.75	3.72	21.93	57.89
	53.34	3.72	21.68	13.38	10.98	16.65	44.29
$[P_{4444}][HEPES]$	39.99	7.90	20.44	22.23	6.48	32.48	41.59
	47.08	7.90	19.63	23.30	6.45	32.48	48.16
$[P_{4444}][MES]$	45.45	6.43	20.00	22.52	4.85	31.73	48.69
	41.05	8.62	19.85	21.43	7.18	29.09	39.58
$[P_{4444}][TES]$	53.39	9.32	20.12	28.71	7.01	36.35	53.68
	44.68	13.26	20.30	27.95	7.91	35.41	42.92
$[P_{4444}][Tricine]$	44.86	10.73	19.99	30.01	6.78	40.25	48.17
	42.69	11.96	20.42	28.02	13.29	33.16	36.25
$[N_{4444}][CHES]$	68.08	2.72	19.94	24.24	0.23	33.44	72.67
	64.87	2.88	20.46	20.80	1.31	28.52	68.54
$[N_{4444}][HEPES]$	62.01	0.12	19.85	29.57	4.69	40.15	69.91
	40.64	4.71	19.74	24.10	7.35	35.60	45.41
$[N_{4444}][MES]$	52.77	2.75	20.15	24.89	4.99	35.18	57.75
	61.81	1.06	19.90	29.71	1.73	42.12	72.77
$[N_{4444}][TES]$	37.37	14.79	18.91	28.35	11.79	33.58	31.73
	74.30	4.36	20.67	34.68	5.01	43.53	79.59
$[N_{4444}][Tricine]$	68.76	1.74	30.16	29.91	0.97	51.21	83.93
	54.89	5.69	28.00	23.39	9.31	35.69	54.58

Annex B

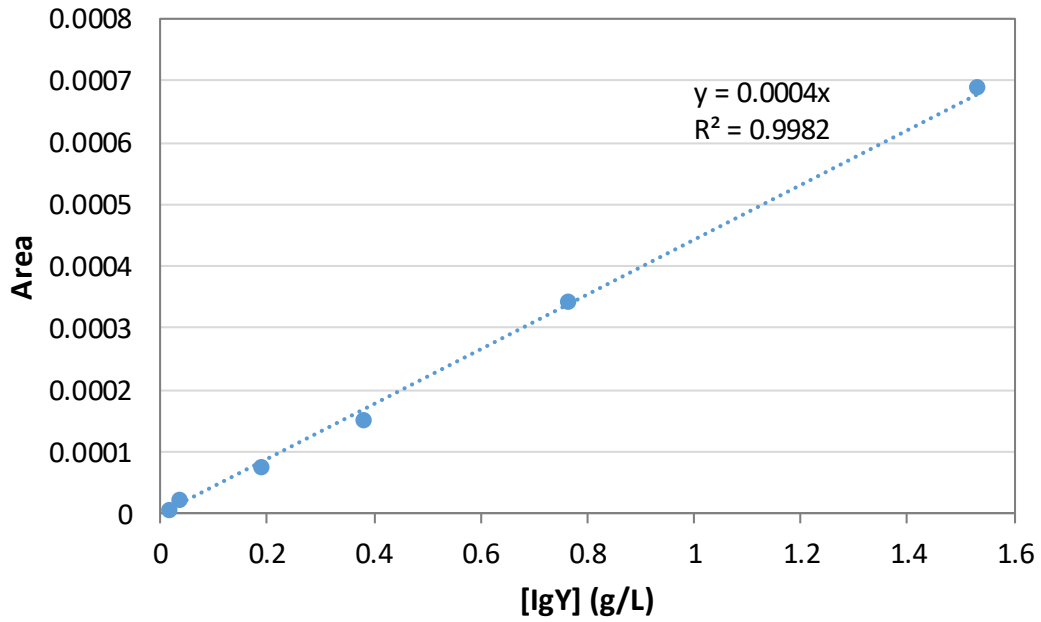

Figure B1. SE-HPLC calibration curve for IgY (purified using the Pierce® Chicken IgY Purification Kit).

TableB1. Composition of K_2HPO_4/ KH_2PO_4 buffers used at different pH values.

pH	KH_2PO_4(g)	$K_2HPO_4 \cdot 3H_2O$(g)	H_2O (g)
5.5	6.50	3.51	25.04
6.0	5.01	5.01	15.00
6.5	4.01	6.00	15.31
7.5	2.01	8.02	15.00
8.0	1.02	8.99	15.00

Table B2. Experimental TLs and TLLs of the ABS composed of PEG + Na_2SO_4 + H_2O at (25 ± 1) °C.

Weight fraction composition/ wt%							
PEG	$[PEG]_{PEG}$	$[Na_2SO_4]_{PEG}$	$[PEG]_M$	$[Na_2SO_4]_M$	$[PEG]_{Na2SO4}$	$[Na_2SO_4]_{Na2SO4}$	**TLL**
200	34.86	6.50	29.98	10.05	8.62	25.64	32.48
300	35.62	4.53	27.14	10.04	4.18	24.96	37.50
400	34.92	3.96	25.12	10.18	4.61	23.18	35.89
600	38.71	2.49	24.29	10.08	0.36	22.66	43.33

Table B3. Experimental TLs and TLLs of the ABS composed of PEG 600 + Na_2SO_4 + H_2O at (25 ± 1) °C.

	Weight fraction composition/ wt%						
	$[PEG]_{PEG}$	$[Na_2SO_4]_{PEG}$	$[PEG]_M$	$[Na_2SO_4]_M$	$[PEG]_{Na2SO4}$	$[Na_2SO_4]_{Na2SO4}$	TLL
PEG range	37.38	2.79	24.28	9.90	0.31	22.90	42.17
	34.53	3.50	21.98	9.94	0.86	20.78	37.85
	31.68	4.31	19.97	10.09	1.69	19.10	33.43
	30.95	4.53	18.04	10.69	2.36	18.18	31.68
Na_2SO_4 range	35.90	3.14	24.09	9.16	0.80	20.96	39.37
	33.61	3.75	24.06	8.30	1.84	18.89	35.19
	27.80	5.56	24.23	7.04	8.53	13.54	20.86
Minimum PEG and Na_2SO_4 concentrations	28.02	5.49	21.87	8.06	7.71	13.99	22.01

Table B4. Experimental weight fraction data for the systems composed of PEG 1000 (1) + K_2HPO_4/ KH_2PO_4 buffer (2) + H_2O (3) at (25 ± 1) °C.

pH 5.5		pH 6.0					
100 w_1	*100* w_2	100 w_1	*100* w_2	100 w_1	*100* w_2	100 w_1	*100* w_2
55.68	1.68	46.81	2.21	20.49	9.67	7.42	18.28
50.29	2.24	41.98	2.45	20.06	9.95	6.98	18.60
47.0	2.75	40.27	2.82	19.77	10.07	6.28	19.08
42.90	3.17	38.43	3.12	19.50	10.17	6.05	19.40
40.42	3.56	37.50	3.44	19.02	10.34	5.33	19.91
38.15	3.94	36.08	3.84	18.68	10.57	5.01	20.26
36.34	4.21	34.84	4.11	18.36	10.7	4.33	20.94
35.14	4.50	33.56	4.41	18.06	10.94	3.96	21.32
34.02	4.81	32.42	4.73	17.86	11.02	3.51	21.73
32.76	5.21	31.34	4.97	17.57	11.23	3.08	22.12
31.70	5.43	30.68	5.30	17.28	11.41	2.67	22.78
30.77	5.69	29.80	5.48	16.79	11.90	2.23	23.27
29.35	6.29	29.27	5.71	16.00	12.40	1.84	24.28
27.91	7.00	28.71	5.93	15.63	12.72	1.46	25.63
27.23	7.19	28.19	6.17	15.16	12.95		
26.20	7.57	27.43	6.34	14.72	13.28		
25.29	7.98	26.45	6.76	14.48	13.50		
24.34	8.34	25.95	6.96	14.01	13.75		
23.26	8.91	25.52	7.14	13.70	13.94		
22.20	9.42	25.04	7.34	12.94	14.43		
21.13	10.03	24.63	7.52	12.58	14.67		
20.37	10.39	23.82	8.15	12.127	15.00		
19.20	11.08	23.44	8.31	11.78	15.20		
18.14	12.20	23.05	8.48	11.04	15.71		
16.92	12.95	22.70	8.60	10.824	15.87		
14.02	15.08	22.333	8.76	10.04	16.37		
12.61	16.05	21.85	9.07	9.43	16.78		
10.46	17.52	21.52	9.20	8.87	17.17		
		21.18	9.32	8.51	17.48		
		20.79	9.56	7.80	17.86		

Table B5. Experimental weight fraction data for the systems composed of PEG 1000 (1) + K_2HPO_4/ KH_2PO_4 buffer (2) + H_2O (3) at (25 ± 1) °C.

pH 6.5				pH 7.5					
100 w_1	*100* w_2	100 w_1	*100* w_2	100 w_1	*100* w_2	100 w_1	*100* w_2	100 w_1	*100* w_2
57.13	1.58	16.38	11.33	56.14	2.01	22.18	7.648	6.771	15.779
50.93	2.16	15.83	11.63	47.47	2.11	21.88	7.738	6.047	16.258
45.56	2.84	15.47	11.87	44.64	2.32	21.17	7.821	5.648	16.665
40.65	3.24	15.08	12.12	42.74	2.63	20.88	7.98	4.84	17.15
37.55	3.94	14.65	12.36	40.72	3.246	20.41	8.17	4.36	17.62
34.46	4.49	14.24	12.62	37.78	3.35	20.09	8.34	3.92	18.10
32.45	4.98	13.85	12.90	36.05	3.76	19.55	8.56	3.45	18.58
30.59	5.40	13.43	13.20	34.30	3.87	19.25	8.751	2.97	19.11
29.43	5.92	12.97	13.51	33.63	4.09	18.91	8.93	2.44	19.67
27.91	6.29	12.48	13.83	32.66	4.23	18.56	9.11	2.04	21.04
26.55	6.63	12.00	14.15	32.02	4.44	18.14	9.32		
25.70	7.02	11.44	14.50	31.44	4.64	17.73	9.54		
24.88	7.39	10.88	14.88	30.56	4.79	17.34	9.74		
23.87	7.55	10.27	15.29	29.65	5.31	16.94	9.96		
23.15	7.88	9.67	15.70	28.83	5.42	16.49	10.20		
22.45	8.21	9.29	16.00	28.02	5.63	15.95	10.47		
21.80	8.48	8.57	16.41	27.58	5.80	15.50	10.71		
21.18	8.81	7.83	16.88	27.12	5.95	15.16	10.94		
20.59	9.09	7.45	17.22	26.64	6.16	14.62	11.22		
20.00	9.23	6.66	17.77	26.193	6.29	14.03	11.53		
19.99	9.40	6.26	18.13	25.76	6.41	13.38	11.85		
19.63	9.43	5.90	18.48	25.36	6.54	13.01	12.12		
19.21	9.65	4.95	19.09	24.98	6.65	12.57	12.41		
18.95	9.79	4.487	19.50	24.61	6.76	11.78	12.83		
18.55	10.01	4.03	19.94	24.29	6.86	11.00	13.25		
18.29	10.20	3.58	20.68	23.89	6.97	10.47	13.60		
18.01	10.38	3.13	21.12	23.40	7.25	9.85	13.96		
17.49	10.64	2.59	21.94	23.09	7.36	9.23	14.33		
17.07	10.90	2.01	23.27	22.77	7.47	8.28	14.86		
16.77	11.111	1.52	25.18	22.46	7.56	7.56	15.29		

Table B6. Experimental weight fraction data for the systems composed of PEG 1000 (1) + K_2HPO_4/ KH_2PO_4 buffer (2) + H_2O (3) at (25 ± 1) °C.

pH 8.0					
100 w_1	*100* w_2	100 w_1	*100* w_2	100 w_1	*100* w_2
56.88	1.65	19.41	8.51	11.47	12.90
48.42	2.20	19.11	8.65	11.29	13.02
43.18	2.60	18.64	8.94	11.17	13.06
39.84	3.03	18.36	9.03	11.00	13.18
37.72	3.44	18.08	9.11	10.87	13.20
35.36	3.82	17.82	9.19	10.71	13.30
33.29	4.046	17.42	9.46	10.70	13.41
32.35	4.40	17.15	9.546	10.37	13.61
31.37	4.76	16.90	9.66	9.50	13.97
30.22	4.92	16.57	9.87	9.05	14.206
29.43	5.20	16.32	9.96	8.70	14.55
28.705	5.47	16.05	10.08	8.27	14.80
27.68	5.65	15.72	10.27	7.78	15.08
26.99	5.91	15.50	10.34	7.32	15.39
26.32	6.11	15.19	10.52	6.81	15.68
25.71	6.33	14.98	10.59	6.33	16.00
24.91	6.41	14.67	10.78	5.79	16.33
24.37	6.55	14.48	10.82	5.40	16.77
23.92	6.725	14.204	11.01	4.79	17.19
23.46	6.90	13.79	11.41	4.28	17.55
22.97	7.08	13.53	11.58	3.38	18.29
22.57	7.23	13.37	11.63	2.77	18.823
22.15	7.42	13.15	11.76	2.12	19.80
21.75	7.59	12.92	11.89		
21.37	7.74	12.75	11.94		
21.03	7.90	12.61	12.00		
20.65	8.04	12.38	12.17		
20.35	8.13	12.25	12.20		
20.00	8.27	12.06	12.32		
19.69	8.41	11.60	12.88		

Table B7. Correlation parameters and respective standard deviations (σ) of equation 15 used to describe the experimental binodal data of the ABS composed of PEG 1000 + K_2HPO_4/ KH_2PO_4 buffer.

pH	$A \pm \sigma$	$B \pm \sigma$	$10^5 (C \pm \sigma)$	R^2
5.5	99.2 ± 4.0	-0.49 ± 0.02	2.7 ± 2.0	0.993
6.0	81.3 ± 2.4	-0.40 ± 0.01	10.2 ± 0.8	0.998
6.5	110.4 ± 2.7	-0.54 ± 0.01	9.01 ± 0.7	0.998
7.5	109.5 ± 6.0	-0.56 ± 0.03	12.5 ± 2.5	0.992
8.0	113.5 ± 3.8	-0.58 ± 0.02	11.8 ± 1.5	0.996

Table B8. Experimental TLs and TLLs of the ABS composed of PEG 1000 + K_2HPO_4/ KH_2PO_4 buffer + H_2O at (25 ± 1) °C.

pH	Weight fraction composition / wt%						
	$[PEG]_{PEG}$	$[buffer]_{PEG}$	$[PEG]_M$	$[buffer]_M$	$[PEG]_{buffer}$	$[buffer]_{buffer}$	TLL
5.5	27.50	6.89	17.92	14.98	5.39	25.57	28.94
	38.46	3.80	24.63	15.06	2.18	33.35	46.80
6.0	33.74	4.43	18.10	14.79	1.82	25.56	38.29
	39.99	2.92	24.82	15.00	0.10	34.69	50.99
6.5	34.27	4.70	18.01	15.03	1.70	25.40	38.59
	42.25	3.20	24.86	14.91	0.34	31.42	50.52
7.5	36.12	3.85	17.97	14.96	0.82	25.47	41.39
	43.75	2.66	25.05	14.98	0.10	21.42	52.28
8.0	37.44	3.60	18.32	14.85	0.95	25.07	42.33
	43.29	2.74	24.98	15.00	0.10	31.69	51.99